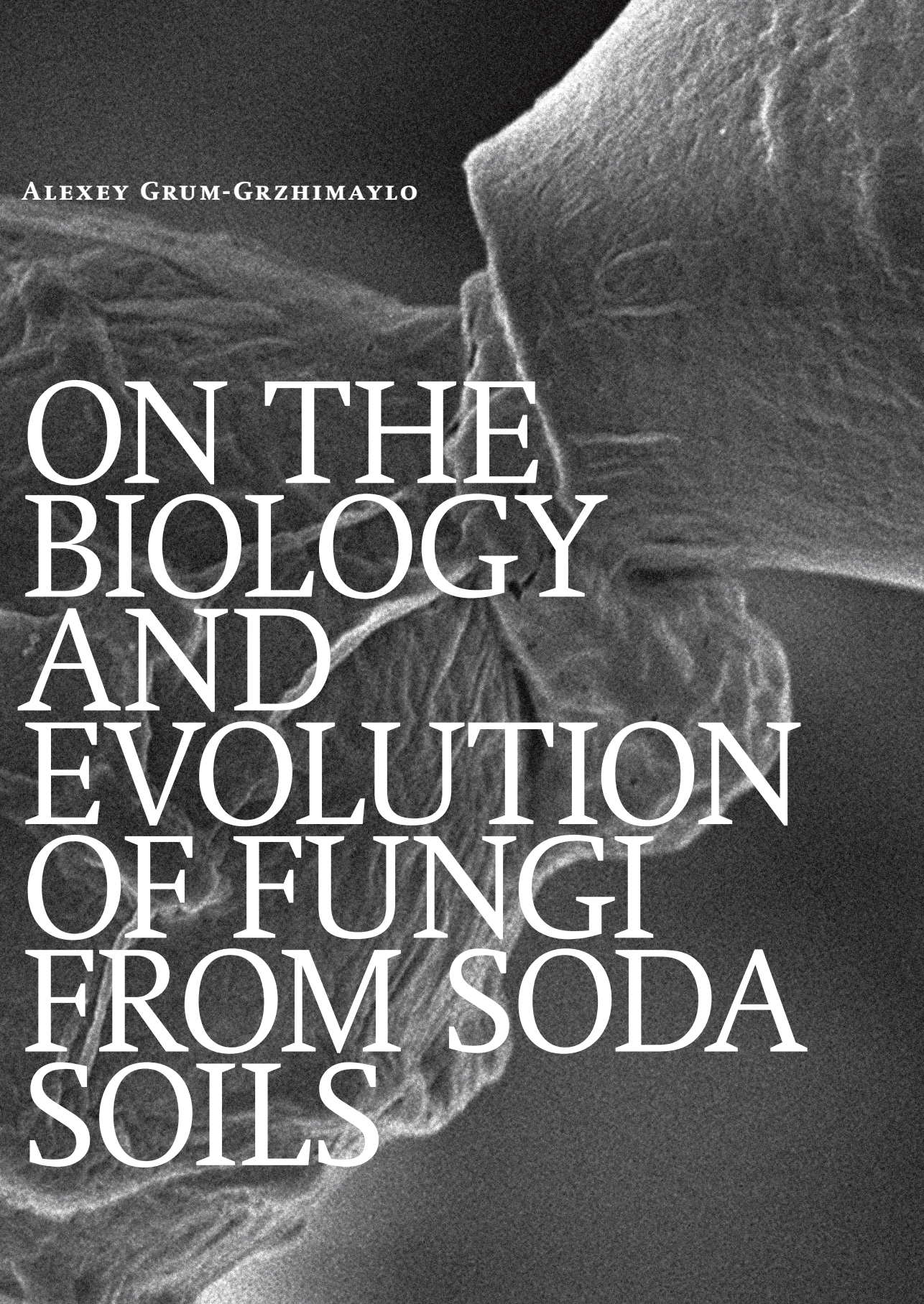

Alexey Grum-Grzhimaylo

ON THE BIOLOGY AND EVOLUTION OF FUNGI FROM SODA SOILS

On the biology and evolution of fungi from soda soils

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On the biology and evolution of fungi from soda soils

Alexey Grum-Grzhimaylo

Thesis

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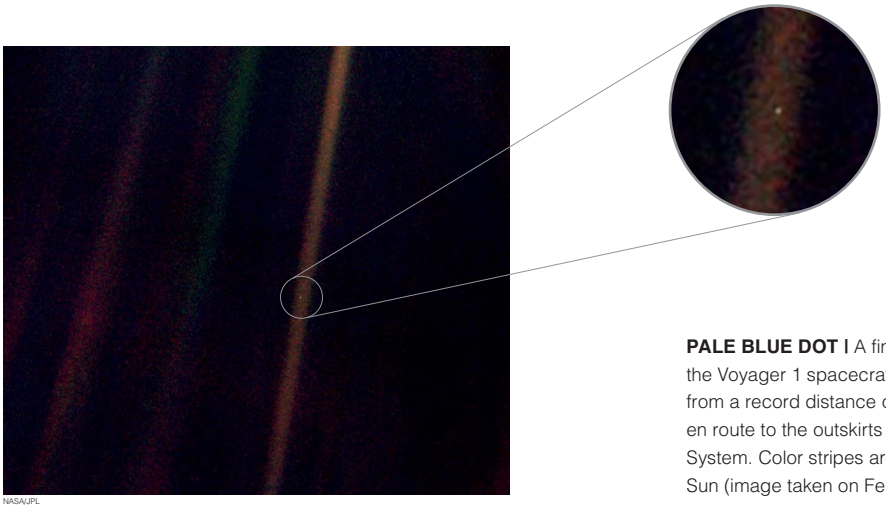


NASA/JPL

PALE BLUE DOT I A final look-back of the Voyager 1 spacecraft at the Earth from a record distance of 4 billion miles en route to the outskirts of the Solar System. Color stripes are glare from the Sun (image taken on February 14, 1990).

"Look again at that dot. That's here. That's home. That's us. On it everyone you love, everyone you know, everyone you ever heard of, every human being who ever was, lived out their lives. The aggregate of our joy and suffering, thousands of confident religions, ideologies, and economic doctrines, every hunter and forager, every hero and coward, every creator and destroyer of civilization, every king and peasant, every young couple in love, every mother and father, hopeful child, inventor and explorer, every teacher of morals, every corrupt politician, every "superstar," every "supreme leader," every saint and sinner in the history of our species lived there — on a mote of dust suspended in a sunbeam. [...]. Our posturings, our imagined self-importance, the delusion that we have some privileged position in the Universe, are challenged by this point of pale light. Our planet is a lonely speck in the great enveloping cosmic dark. In our obscurity, in all this vastness, there is no hint that help will come from elsewhere to save us from ourselves. [...]. To me, it underscores our responsibility to deal more kindly with one another, and to preserve and cherish the pale blue dot, the only home we've ever known."

— CARL SAGAN, *PALE BLUE DOT,* **1994**

Contents

Chapter I

Introduction

Extremophiles

Extremophiles are organisms that thrive and flourish in habitats that one would think to be too hostile to sustain life. In a short communication published in **1974**, **MACELROY** proposed the term 'extremophiles' as "*descriptive for organisms able to populate environments hostile to mesophiles, or organisms which grow only in intermediate environments*". The extremophiles is an artificial ensemble reflecting its anthropocentricity – for an extremophilic organism the 'extreme' environmental conditions are just right for the optimal performance, while being clearly inhabitable from a human standpoint. It is natural to presume that extremophilic organisms populate extreme environments. As of now, there is no consensus opinion on the definition for an extreme environment. From a human point of view, a combination of physicochemical parameters of an environment which eliminate mammalian life would be considered extreme (**SELBMANN ET AL. 2013**). On Earth, there is a plethora of fluctuating physicochemical properties that can account for an extreme environment: temperature, pressure, acidity, salts and heavy metals concentrations, irradiation levels, water activity, all can be anomalously high or low, deviating from certain optimal levels from our perspective. And yet, habitats with the above named extreme environmental skews have been shown to support life, often holding extensive biodiversity after all. A less stringent occurrence is extremotolerants (or extremotrophs) – organisms that survive exposure to extreme conditions, however not necessarily performing best at these conditions (**MUELLER ET AL. 2005**). One should cautiously tag a given organism as extremophile even if isolated from an extreme environment, as many of them are dormant and merely present in these habitats, rather than being fully metabolically active at given physicochemical extremes (**HORIKOSHI & BULL 2011**).

Most studies on extremophiles conducted thus far focus on the organisms that belong to the prokaryotic (bacteria and archaea) domain of life. Their longer evolutionary history and plastic genomes resulting in quick adaptability, which facilitated the establishment of stable populations in extreme niches (**ROCHA 2008**). Nonetheless, there is an increasing body of evidence that eukaryotes also can adopt extremophilic lifestyles. Examples of extreme environments include permafrost glaciers, thermal springs, hot dry deserts, deep-sea trenches, saline and alkaline lakes, acidic sulfuric pools, mine drainage and geysers (**FIG. 1**). In some of these habitats, several abiotic factors intermingle resulting in a combination of stresses exemplified in polyextreme environments. Elaborate descriptions of every group of extremophiles are beyond the scope of the presented thesis; however, I will provide few selected examples to illustrate some remarkable physiological capacities of microorganisms.

Temperature and pressure

Temperature of the salty sea ice ranges from -1.8 to -30 °C permitting liquid water state in brine pockets of ice at about -10 °C (**ASSUR 1958**). Thin liquid water films have been shown to exist at dust grains contacts at temperatures as low as -20 °C (**JAKOSKY ET AL. 2003**). From an arctic sea-ice column excavated in Alaska, an extreme psychrophilic (literally, 'cold-loving') rod-shaped bacterium named *Phychromonas ingrahamii* was recovered with a growth optimum at 5

°C and still capable of exponential growth at least as low as -12 °C (**BREEZEE ET AL. 2004**). The genome sequence of *Phychromonas ingrahamii* was later determined and analyzed, helping to explain the extreme adaptation. Water-keeping extracellular polysaccharides, betaine choline osmolyte accumulation, TRAP transporters, chaperones and stress proteins along with many small protein sets of unknown function have all been attributed to the psychrophilic trait of this peculiar bacterium (**RILEY ET AL. 2008**). Many other species from various taxonomic groups, including filamentous fungi and other eukaryotes, have been shown to thrive in cryo-ecosystems (**ROBINSON 2001, MUELLER ET AL. 2005, WANG ET AL. 2014**).

Although high temperatures cause destructive effects on DNA, proteins and lipid membranes, rich diversity of organisms have been demonstrated to populate environments such as hot geysers, deep-sea hydrothermal vents and black smokers. In **2007**, researchers explored the Ashadze site on the Mid-Atlantic Ridge, one of the deepest (4100 m) active hydrothermal vent fields known, which resulted in the recovery of a hyperthermophilic anaerobic archaeon, *Pyrococcus yayanosii* (**BIRRIEN ET AL. 2011**). Its cocci cells were growing optimally at 95 °C at hydrostatic pressure of 52 MPa (atmospheric pressure at the sea level is about 0.1 MPa), additionally tagging this organism as a piezophile (literally, 'pressure-loving'). Yet, *Pyrococcus yayanosii* is out-performed by a current record-holder, hyperthermophilic methanogenic archaeon *Methanopyrus kandleri* with a growth optimum of 105 °C at hydrostatic pressure between 20 and 30 MPa. Strikingly, the cells were still proliferating at 122 °C (**TAKAI ET AL. 2008**). Thermophilic adaptations include an adjusted ratio of saturated versus unsaturated lipids in the membranes (**ROTHSCHILD & MANCINELLI 2001**); monovalent and divalent cations that increase stability of DNA, protecting it from depurination and hydrolysis (**MARGUET & FORTERRE 1998**); and various adaptations to the overall protein stability. Oligomerization, tighter packing of the hydrophobic core, increased number of large hydrophobic residues, disulphide bonds, salt bridges, and surface charge appear to be important constituents for thermophily (**REED ET AL. 2013**). Notably, all known hyperthermophiles are either archaea or bacteria; the upper temperature limit for eukaryotes is substantially lower at about 60 °C, which is suitable for some algae, fungi and protozoa (**ROTHSCHILD & MANCINELLI 2001**).

Water activity

Even completely opposite temperature-wise extremes such as Antarctic ice-free dry valleys and Atacama Desert (Chile) as one of the hottest and driest places on Earth, do share a common feature – both are limited in water. Low water availability also arises at high ambient salts or sugars content. Collectively, these environments are said to have low water activity (a_w). Xerophiles (literally, 'dry-loving') are organisms that live at extremely low water activity. Surprisingly enough, it was previously stated that prokarya have less capacity to withstand desiccation than eukaryotic organisms. In most xerophilic bacteria and archaea cell proliferation was seen at a_w down to 0.755 (saturated NaCl solution at 25–35 °C temperature range has a_w 0.755), and some most xerophilic fungi are capable of germination and growth at a_w as low as 0.605 (**PITT 1975, BROWN 1976, WILLIAMS & HALLSWORTH 2009, RUMMEL ET AL. 2014**). Superior xerophilic capabilities of

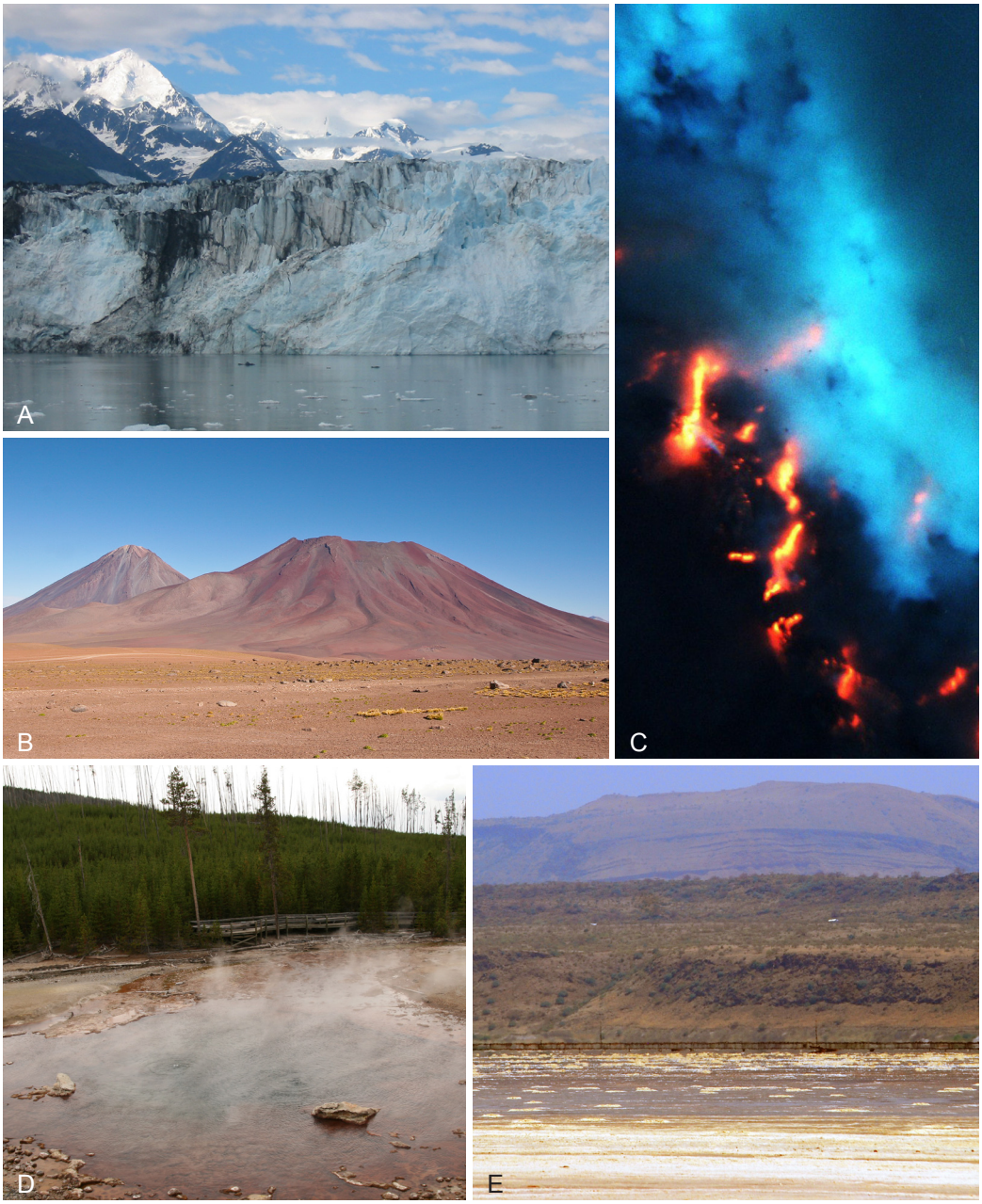


FIG. 1. Extreme habitats that support a substantial diversity of life forms. **A.** Permafrost arctic ice at Alaska, USA (photo by Valerie Lam). **B.** The driest place on Earth – the Atacama Desert, Chile (photo by Danielle Pereira). **C.** Deep-sea hydrothermal vent at Fiji area, Pacific Ocean (photo credit: NSF & NOAA). **D.** Acidic hot-spring, Echinus Geyser, USA (photo by Bernt Rostad) **E.** Saline alkaline Magadi Lake, Kenya (photo by Sofiya Bondarenko).

fungi over prokaryotes later proved false, as it turned out to be merely an artifact created by the solubility limit for NaCl rather than physiological properties of prokaryotes. Hence it was demonstrated that all the three domains of life have somewhat similar adaptabilities towards desiccation – the revised lower a_w growth limits for xerophilic prokaryotes and fungi are now 0.611 and 0.632, respectively (**STEVENSON ET AL. 2014**), with the growth optimum of many of these species around a_w 0.8. Some of the most xerophilic fungi *Xeromyces bisporus* and *Zygosaccharomyces rouxii* are found in high-sugar environments such as dried fruits (**PETTERSSON ET AL. 2011, LIEVENS ET AL. 2015**). Many xerophilic fungi cause food spoilage – *Aspergillus penicillioides*, *Bettsia fastidia*, *Eurotium* species, *Wallemia sebi* and *Xerochrysium xerophilum* have been recovered from low water-activity foods such as dried fish and meat, grains, nuts and spices, jams, chocolate and others (**PITT 1975, HOCKING 2003, PITT & HOCKING 2009, PITT ET AL. 2013**).

Salinity

Notably, low water-activity habitats are often salty, i.e. enriched with NaCl. A large body of research has been devoted to the study of halophilic (literally, 'salt-loving') organisms, those that require high Na^+ concentrations to thrive. Rich diversity of halophiles occurs in natural salty habitats such as hypersaline lakes (e.g. Dead Sea in the Middle East and Great Salt Lake in the USA), coastal salterns, evaporation ponds (**JAVOR 1989**) as well as in artificial solar salterns build for salt production. Halophiles are nested within many taxonomic groups including archaea, bacteria, algae, protozoa, fungi, invertebrates, and even fishes. Extremely halophilic archaea *Halobacterium* sp. grows best at salinities from 3.4 M to saturation (5.1 M) occasionally developing dense blooms that may colour water or salt brines in red due to carotenoids (**DASSARMA & DASSARMA 2012**). Well-known eukaryotic halophiles are species of unicellular green algae *Dunaliella* (**JAVOR 1989**). Fungi are capable of thriving at extremely high salinities as well. Halotolerant black yeast *Hortaea werneckii* can grow over a wide range of salinity from none to saturation, however this fungus performs best at salinities between 0.8 M and 1.7 M NaCl. Another prominent example is a basidiomycete *Wallemia ichtyophaga* which does not grow without salts at all (**ZALAR ET AL. 2005, PLEMENITAŠ ET AL. 2014**). Halophilic organisms synthesize or uptake an array of osmoprotectants to facilitate the extrusion of salts from the cell: arabitol, carboxamines, ectoine, glycine betaine, K^+ (in haloarchaea, **LARSEN 1967**), N-α-carbamoyl-L-glutamine-1-amide, N-acetylated diamino acids, N-acetylglutaminylglutamine amide, N-ε-acetyllysine, oligosaccharides, polyols, proline, sucrose, trehalose have been detected in various groups of halophiles (**DASSARMA & DASSARMA 2012**). General protein composition biases towards having a larger fraction of acidic residues, and overall lower hydrophobicity along with other hallmarks have been proposed to contribute to the halophilic lifestyle (**PAUL ET AL. 2008**).

pH

Acid habitats are not often encountered in nature, and still there are ecotopes with very low pH values, e.g. acid-mine drainages, hot springs, sulfuric ponds, sulfataric volcanic fields, from which acidophilic (literally, 'acid-loving') organisms have been recovered (**EDWARDS ET AL.**

2000, JOHNSON & HALLBERG 2003). Some unicellular prokaryotes, algae, and fungi have evolved the ability to withstand devastating effects of extremely low pH. The aerobic heterotrophic bacteria, *Picrophilus oshimae* and *Picrophilus torridus*, have been recovered from extremely acid Japanese sulfataric soils and displayed growth optimum at pH 0.7 and 60 °C (**SCHLEPER ET AL. 1995A, 1995B**). The algae *Dunaliella acidophila* and *Cyanidium caldarium* are capable of growing at pH approaching 0, however the optima lie at about 1–3 (**DOEMEL & BROCK 1971, BEN-AMOTZ & AVRON 1992**). Several fungal species, *Acidomyces acidophilus*, *Acontium velatum*, *Cephalosporium* sp., *Hortaea acidophila*, *Scytalidium acidophilium*, *Teratosphaeria acidotherma*, *Trichosporon cerebriae* can grow at pH as low as 0–1, but having optima at higher pH (**STARKEY & WAKSMAN 1943, SLETTEN & SKINNER 1948, BAKER ET AL. 2004, HÖLKER ET AL. 2004, SELBMANN ET AL. 2008, YAMAZAKI ET AL. 2010**). As biological processes tend to occur in the middle of the pH range, acidophiles have to be able to keep cytoplasm around neutral. Indeed, it was demonstrated that acidophiles have near-neutral cell interiors (**BEARDALL & ENTWISLE 1984**), which means they can sustain an impressive proton gradient of about a million times $[H^+]$ difference across the internal and external milieu of the cell. Much less permeable membranes, reduced pore size for membrane channels, predominance of secondary transporters, reversed membrane potential ($\Delta\Psi$), efficient means for proton efflux (antiporters, H^+-ATPases, symporters), cytoplasmic buffer molecules that sequester protons (free amino acids, phosphoric acid), organic acids degradation metabolic pathways, highly expressed chaperones are believed to contribute to the survival at extremely low ambient pH (**BAKER-AUSTIN & DOPSON 2007**). On the other side of the pH scale, alkalinity yet presents another stress. Various microorganisms have been shown to live at high pH values as well. Prokaryotic and eukaryotic alkaliphiles (literally, 'alkali-loving') with growth optima at pH > 9, can be readily isolated from soda lakes and soda soils – stable natural alkaline habitats on Earth. A more detailed treatment of this extremophilic group is given below, as alkaliphiles are the focus of the presented thesis.

...and other oddities

Many peculiar microorganisms have adapted to withstand other types of stresses utilizing efficient means to neutralize the environmental effects and tolerate them at a certain life-stage or at dormant state (eggs, sclerotia, seeds, spores). A well-known extraordinary capacity of the bacterium *Deinococcus radiodurans* to deal with doses of ionizing radiation lethal to other life-forms, is more efficient at logarithmic growth phase rather than at stationary, and also depends upon other conditions (**MINTON 1994, VENKATESWARAN ET AL. 2000**). *Deinococcus radiodurans* possesses a unique ability to quickly repair and reassembly of the broken DNA caused by UV- or γ-illumination (**BATTISTA 1997**). Metallotolerance has been described in bacteria which display efficient means in effluxing or detoxifying the excess of heavy metals such as As^{3+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+} (**NIES 2003**). Tardigrades (or water bears), small invertebrates tagged as one of the toughest organisms known, can survive vacuum, temperatures in a range from -253 °C to 151 °C, extreme pressures of 600 MPa, and X-rays (**SEKI & TOYOSHIMA 1998**). Out of this rich range of extremes, the current thesis is dedicated to study the fungal diversity of alkaline soils. Hence I sought to provide a more elaborate overview of alkaline habitats.

Soda lakes and other alkaline habitats

Soda (or alkaline) soils and lakes are the most alkaline natural habitats on Earth. With pH typically ranging 9–11, these environments are also usually very salty – with high Na^+ concentrations. As the name implies, those habitats possess a large amount of soda (sodium carbonate, or hydrated complexes of this salt) that generates high pH values. Soda soils are usually restricted to arid or semi-arid savanna inland areas and often nest alkaline water basins called soda lakes, fluctuating in size throughout the season due to evaporative concentration or rain precipitation (**JONES ET AL. 1998, JONES & GRANT 2000**). However, there are alkaline habitats where high alkalinity is not generated by soda. The genesis of alkaline lakes depends on the local geology and rock contents of the area. At the sites with Ca^{2+} and Mg^{2+}-rich silicates, weathering processes lead to highly alkaline oligotrophic habitats generated by $Ca(OH)_2$ of low buffering capacity due to limited solubility of $Ca(OH)_2$ (**GRANT ET AL. 1990**). In addition, at the conditions of high Ca^{2+} and Mg^{2+}, alkaline carbonates are trapped by those cations, and subsequently removed from the solution as insoluble minerals (**JONES ET AL. 1998**). Conversely, in habitats with low Ca^{2+}/Mg^{2+} contents, the dominant anion becomes CO_3^{2-}, which leads to the formation of soda – Na_2CO_3 – determining high pH with strong buffering capacity. Restricted water outflow from a drainage basin is another important factor that promotes the genesis of soda lakes (**ULUKANLI & DIĞRAK 2002**). In several areas, removal of carbonates results in the accumulation of NaCl leading to the formation of neutral or even slightly acid salt lakes, such as the Great Salt Lake (Ohio, USA) or the Dead Sea in the Middle East. The slightly acidic pH (6–7) of the Dead Sea is a consequence of the high magnesium content (**GRANT & SOROKIN 2011**). It is worth noting that alkaline habitats can arise as a result of human industrial processes such as cement manufacturing, electroplating, paper manufacturing, and indigo reduction in alkali – an ancient-time Japanese industrial process, along with few others that generate alkaline wastes (**GRANT ET AL. 1990, HORIKOSHI 1999**). Natural alkaline habitats also include limestone caves and physiology-driven local alkalinized fractions of neutral soils, as a result of nitrogen circulation, proteins decay, urea hydrolysis, photosynthesis. Guts of some insects (e.g. termites) and sea-borne environments are known to be alkaline as well (**STAUDENMAYER 1940, WATERHOUSE 1949, YUMOTO ET AL. 2011**).

As mentioned earlier, alkaline saline soils and lakes are usually spread across the arid continental interiors that are often hard to reach, therefore a relatively limited number of studies has been conducted on them. Nonetheless, there are few areas which have been extensively investigated – one of them is the Kenyan-Tanzanian Rift Valley which runs through East Africa. This is an area with high tectonic activity and active volcanism, which created a series of shallow depressions many of which are soda lakes (**GRANT & SOROKIN 2011**). At least one active volcano is believed to participate in the formation of soda lakes in that area as it delivers soda-rich lava to the surface (**BAKER 1958, EUGSTER 1970**), however alkaline lava contribution seems unlikely to be the universal mechanism for soda lakes genesis (**GRANT ET AL. 1990**). The Kenyan-Tanzanian Rift Valley is typical low Ca^{2+} and Mg^{2+} environments dominated by alkaline trachyte lavas – rich in Na^+, poor Ca^{2+}/Mg^{2+}, resulting in quick accumulation of Na^+, Cl^-, HCO_3^-, CO_3^{2-} (**THOMPSON & DODSON 1963, MCCALL 1967**). Various soda lakes have different salinities and vary from 5 % (w/v) in more

dilute lakes (Lake Bogoria, Lake Elmenteita, Lake Nakuru) to nearly saturated water solution (> 30 %) such as in southern lakes of the Great Rift Valley – Lake Magadi and Lake Natron with the pH values approaching 12.

A prominent reservoir of soda lakes and soils (solonchaks) is located in the Western Siberia, Altai, Trans-Baikal area (Russia) and Mongolia, many of which are unnamed, ephemeral, thus impose substantial difficulties to navigate around without a local guide (**SOROKIN, PERSONAL COMMUNICATION**). Mono Lake is a well-known example of saline alkaline habitat in the Sierra Nevada Mountains (California, USA). This lake is meromictic (i.e. lacking seasonal water mixing), therefore has strong geochemical gradients with a simple food web, making this habitat a good site for studying bacterial communities of alkaline lakes (**HUMAYOUN ET AL. 2003**). Lonar Lake of India is another well-studied saline soda lake that formed in a crater basin originated from a meteorite impact about 50000 years ago (**FREDRIKSSON ET AL. 1973, WANI ET AL. 2006**). Descriptions of a large number of known saline soda lakes is beyond the scope of the presented thesis, and the reader can be referred to **GRANT & SOROKIN (2011)** for the overview on the world distribution of soda lakes. To summarize, soda lakes have a worldwide distribution and reflect local geologies resulting in various physicochemical properties determining the establishment of biological communities.

Life in soda lakes

Fluctuations in abiotic factors of different soda lakes across the world ultimately determine the biota content at a given soda lake. Despite this variation, I will roughly portray major trophic groups found in alkaline lakes worldwide. Counter-intuitively as it may sound, the extreme environment of saline soda soils and lakes harbor an extensive diversity of life forms. As suggested by **ZAVARZIN (1993)**, soda lakes are essentially closed ecosystems and should exhibit complete nutrient recycle, if we though ignore the contribution by flocks of grazing flamingoes (**JONES ET AL. 1998**). Commonly located in arid warm geographical locations with high solar illuminations and excess of CO_2, saline soda lakes adopt excellent conditions for photosynthesis, and have been shown as highly productive environments (**TALLING ET AL. 1973**). For example, a study by **MELACK & KILHAM (1974)** have demonstrated extraordinary high rates of phytoplankton photosynthesis rate in Lake Nakuru of East Africa – about 36 g O_2 m^{-2} per day with the eutrophic zone usually less than 1 m depth (optimal around 0.5 m). That magnitude of photosynthesis rate is about 22 times higher than the mean gross photosynthetic production estimate of the lakes and streams across the world, as given by **WHITTAKER & LIKENS (1973)**. Saline soda lakes are thus ranked among the most productive aquatic habitats on Earth (**DUCKWORTH ET AL. 1996**). Microbial biodiversity studies have shown that saline alkaline lakes host microorganisms from virtually all major physiological groups, which turns these habitats as possible relict centers of microbial communities that led to inland microflora diversification, a concept postulated and actively promoted by **ZAVARZIN ET AL. (1993, 1999)**.

The bulk of primary production in soda lakes can build up in both oxic and anoxic layers,

i.e. in the water body or in the sediment microbial mat. In the dilute soda lakes, organic matter synthesis is believed to be driven by permanent or occasional blooms of uni- or multicellular cyanobacterial species, most commonly restricted to members of *Arthrospira* (= *Spirulina*), *Chroococcus*, *Cyanospira*, *Synechococcus* (**MELACK 1979, JONES ET AL. 1998, JONES & GRANT 2000**). Just as carbon production is an important property of cyanobacteria, nitrogen fixation and generation of oxygen too are essential processes governed by these microorganisms. Eukaryotic diatom algae communities of *Cyclotella*, *Navicula*, *Nitzschia* may also dominate the environments throughout the season, however, generally at lower salinities, as it was demonstrated by studying East African soda lakes (**HECKY & KILHAM 1973**). In the lower zones of the microbial mat, anoxygenic photosynthetic purple bacteria of the genera *Allochromatium*, *Ectothiorhodospira*, *Rhodovulum*, and *Thiocapsa* have been found, which may account for a substantial part of the primary production (**GRANT ET AL. 1990, KOMPANTSEVA ET AL. 2005**). In fact, **KOMPANTSEVA ET AL. (2005)** showed that the shallow water basin (35–45 cm) of the saline alkaline Lake Khilganta located in the Trans-Baikal area, exhibits predominantly (96 %) anoxygenic photosynthesis governed by the purple bacteria that occupy the sediment mat. Novel species of these bacteria from genera *Rhodobaca* and *Rubribacterium* have been recorded from soda lakes in Eastern Siberia (**BOLDAREVA ET AL. 2008, 2009**), and surely more are awaiting to be discovered. Cyanobacteria occur rarely in more concentrated soda lakes such as Lake Magadi in Kenya, possibly only during periods of water dilutions after heavy rainfalls (**GRANT ET AL. 1990**). Purple bacteria are believed to be responsible for the primary production in those types of soda lakes. Noteworthy, the massive microbial blooms of saline soda lakes colour the environment in shades of green or red (**GRANT & SOROKIN 2011**).

Primary producers support the rest of the microbial community, members of which break down complex carbon compounds. Both aerobic and anaerobic heterotrophic bacteria of various taxonomic affinities produce large set of hydrolytic enzymes that degrade organic matter. The halomonads (proteobacteria) are probably the most important group of heterotrophs, along with bacilli and actinobacteria which are readily recovered from many saline soda lakes across the world (**LI ET AL. 2005**, **DELGADO ET AL. 2006**, **BORSODI ET AL. 2008**, **JOSHI ET AL. 2008**). Simpler compounds like various sugar types, amino acids and fatty acids can be further utilized by sediment anaerobic fermenters which produce acetate, ammonia, butyrate, ethanol, hydrogen, lactate, methanol, and propionate. Sulfate-reducing and sulfate-oxidizing bacteria drive one of the most active element cycles in soda lakes – the sulfur cycle. However, there is a substantial skew in the recovery rates of these two physiological groups. While sulfate-oxidizing species are readily being isolated from saline soda lakes (**SOROKIN & KUENEN 2005**), only a limited number of sulfate-reducing bacteria have been brought to culture, e.g. *Desulfonatronovibrio* and *Desulfonatronum* (**FOTI ET AL. 2007**, **SOROKIN ET AL. 2011**). Nitrogen cycle is exemplified with ammonia production by anaerobic fermenters and its oxidation by nitrifiers and metanotrophs that produce nitrate, which is further exploited by chemoorganotrophs (**REES ET AL. 2004**). Decomposition of organic matter creates substrates for methanogens, such as *Methanosalus zhilinaeae*, a species that produces methane which is in turn oxidized by methanotrophs (**ZHILINA**

& ZAVARZIN 1994, GRANT & SOROKIN 2011). Notably, extremely salty and alkaline lakes such as Lake Magadi (Kenya), Wadi Natrun (Egypt) and others, harbour haloarchaeal species that are obligate alkaliphiles and halophiles. During intense blooms, these microorganisms colour the brines red due to carotenoid accumulation (**GRANT & SOROKIN 2011**). In summary, a plethora of trophic groups such as phototrophs, acetogens, sulfate-oxidizers, sulfate-reducers, nitrifiers, hydrogenotrophs, carboxytrophs, methanotrophs, and methanogens have been shown to thrive in saline soda lakes that sustain carbon, nitrogen and sulfur cycles and interlink them.

The diversity of species in saline soda lakes is not only confined to bacteria or archaea. In addition to already noted before diatom algae, other eukarya groups such as alveolates, cercozoa, ciliates, cholorophytes, cryptophytes, euglenoids, pavlovophytes, rotifers, and fungi have also been detected using culture methods and pool DNA-based analysis (**LOPEZ-ARCHILLA ET AL. 2004, AMARAL-ZETTLER 2012, ANTONY ET AL. 2013, LANZÉN ET AL. 2013**). However, virtually no further studies are available on those groups of organisms from soda lakes. The vast majority of them were not brought into culture and not tested, therefore the open question remains whether they are metabolically active at soda lakes. More complex, multicellular diversity of alkaline lakes may be represented by species of copepods (**SCHNEIDER ET AL. 2012**), brine shrimps *Artemia*, or brine fly such as *Ephydra hians* that can live underwater for a long duration of time. Occasional abundant crustacean or algal biomass attracts many bird species feeding on it (**MAHONEY & JEHL 1985**), notably huge flocks of grazing flamingoes that inhabit East African soda lakes (**GRANT ET AL. 1990**). Remarkably, a single fish species, tilapia *Oreochromis alcalicus* inhabits the extremely alkaline Magadi Lake (Kenya) and possesses interesting adaptation to high pH water. As ammonia diffusion is impaired at alkaline waters, toxic amounts of it may accumulate in the cells. It is postulated that the fish have adapted to alkaline waters by the ability to secrete virtually all its ammonia as less toxic urea, a property that normal fish rarely show (**RANDALL ET AL. 1989, WOOD & SHUTTLEWORTH 1995**). Amount of vegetation at saline soda lakes varies from a couple of grass species (*Cynodon* and *Dactyloctenium* at Magadi Lake), to a diversity of halophytic grasses mostly from family *Chenopodiaceae*, e.g. at Siberian saline soda lakes (**BONDARENKO & BILANENKO, PERSONAL COMMUNICATION**).

Alkaliphiles

As one may expect, saline alkaline soils and lakes are the likely source for alkaliphilic organisms. Indeed, many species from various taxonomic groups recovered from these habitats display an alkaliphilic phenotype. Researchers noted the ability of some microorganisms to withstand alkali long before the development of the alkaliphily concept. Arguably, the first reference to alkaliphilic bacterium dates back to the end of the 19th century where **CHESTER (1897)** reported the *Sporosarcina pasteurii* (= *Bacillus pasteurii*) requiring alkaline environment for growth. **MEYERHOF (1916)** and later **MEEK & LIPMAN (1922)** showed that the nitrification process goes on optimally at alkaline pH. In the latter somewhat historical study, the authors write, "*The data […] furnish most interesting and unexpected results. […]. We note the very striking fact that both nitrite- and*

nitrate-forming organisms from the garden soil can withstand extremely high concentrations of hydroxyl ion, in one case pH 13 and in the other 13.1. This exceeds the resistance of any living organism, of which we have knowledge, to the effects of alkalinity". This quotation contains another important aspect in the research of alkaliphiles – the fact that these organisms can be recovered from neutral habitats such as garden soil, possibly due to local alkalization as a result of nitrogen circulation, protein decay, urea hydrolysis, or activity of other microorganisms (**YUMOTO ET AL. 2011**). Another prominent point in the investigation by **MEEK & LIPMAN (1922)** was that "*living cells modify very rapidly the reaction of the media into which they are introduced*". This property has indeed been often encountered in alkalitolerant/alkaliphilic microorganisms throughout the history of its research. Several subsequent studies showed alkaline tolerance for other bacterial species (**DOWNIE & CRUICKSHANK 1928, GIBSON 1934**). The ability of a bacillus-like strain to grow optimally at pH 8.6–11, led **VEDDER (1934)** to introduce a new name, *Bacillus alcalophilus* that reflects a novel physiology. In **1959**, **KUSHER & LISSON (1959)** report a possibility of the evolution of the alkalitolerance trait in the laboratory setting through serial transfers. The authors demonstrated that *Bacillus cereus* is capable of development of alkali resistance after about 50 transfers during more than one month, up to the level of tolerating pH 10.3. However, the cells could not develop resistance to higher pH over the course of a next month. Interestingly, the evolved alkalitolerant cells had changed the morphology as compared to the ancestral type, and retained the alkalitolerant phenotype for several subsequent transfers on neutral pH medium. A couple of years later, **CHISLETT & KUSHNER (1961A, 1961B)** encounter another species, *Bacillus circulans* that grew at pH up to 11. An interesting application of alkaliphilic bacteria includes a process known as indigo fermentation, probably the first industrial process which uses alkaliphilic bacteria. However, it took a long time before the realization that actually bacteria are responsible for the indigo reduction in the presence of sodium carbonate (**TAKAHARA & TANABE 1960**). The studies mentioned above are arguably the only ones that touch upon the alkaliphilic phenomenon before the systematic investigations have been started in **1968** by a renowned researcher in the field, Koki Horikoshi.

Already in **1958**, Horikoshi noted that one of his cultivation flasks got contaminated with *Bacillus circulans* which lyzed mycelium of *Aspergillus oryzae* (**HORIKOSHI & IIDA 1958**). But he did not realize that it was the bacterium that produced ammonia and alkalinized the medium to pH 9 which promoted the growth of the bacterium and its enzyme production. Another ten years passed before Horikoshi conceived a huge potential of these organisms – the moment he exquisitely portrays as a voice whispering at his ear: "*There could be a whole new world of microorganisms in different unexplored cultures*". He prepared alkaline selective media containing 1 % of sodium carbonate and inoculated it with various soil samples from around the institute he was working at that time. To his great surprise, all test tubes flourished with microorganisms (**HORIKOSHI 2011A**). Horikoshi first coined the term 'alkaliphile' and pinned it to those species that grew best at alkaline pH and studied them in a greater detail. First purification of an alkaline enzyme (protease) appeared in **1971** (**HORIKOSHI 1971**), and many industrially valuable substances have been obtained and brought to commercial scale since then. Alkaline enzymes

have become indispensable in several industries such as laundry, dehairing, biobleaching, paper manufacturing, and some others (**HORIKOSHI 1999, 2006**). A great body of research has been accumulated on the various aspects of the morphology, taxonomy, physiology, distribution, biochemistry, genetics, and genomics of alkaliphilic bacteria revealing interesting aspects of the biology and adaptations to high pH, which I will briefly portray in the next section.

Thousands of new alkaliphilic bacteria and archaea have been recovered from all over the world and the number continues to rise. But what about eukaryotic alkaliphiles? Information on the eukaryotic alkaliphiles is extremely scarce and the reasons for this are multifaceted. Importantly, until relatively recently there was a notion that only prokaryotes are capable of displaying alkaliphilic phenotype, which explains a bias toward the abundance of studies on prokaryotes. Second, isolation bias might contribute too, as prokaryotes usually appear first during the recovery and may take over the rest of the community in the medium. Various algae species that bloom in saline soda lakes presumably display alkaliphilic/alkalitolerant and halophilic/halotolerant phenotype, however not yet tested in the laboratory (**SECKBACH ET AL. 2007**). *Dunaliella parva* is an example of alkalitolerant green alga (**GIMMLER & DEGENHARD 2001**). Yeasts and filamentous fungi too have been shown to withstand high ambient pH. Early studies by **THOM (1910)** and **JOHNSON (1923)** addressed the effect of pH on several moulds recovered from neutral soils. **THOM (1910)** isolated few penicillia moulds that grew in alkaline media, and he showed that hydroxyl ions have much more poisonous effect than hydrogen ions. This observation made a century ago, corroborates a common notion that fungi generally prefer slightly acidic pH for optimal growth. **JOHNSON (1923)** demonstrated that *Fusarium bullatum*, *F. oxysporum*, and *Penicillium variabile* and some other species were capable of growing at alkaline pH, but acidified the medium with time. **GOTO ET AL. (1981)** obtained a spontaneous mutant of alkalitolerant black yeast *Exophiala alcalophila* that grew well at pH 10.5. A number of alkalitolerant yeast strains were screened by **AONO (1990)** who showed that many of them were capable of growing at pH above 10. **LISICHKINA** with collaborators **(2003)** first obtained several alkalitolerant yeast species from saline soda soils, with the *Cryptococcus laurentii* being the most dominant across the soil samples, however none of the obtained isolates displayed obligate alkaliphilic phenotype. A number of filamentous fungi that have an alkalitolerant phenotype were screened for the production of potentially valuable enzymes (**KLADWANG ET AL. 2003, DUTTA ET AL. 2008**). Information on the alkaliphilic filamentous fungi though is very limited, yet such species do exist – several acremonia, *Aspergillus nidulans* strain KK-99, *Cephalosporium* sp., *Chrysosporium* sp., *Verticillium* sp., and few others (**NAGAI ET AL. 1995, 1998, KANG ET AL. 1996, TANEJA ET AL. 2002, ELÍADES ET AL. 2006**). A prominent example is the recovery of *Acremonium alcalophilum* from alkaline compost feces and its detailed morphological investigation (**OKADA ET AL. 1993**). The authors demonstrated its strong capacity for the production of alkaline cellulases. The genome of *Acremonium alcalophilum* was ultimately sequenced and assembled in **2011** at Joint Genome Institute (Walnut Creek, USA) by Adrian Tsang with collaborators. The detection of fungal mRNAs in saline soda lakes is intriguing, however these fungi were never brought to axenic cultures and investigated.

Physiology of alkaliphiles

Discovery of something unusual naturally renders questions about how a certain phenomenon came to be and how it is different if compared to normal. Already in the early pioneering works on the alkalitolerant bacteria, the authors suggested few scenarios on how this adaptation may arise (**KUSHNER & LISSON 1959**). To date, numerous studies have investigated the survival strategies that enable bacteria to thrive in extremely alkaline environments. Alkaliphily in bacteria is a complex trait and not determined by a single prerequisite, but rather is a combination of structural, metabolical, physiological and bioenergetical adaptations. It was shown that both neutrophilic and alkaliphilic *Bacillus* are capable of maintaining internal pH around neutral during optimal growth (**KRULWICH & GUFFANTI 1992, STURR ET AL. 1994**). Thus, perhaps the most obvious question is how alkaliphiles maintain their intracellular pH, given its large contrast between inner and outer milieu. However, quite strikingly, there is also a variation in tolerating cytoplasmic pH among bacteria. For example, an extreme alkaliphile *B. pseudofirmus* OF4 was still capable of growth at external pH of 11.4 while internal pH was shown to be 9.6 – a value that none of the neutralophiles can withstand, as their growth arrests at cytoplasmic pH of 8 (**KRULWICH ET AL. 2011B**). It was therefore hypothesized that extreme alkaliphiles have adaptations to higher-than-neutral cytoplasmic pH. Evidence supporting this hypothesis was obtained by studying the stability of cytoplasmic enzyme, phosphoserine aminotransferase in two alkaliphiles (**DUBNOVITSKY ET AL. 2005, KAPETANIOU ET AL. 2006**). Yet it is still unclear how alkaliphiles can cope with extremely high cytoplasmic pH (**KRULWICH ET AL. 2011B**). Generally speaking, for an alkaliphilic bacterium at its optimal alkaline ambient pH, cytoplasmic pH is always about 1.8–2.3 pH units lower than outside. It was shown that *B. pseudofirmus* OF4 with cytoplasmic pH of 7.5, had an optimal growth rate only when ambient pH was exceeding 7.5, typically 8.5–9.5 (**KRULWICH ET AL. 2011A**). Therefore, alkaliphiles should have efficient means to neutralize cytoplasmic pH under alkaline conditions.

The cell wall is the first barrier that ambient pH faces upon. In alkaliphilic bacteria, the presence of acidic polymers such as teichuronic and poly-γ-D-glutamic acids in the cell wall can reduce the pH by about a unit (**AONO ET AL. 1999, HORIKOSHI 2011B**). These acids are negatively charged and therefore promote retaining effect on protons (and sodium ions) at the cell wall surface, which decreases the pH value at the plasma membrane to some extent. **TSUJII (2002)** estimated that these acidic polymers acidify the inside of the cell wall by 1–1.5 pH units. Alkaliphilic *B. pseudofirmus* OF4 additionally has so-called acidic S-layer polymer that was also demonstrated to contribute to the alkaliphilic adaptation (**GILMOUR ET AL. 2000**), however, this polymer was not found in other alkaliphilies. Another peculiar passive adaptation was revealed after comparative genomics studies between alkaliphiles and neutrophiles – low isoelectric point (pI) of the proteins that are exposed to high ambient pH. Those include exo-cellular enzymes and cell-wall or plasma-membrane transporters. Exposed proteins with low pI values would have negative charge at high pH which acts as proton and sodium attractant and their surface retention (**JANTO ET AL. 2011**). Although passive mechanisms support the adaptation to high alkalinity, yet the central role for pH homeostasis in bacteria has been attributed to the electrogenic Na^+/H^+

antiporters that actively uptake protons from the external milieu in exchange for cytoplasmic sodium, in 2:1 ratio. These antiporters are energized by the transmembrane potential ($\Delta\Psi$) generated by the components of respiratory chain and other primary ion pumps (**ROTTENBERG 1979**); therefore generation of substantial $\Delta\Psi$ under alkaline conditions is an essential process that drive active pH homeostasis by antiporters (**KRULWICH ET AL. 2011B**). In neutrophilic bacteria, a major role for pH homeostasis is believed to be taken over by K^+/H^+ antiporters, while Na^+/H^+ antiporters seem to be essential specifically in alkaliphiles making sodium a crucial attribute for pH homeostasis (**PADAN ET AL. 2005, KRULWICH ET AL. 2011A**). Extraordinary high levels of Na^+/H^+ antiporter expression at high pH and its functioning is concomitant with intracellular sodium which re-enters the cells via Na^+/solute uptake symporters and Na^+ channels – either flagella-associated or voltage-gated (**KRULWICH ET AL. 1985, ITO ET AL. 2004A, 2004B**). In addition, electrochemical proton gradient generated by respiratory chain is utilized by the upregulated F_1F_o-ATPases in the synthetic direction contributing to alkaliphile pH homeostasis. Non-respiring alkaliphiles have their F_1F_o-ATPases work in hydrolytic direction that generates a $\Delta\Psi$, and utilize Na^+-coupled F_1F_o-ATPases to avoid proton loss, instead of H^+-coupled F_1F_o-ATPases (**FERGUSON ET AL. 2006**). Detected in anaerobic alkaliphiles, Na^+-coupled F_1F_o-ATPases have never been shown to function in the synthetic direction. From bioenergetics perspective, why alkaliphiles use only H^+-coupled F_1F_o-ATP synthases given the sub-optimal conditions of low proton-motive force at high ambient pH, rather than using Na^+-coupled F_1F_o-ATP synthases poses a big puzzle at the moment (**KRULWICH ET AL. 2011A**). Remodeled metabolic pathways are believed to contribute to the pH homeostasis supporting primary mechanisms highlighted above. Increased activity of deaminases and catabolic pathways that produce organic acids offer effective means of neutralizing cytoplasmic milieu (**BLANKENHORN ET AL. 1999, YOHANNES ET AL. 2004**).

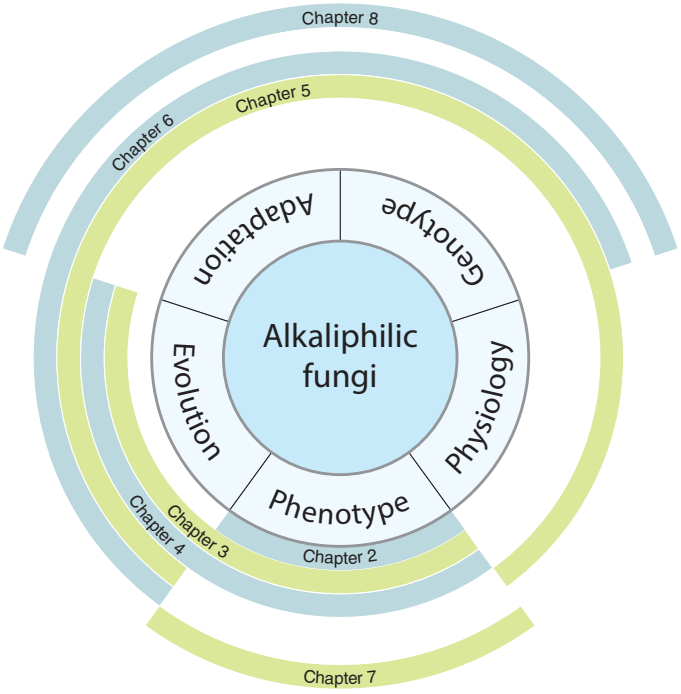
At the start of my PhD-project, no studies on the adaptations in alkaliphilic filamentous fungi were available. However, there is considerable literature on pH homeostasis strategies and how elevated ambient pH affects neutrophilic fungi. Similarly to bacteria, filamentous fungi also seem capable of maintaining intracellular pH around neutral at elevated ambient pH (**HESSE ET AL. 2002, BAGAR ET AL. 2009**). In fungi, P-type Na^+- and H^+-ATPases located on the plasma membrane are believed to take the primary role in sodium and pH homeostasis of the cells and generation of a $\Delta\Psi$ across the cytoplasmic membrane that in turn drives secondary transport systems (**HESSE ET AL. 2002**). The *ena1* gene encoding for a P-type Na^+-ATPase is a key player and the most studied efflux pump, which confers proper ion homeostasis and sodium tolerance (**BENITO ET AL. 2009**). In *Fusarium oxysporum*, *ena1* expression is up-regulated in response to a combination of high sodium and elevated pH conditions (**CARACUEL ET AL. 2003**). Intracellular pH is thought to be additionally tuned by the plasma membrane or endosomal electroneutral Na^+/H^+ antiporters (**BRETT ET AL. 2005**). Within the cell, mitochondrial F_1F_o-ATPases and vacuolar V-type ATPases also seem to contribute to the maintenance of a proper intracellular pH gradient (**HESSE ET AL. 2002**). The molecular response upon exposure to high ambient pH has been extensively studied over the last decades on the model fungus *Aspergillus nidulans*. Its ability to grow over a wide range of environmental pH values (from 2.5 to 9), makes it an appropriate organism to study the pH

effect on gene expression. *Aspergillus nidulans* excretes sets of enzymes and metabolites that function optimally at the ambient pH the fungus is growing in a given moment (**DENISON 2000**). Thus it must have a system that exhibits a pH-sensing function followed by a proper response. It was shown that the key role of the pH-signaling transduction cascade is attributed to the PacC protein – a multi-modular transcriptional factor that is activated under high ambient pH (**PEÑALVA ET AL. 2008**). The pH-'sensing' function in filamentous fungi is carried out by the seven-transmembrane domain PalH receptor along with its helper, three-transmembrane domain PalI. At high ambient pH, a rather complex sequence of events and intermediate players passes the downstream signal which activates PacC (**HERRANZ ET AL. 2005, GALINDO ET AL. 2012, MAEDA 2012**). The activation of the PacC transcription factor is achieved by double proteolytic cleavage yielding a short form of PacC (27 kDa in *A. nidulans*) that migrates to the nucleus and affects the expression of a large number of genes which confer alkalitolerance. The activated PacC binds to the promoters of 'alkaline-related' genes via a zinc-finger domain up-regulating their expression, and down-regulating the expression of 'acid-related' genes (**ESPESO & ARST 2000**). PacC therefore is a bi-functional switcher that regulates global expression profiles in response to ambient pH. Conversely, at acid pH, PacC remains unprocessed (i.e. inactive) and 'acid-related' genes are constitutively expressed. Remarkably, a recent study by **FRANCO-FRÍAS ET AL. (2014)** demonstrated that PacC regulates the expression of up to 20 % of the transcriptome of *Ustilago maydis*. A study by **TRUSHINA ET AL. (2013)** done on *Trichoderma virens* provides a more modest number of about 1 % of genes (that is still over a hundred of genes) that are affected by the PacC factor. It should be noted though that not all the pH-dependent genes are regulated by the PacC cascade. Given a global regulatory role of PacC of the pH response and its strong conservation among various physiological groups of filamentous fungi, it is intriguing to speculate on the details of this cascade in alkaliphilic fungi.

Outline of the thesis

Given a substantial body of research devoted to bacteria and archaea living in saline soda soils and lakes, I sought to find and study fungi – the biota component of saline soda lakes which was never addressed before. The presented thesis aimed to recover and study filamentous fungi from saline soda soils of several geographical locations (Armenia, Kazakhstan, Kenya, Mongolia, Russia, and Tanzania). First of all, I show that filamentous fungi are indeed present in soda soils. In **CHAPTER 2**, **CHAPTER 3** and **CHAPTER 4**, I will combine morphological examinations, growth tests and DNA-based information to thoroughly study each fungal isolate recovered from the soda soils samples. Using above mentioned approaches, I establish what fraction of the overall fungal diversity in soda soils include fungi with alkaliphilic phenotype and if there are certain morphological traits that are pertinent to this phenotype. In addition, DNA-based data are used to address the evolution of the alkaliphilic trait – whether this trait in fungi is monophyletic or not. Next, in **CHAPTER 5**, I chose a model alkaliphilic fungus to obtain insights on its role in soda soils by looking at the enzyme sets encoded in its genome and carrying out

the enzyme assays under different conditions. I also address how gene expression is regulated at alkaline conditions. In **CHAPTER 6**, I focus on the conserved pH-sensing PacC cascade and how it may function at extremely high ambient pH. The next two chapters provide insights on the oddities of the alkaliphilic fungus that were not anticipated when this PhD-project initiated. **CHAPTER 7** describes experiments on the mycoviruses we found in our model alkaliphilic fungus. **CHAPTER 8** accounts a horizontal gene transfer event from bacteria to the alkaliphilic fungus. Putative evolutionary impacts of these events are addressed as well. The discussion in **CHAPTER 9** contemplates on the results obtained throughout the thesis. The schematic outline of the research chapters of the thesis is portrayed below.



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Chapter II

Sodiomyces alkalinus, a new holomorphic alkaliphilic ascomycete within the *Plectosphaerellaceae*

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Abstract

In this study we reassess the taxonomic reference of the previously described holomorphic alkaliphilic fungus *Heleococcum alkalinum* isolated from soda soils in Russia, Mongolia and Tanzania. We show that it is not an actual member of the genus *Heleococcum* (order *Hypocreales*) as stated before and should, therefore, be excluded from it and renamed. Multilocus gene phylogeny analyses (based on nuclear ITS, 5.8S rDNA, 28S rDNA, 18S rDNA, RPB2 and TEF1-alpha) have displayed this fungus as a new taxon at the genus level within the family *Plectosphaerellaceae*, *Hypocreomycetidae*, *Ascomycota*. The reference species of actual *Heleococcum* members showed clear divergence from the strongly supported *Heleococcum alkalinum* position within the *Plectosphaerellaceae*, sister to the family *Glomerellaceae*. Eighteen strains isolated from soda lakes around the world show remarkable genetic similarity promoting speculations on their possible evolution in harsh alkaline environments. We established the pH growth optimum of this alkaliphilic fungus at c. pH 10 and tested growth on 30 carbon sources at pH 7 and 10. The new genus and species, *Sodiomyces alkalinus* gen. nov. comb. nov., is the second holomorphic fungus known within the family, the first one being *Plectosphaerella* – some members of this genus are known to be alkalitolerant. We propose the *Plectosphaerellaceae* family to be the source of alkaliphilic filamentous fungi as also the species known as *Acremonium alcalophilum* belongs to this group.

Key words

alkaliphilic fungi, growth, *Heleococcum alkalinum*, molecular phylogeny, scanning electron microscopy, taxonomy

Introduction

The fungal kingdom is highly diverse and versatile, with members able to grow under various environmental conditions. Although the majority of fungi are considered as neutrophiles, showing optimal growth in moderate habitats (e.g. 25–30 °C, pH 5–7), some of them have adapted to thrive in extreme environments where abiotic conditions are so harsh that most organisms cannot survive. One such stressful condition is high alkalinity, to which some fungi have become adapted. Alkaliphilic fungi, i.e. fungi that are capable of growing at high pH, above pH 9 (**HORIKOSHI 1999**), have been little studied. Only a handful of filamentous alkaliphilic fungi have been reported to date (**NAGAI ET AL. 1995, 1998**). The natural habitats for this kind of fungi are believed to be soda soils and soda lakes, as are often encountered in arid and semi-arid areas. These sites represent an unusual, naturally occurring stable ecotope where the overall biodiversity is compromised due to significant ambient stress, namely, high salt-osmotic pressure and high pH. Besides these natural sites, there are also sites created by human industrial processes like concrete and paper manufacture. These industries are known to create alkaline wastes that are potential habitats for alkaliphilic fungi (**MUELLER ET AL. 2004**). Already in

1923, **JOHNSON (1923)** showed the ability of *Fusarium oxysporum*, *F. bullatum* and *Penicillium variabile* to grow at the extremely high pH of 11. Probably the most notable study on alkaliphilic fungi was the isolation and description of mitosporic *Acremonium alcalophilum*, which has a growth optimum at around pH 9, by **OKADA ET AL. (1993)**. More recently, **KLADWANG ET AL. (2003)** isolated a number of alkalitolerant species in Thailand and **ELÍADES ET AL. (2006)** reported eight species of alkaliphilic and alkalitolerant soil fungi from Argentina, taxonomically distributed through *Bionectriaceae*, *Trichocomaceae*, *Sporormiaceae*, *Ceratostomataceae* and *Sordariaceae*. However, overall, information on the biodiversity of alkaliphilic filamentous fungi is scarce.

Fungi growing at extreme pH values are of scientific interest for the general study of fungal adaptive evolution as well as for the evaluation of their potential in producing commercially valuable substances. Obviously, the fungi adapting to alkalinity must have metabolic pathways that have become modified with respect to those seen in related neutrophilic fungi. For instance, enzymes that are being secreted into the ambient environment should work optimally in alkalinity in order to provide sufficient amounts of nutrients (**KLADWANG ET AL. 2003**). In addition, adaptations to alkaline environments are required for structures involved in exporting metabolites like toxins and antibiotics, for domains of the membrane transporters exposed to ambient environment and for regulation of gene expression by ambient pH. Alkaliphilic fungi are likely to possess unique properties that have not been well elucidated so far.

In **2005**, a new alkaliphilic holomorphic fungus from hyper-saline soda soils (pH around 10) was isolated, described and placed among members of the genus *Heleococcum* as *H. alkalinum*. The genus *Heleococcum* (order *Hypocreales*) seemed appropriate based on morphological and ecological features (**BILANENKO ET AL. 2005**). *Heleococcum* species are known to be soil saprobes, all producing acremonium-like anamorphs (except *H. aurantiacum*) along with bright-coloured cleistothecial ascomata (**JØRGENSEN 1922**, **TUBAKI 1967**, **UDAGAWA ET AL. 1995**).

Upon considering the ecological distinctiveness of *H. alkalinum* and also some differences in morphological features compared to other *Heleococcum* species, we decided to investigate the taxonomic position of this species with the help of molecular phylogeny. We used multiple molecular phylogenetic markers including ribosomal rDNA (18S, 28S, ITS and 5.8S) and proteincoding genes for the second largest subunit of RNA polymerase II (RPB2) and for transcriptional elongation factor 1-alpha (TEF1-alpha). Results demonstrated that this fungus does not belong in *Heleococcum* but instead belongs to a new genus within the family of the *Plectosphaerellaceae*. We confirm its alkaliphilic nature and speculate on its possible role in alkaline ecotopes, based on growth experiments carried out on media containing various carbon sources at different pH levels. We also discuss the significance of the low levels of genetic variation observed among strains isolated from soda lakes located thousands of kilometres apart.

MATERIALS AND METHODS

Strains and media

In this study we used 18 strains of *Heleococcum alkalinum* (**BILANENKO ET AL. 2005**) isolated from soils near soda lakes in Russia, Mongolia and Tanzania (**TABLE 1**). All strains were grown at 27 °C on alkaline agar medium (AA) containing per litre: 1) Na_2CO_3 – 24 g, $NaHCO_3$ – 6 g, NaCl – 5 g, KNO_3 – 1 g, K_2HPO_4 – 1 g; 2) malt extract (Merck) – 17 g, yeast extract (Difco) – 1 g, agar (Difco) – 20 g. Components 1 and 2 were autoclaved separately for 20 min at 120 °C and mixed together after cooling which resulted in a final pH of c. 10. Reference strains were members of other species placed in the genus *Heleococcum*, including *H. japonense* CBS 397.67 and *H. aurantiacum* CBS 201.35. In addition, we included some pertinent *Acremonium* cultures, *A. alcalophilum* CBS 114.92 and *A. antarcticum* CBS 987.87, that were obtained from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). The *Acremonium* and *Heleococcum* isolates were maintained on standard malt extract agar (MEA), oat meal agar (OA) (**MUELLER ET AL. 2004**) or our own AA medium at 27 °C.

TABLE 1. Strains of *Sodiomyces alkalinus* (former *Heleococcum alkalinum*) used in the study. Locations, characteristics of soil samples and isolation date are indicated.

Strain	CBS no.	Location	Place	Soil pH	Total salts (g/kg)	Depth (cm)	Saltification type	Date
F7	CBS 132729	Kunkur steppe, Chitinskaya area, Russia	Low-salt soda lake	-	-	0 to 5	Soda	1999
F8	CBS 133680	Kunkur steppe, Chitinskaya area, Russia	Low-salt soda lake	-	-	0 to 5	Soda	1999
F9	CBS 133681	Kunkur steppe, Chitinskaya area, Russia	Low-salt soda lake	-	-	0 to 5	Soda	1999
F10	CBS 132730	Tanzania	Natron lake	10 (water)	-	0 to 5	Soda	1999
F11	CBS 110278^T	Choibalsan area, North-East Mongolia	Shar-Burdiyn lake	10,7	49	0 to 5	Soda	1999
F12	CBS 132731	North-East Mongolia	Barun-Undziyn lake	10,5	82	0 to 5	Soda	1999
F13	CBS 132732	Kulunda steppe, Altai, Russia	Solyonoe lake	10	187	0 to 5	Chloride	Aug. 2002
F14	CBS 133682	Kulunda steppe, Altai, Russia	Karakul' lake	9,8	144	0 to 5	Soda	Aug. 2002
F15	CBS 133683	Kulunda steppe, Altai, Russia	Mirabilit lake	9,7	165	0 to 5	Soda-chloride-sulfate	Aug. 2002
F16	CBS 133684	Kulunda steppe, Altai, Russia	Petuhovskoe lake	10,2	163	0 to 5	Soda	Aug. 2002
F17	CBS 133685	Kulunda steppe, Altai, Russia	Bezimyannoe lake	9,9	310	0 to 5	Soda	Aug. 2002
F18	CBS 132733	Kulunda steppe, Altai, Russia	Tanatar lake	10,2	73	0 to 5	Soda	Aug. 2002
F19	CBS 133686	Kulunda steppe, Altai, Russia	Mirabilit lake	9,6	100	0 to 5	Soda-chloride-sulfate	Aug. 2002
F20	CBS 133687	North Gobi, Mongolia	Bayan-Dzag area	9,3	43	1 to 2	Sulfate-soda	Aug. 2003
F21	CBS 133688	North Gobi, Mongolia	Bayan-Dzag area	9,2	6	10 to 18	Sulfate-soda	Aug. 2003
F22	CBS 133689	Kulunda steppe, Altai, Russia	North	10	22	0 to 5	Soda	Aug. 2005
F23	CBS 133690	Kulunda steppe, Altai, Russia	Karagay lake	9,9	43	0 to 5	Soda	Aug. 2005
F24	CBS 133691	Kulunda steppe, Altai, Russia	Gor'koye lake	10,4	30	0 to 5	Soda	Aug. 2005

Growth at different pH

To elucidate the pH growth optimum of the strains we used malt extract-based medium buffered at pH levels ranging from 4 to 11.4. Acetic acid buffers were used to create pH 4 and 5.2, while phosphate buffers were used for pH 5.9, 7 and 7.8. Carbonate buffers were employed to set pH 8.7 and 9.8. Finally, a Na_2HPO_4/NaOH buffer system was used to generate pH 10.5 and 11.4. The final buffer concentration in all media was set to 0.1 M. The core nutrient component of media contained per litre: malt extract (Merck) – 17 g, yeast extract (Difco) – 1 g, agar (Difco) – 20 g. Buffers and nutrient components were autoclaved separately for 20 min at 120 °C and mixed afterwards, making up complete media. Strains were inoculated in so-called race tubes (**PERKINS & POLLARD 1986**). Four tubes were inoculated per strain in media at each of these pH

values: 4, 5.2, 5.9, 7, 7.8, 8.7, 9.8, 10.5 and 11.4. Linear growth was measured once or twice a week for c. 3 mo.

Growth on different carbon sources

To analyse the capacity of *H. alkalinum* to use diverse carbon sources at neutral and alkaline conditions, we employed 31 media buffered at pH 7 and 10 based on various carbon sources ranging in complexity (including no-C source as a control). The final buffer concentration (phosphate buffer for pH 7 and carbonate buffer for pH 10) was chosen to be 0.1 M. All media had final salts concentrations per litre of NaCl – 5 g, KNO_3 – 1 g, K_2HPO_4 – 1 g. The salt component was autoclaved separately. Simple soluble sugars, namely, D(+)-glucose, D(-)-fructose, D(+)-galactose, D(+)-mannose, D(+)-xylose, L(+)-arabinose, L-rhamnose, D(+)-glucuronic acid, D(+)-cellobiose, D(+)-maltose, D(+)-lactose, D(+)-raffinose, sucrose, were used at 25 mM final concentration. They were gently (15 min at 110 °C) autoclaved separately from salt and agar solutions and were subsequently added to the final media after cooling. A 1 % concentration was chosen for the following complex carbon sources: arabinogalactan, beechwood xylan, birchwood xylan, oat spelt xylan, guar gum, soluble starch, apple pectin, inulin, hydrolytic lignin, alpha-cellulose and chitin. A 3 % concentration was used for: sugar beet pulp, citrus pulp, soybean hulls, cotton seed hulls, alfalfa meal and corn gluten. All media were supplemented with solution of metal traces (**VAN DIEPENINGEN ET AL. 2008**). Complex substrates were autoclaved together with agar. Growth experiments on carbon sources were conducted at 27 °C in six replicates with initial inoculation of c. 1 400 spores per replicate. Growth patterns were recorded through 12 d.

Morphological studies with cryo-SEM and SEM

A piece (0.5 x 0.5 cm) of mycelium with agar was cut out from the plate directly and glued on a brass Leica sample holder with carbon paste (Leit-C, Neubauer Chemikalien, Germany). It was further frozen in liquid nitrogen and simultaneously fitted into the cryo-sample loading (VCT 100) system. The Leica sample holder was transferred to a non-dedicated cryo-preparation system (MED 020/VCT 100, Leica, Vienna, Austria) onto a sample stage at -93 °C. In this cryo-preparation chamber, the samples were freeze dried for 3 min at -93 °C at $1.3 \cdot 10^{-6}$ mbar to remove water vapour contamination from the surface. The sample was sputter-coated with a layer of 10 nm tungsten at the same temperature and transferred into the field emission scanning microscope (Magellan 400, FEI, Eindhoven, The Netherlands) on the sample stage at -122 °C at $4 \cdot 10^{-7}$ mbar. The analysis was performed with SE at 1 and 2 kV, 13 pA. All images were recorded digitally.

We also examined fungal samples by scanning electron microscopy after 1 h of 2.5 % glutaraldehyde fixation and series of 0.1 M phosphate buffer (pH 7.2) washing steps (20 min each) followed by dehydration through an ethanol series (30 %, 50 %, 70 % and 96 % concentration) and acetone before critical point drying in CO_2, carbon and metal coating. Specimens were observed under a JEOL (Japan) scanning electron microscope. Light microscopic studies were done with a Nikon Eclipse 80i. Taxonomic novelties were deposited in MycoBank (**WWW.MYCOBANK.ORG; CROUS ET AL. 2004**).

DNA extraction

All strains used in the study were grown in Petri dishes containing either AA or MEA depending on which yielded better growth performance for the specific strain. Plates were incubated at 27 °C for c. 5 d on a cellophane membrane. Total genomic DNA (free of RNA) was extracted from mycelium harvested from the cellophane surface with DNeasy Plant Mini kit (Qiagen Inc., Chatsworth, California, USA). DNA quality and concentration was verified with a NanoDrop 2000.

Polymerase chain reaction and sequencing

Amplification and sequencing were performed for five nuclear loci: ITS1–ITS4 region including 5.8S rDNA, LR0R–LR9 region of 28S rDNA, NS1–NS8 of 18S rDNA, 5–7 region of RPB2 and 983–2218 region of TEF1-alpha. The final volume of the PCR mix was 25 µL which contained 5x GoTaq Green buffer (Promega, USA), 400 µM dNTP, 0.4 µM of each primer, 0.02 U GoTaq polymerase (Promega, USA), 5–100 ng template genomic DNA, 1 mM $MgCl_2$. PCR conditions were as follows: for ITS 5 min at 94 °C; 33 cycles of 1 min at 94 °C, 1 min at 51 °C, 1 min at 72 °C; for 28S rDNA 5 min at 94 °C; 33 cycles of 1 min at 94 °C, 1 min at 49 °C, 2 min at 72 °C; for 18S rDNA 5 min at 94 °C; 33 cycles of 1 min at 94 °C, 1 min at 52 °C, 2 min at 72 °C; for 5–7 region of RPB2 gene 5 min at 94 °C; 9 cycles of 1 min at 94 °C, 1 min at 60 °C to 50 °C (with 1 degree decrement each cycle), 1.5 min at 72 °C followed by 32 cycles of 1 min at 94 °C, 1.5 min at 50 °C, 1.5 min at 72 °C; for TEF1-alpha gene 5 min at 94 °C; 9 cycles of 1 min at 94 °C, 1 min at 66 °C to 56 °C (with 1 degree decrement each cycle), 1.5 min at 72 °C followed by 32 cycles of 1 min at 94 °C, 1.5 min at 56 °C, 1.5 min at 72 °C. All reactions were kept for 7 min at 72 °C for final extension step. Standard fungal primers were used for PCR listed in the overview by **BINDER & HIBBETT (2003)** available at **HTTP://WWW.CLARKU.EDU/FACULTY/DHIBBETT/PROTOCOLS_FOLDER/PRIMERS/PRIMERS.PDF**. The amplification products were visualized on a 1 % agarose gel stained with ethidium bromide, purified using GeneElute PCR Clean-Up Kit (Sigma) according to the manufacturer instructions and subsequently sequenced by the chain termination method at Eurofins MWG Operon (Germany) service. LR7; NS4 and NS6 primers for 28S rDNA and 18S rDNA regions, respectively, were supplemented for sequencing in addition to original PCR primers. Raw sequence chromatograms were viewed and edited using CodonCodeAligner v. 3.7.1 (CodonCode Corporation, Dedham, MA, USA) and DNAStar Lasergene EditSeq v. 7.1.0 (DNASTAR Inc., Madison, WI, USA).

Phylogenetic analyses

Newly generated sequences were deposited in GenBank with accession numbers listed in **SUPPLEMENTARY TABLE 1**. Sequences of ITS1 & 2 (including 5.8S rDNA gene), 28S rDNA gene (LSU), 18S rDNA gene (SSU) and protein coding genes RPB2 and TEF1-alpha were used for phylogenetic analysis. Reference sequences of *Sordariomycetes* members needed for phylogenetic reconstruction were obtained from GenBank along with our new data.

TABLE 2. Loci and substitution models used in the phylogenetic analyses. Information on included base pairs is provided.

Phylogenetic analysis	Locus	Nucleotide substitution model	Characters	Phylogenetically informative characters	Uninformative variable characters	Invariable characters
1	18S rDNA	TIM+I+G (or GTR+I+G for MrBayes)	1652	328	185	1139
	28S rDNA	GTR+I+G	1381	421	158	801
	RPB2	GTR+I+G	954	571	43	340
	TEF1alpha	GTR+I+G	867	310	70	487
	5.8S rDNA	GTR+G	159	26	9	124
	TOTAL		**5013**	**1656 (33 %)**	**465 (9 %)**	**2891 (58 %)**
2	ITS with 5.8S rDNA	HKY+I+G	472	135	21	316
	28S rDNA	GTR+G	843	154	48	641
	TOTAL		**1315**	**289 (22 %)**	**69 (5 %)**	**957 (73 %)**

Separate datasets of each gene were constructed with a multiple sequence alignment online service MAFFT v. 6 (**KATOH & TOH 2008**) and were further reviewed and edited manually in BioEdit v. 7.1.3.0 (**HALL 1999**). Ambiguously aligned regions were removed from the data matrix. For better illustration of phylogenetic relations among taxa we generated two matrices with different datasets and resolution power: 1) we concatenated five gene alignments (SSU, LSU, RPB2, TEF1-alpha and 5.8S rDNA) using Mesquite 2.74 (**MADDISON & MADDISON 2010**) for a combined large scale taxa phylogenetic analysis showing subphyla and orders of *Sordariomycetes*. The matrix was subdivided into nine partitions (three for ribosomal genes and six for each codon position in two protein-coding genes). 2) For the second, low-taxon level phylogeny, we combined ITS regions including 5.8S rDNA and 28S rDNA on another taxa set for a detailed resolution of *Plectosphaerellaceae* family members within *Sordariomycetes* clade. Both analyses are deposited in TreeBase (**SUBMISSION ID 12948**).

Nucleotide substitution models for partitions were tested in the jModelTest v. 0.1.1 (**RANNALA & YANG 1996**, **POSADA & BUCKLEY 2004**, **POSADA 2008**) software package. The Akaike Information Criterion (AIC) implemented in jModelTest was used to select for best fit models after likelihood score calculations were done. Evolutionary models listed in **TABLE 2** were further used for ML and BI analyses.

Maximum likelihood (ML) and Bayesian Inference (BI) analyses were used for phylogeny estimation. ML evaluation was conducted in GARLI v. 2.0 (**ZWICKL 2006**) with random starting trees through partitions. The number of search runs was set to 5 for a large scale taxa matrix and 10 for low-taxon level taxon matrix. Bootstrap analyses were replicated 200 and 100 times, respectively. A 50 % majority-rule consensus tree was generated in the SumTrees v. 3.3.1 application in the DenroPy v. 3.11.0 package (**SUKUMARAN & HOLDER 2010**) running under Python 2.6 platform. Bayesian (BI) analysis was performed as implemented in MrBayes v. 3.1.2 (**HUELSENBECK & RONQUIST 2001**). The first partition in the large scale taxon matrix was fitted into GTR+I+G, since that was the parameter closest to best-fit TIM+I+G, which is not included in MrBayes. Metropolis-coupled Markov chain Monte Carlo (MCMCMC) searches were run for 5M generations sampling every 100th tree. Two independent analyses with four chains each (one cold and three heated) were run until stationary distribution was achieved. Convergence of the

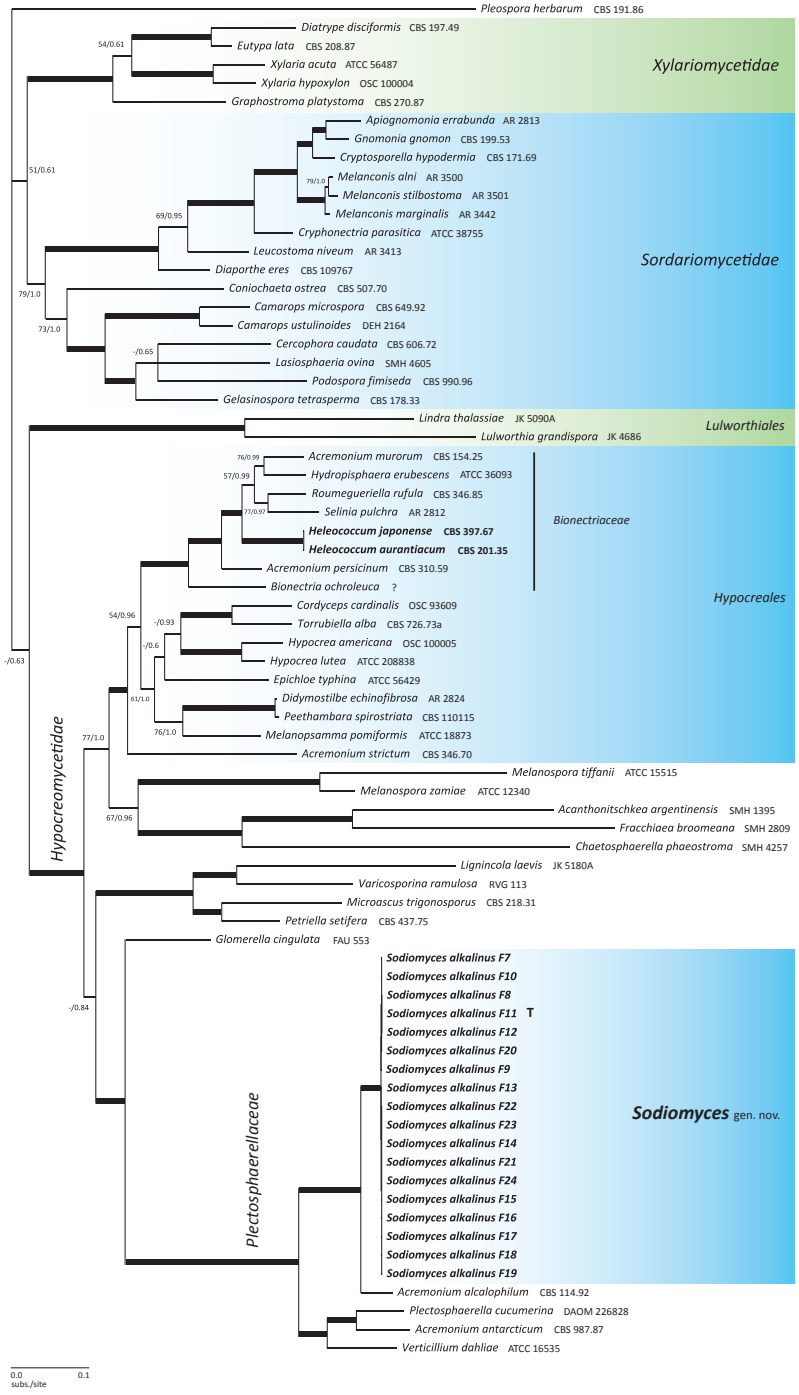

FIG. 1. Position of *Sodiomyces alkalinus* clade within *Sordariomycetes*. Bayesian 50 % majority-rule consensus tree based on five-gene data matrix (SSU+LSU+RPB2+TEF1-alpha+5.8S rDNA). Actual members of *Heleococcum* genus marked bold within *Bionectriaceae* family. Thickened branches indicate ML > 90 and BI posterior probability (PP) > 0.95.

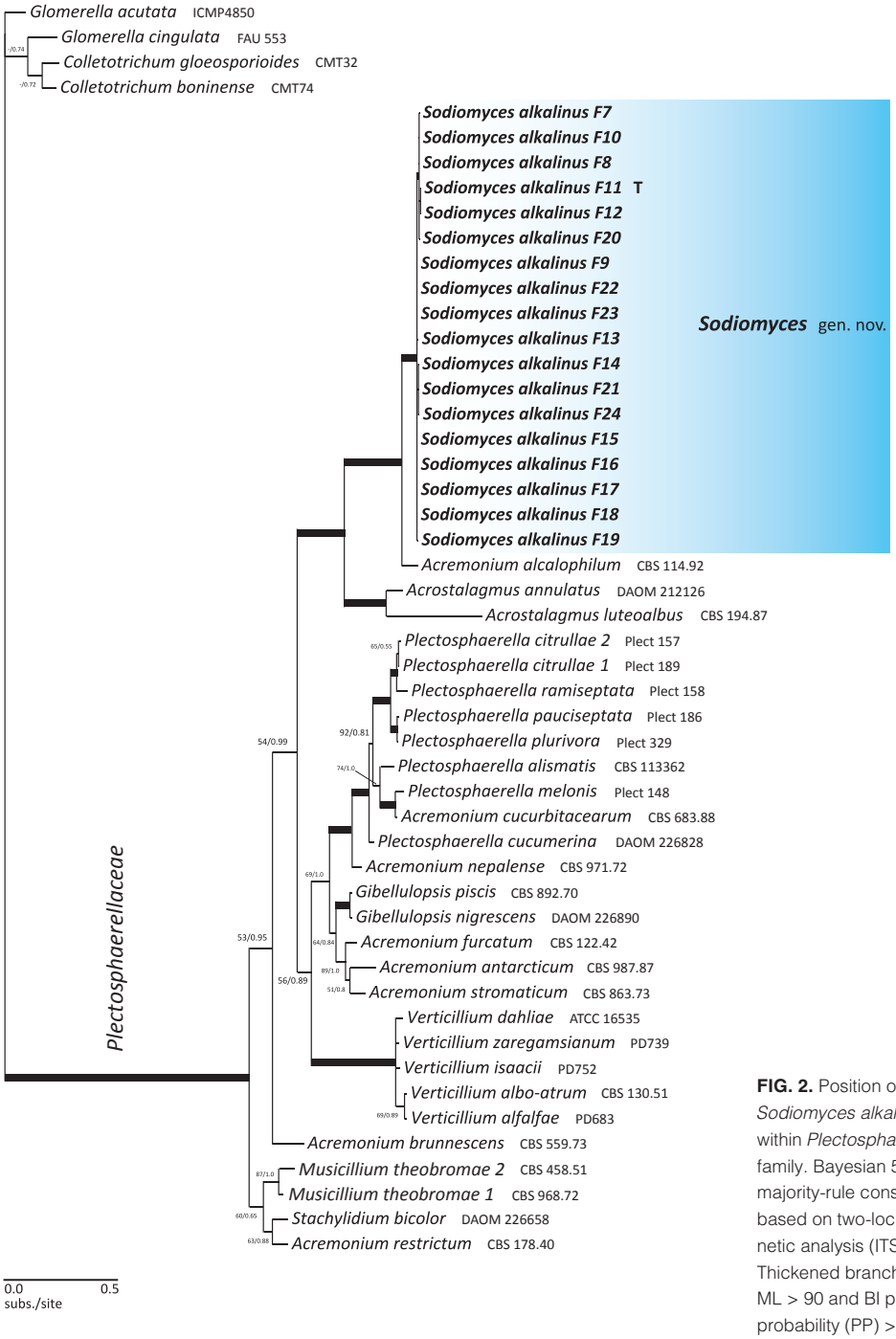

FIG. 2. Position of *Sodiomyces alkalinus* strains within *Plectosphaerellaceae* family. Bayesian 50 % majority-rule consensus tree based on two-locus phylogenetic analysis (ITS+LSU). Thickened branches indicate ML > 90 and BI posterior probability (PP) > 0.95.

run logs was analysed in TRACER v. 1.5 (**HTTP://BEAST.BIO.ED.AC.UK/TRACER**). The first 15 000 (30 %) 'burn-in' trees were excluded from further analysis. The rest was summarized to produce a 50 % majority-rule consensus tree with recovery of posterior probabilities (PP).

Results

Isolation of strains and genetic identity

Eighteen strains were isolated from soda-soil samples collected at different sites in Russia, Mongolia and Tanzania. They have been deposited in the CBS collection (Utrecht, The Netherlands) (**TABLE 1**). All of them readily produce abundant ascomata and show intense conidiation on an alkaline agar medium of pH 10 and all share the same morphological characteristics. In addition, all strains have nearly identical sequences for the five loci studied: SSU, LSU, RPB2, TEF1-alpha and 5.8S rDNA. Even the ITS regions and third codon positions of protein-coding genes (RPB2 and TEF1-alpha), known to have relatively high rate of mutation accumulation, have minor or no nucleotide differences. We found nucleotide substitution mutations in 18 sites across a 6189-base alignment derived from the studied loci. Most of the substitutions were at 3rd codon positions in the RPB2 gene.

Phylogenetic analyses

In the first analysis, the dataset for the five-gene phylogeny consisted of 70 taxa within the *Sordariomycetes*. The data matrix comprised 5 013 characters, of which 1 656 (33 %) were phylogenetically informative, 2 891 invariable and 465 noninformatively variable. The negative log likelihoods (-Ln) for ML BP and BI were 54607.294 and 55042.994. MCMCMC runs converged and had a deviation of around 0.008 at the end of the run. Maximum likelihood bootstrap and posterior probabilities (PP) are provided on the corresponding internodes on the 50 % majority rule Bayesian tree shown (**FIG. 1**). In both our phylogenetic analyses, clades supported with ML BP/BI PP exceeding 90/0.95 were considered very strong and are displayed as thickened braches. Our five-locus phylogeny provides firm topological support for three major monophyletic subphylum-level clades of *Sordariomycetes* outlined previously (**ERIKSSON 2006, ZHANG ET AL. 2006**), namely, *Xylariomycetidae* (100/1.0), *Sordariomycetidae* (79/1.0) and *Hypocreomycetidae* (97/1.0). The marine fungi order *Lulworthiales* displayed a strong supported clade (100/1.0) with no clear assessment to any of the other subphyla within the *Sordariomycetes*. The taxonomic position of the *Lulworthiales* has been a matter of discussion since the description of the order, and it currently has incertae sedis status (**KOHLMEYER ET AL. 2000, HIBBETT ET AL. 2007**). The monophyletic order *Hypocreales* within the *Hypocreomycetidae* forms a well-supported clade (92/1.0) in which the genus *Heleococcum* is located. We used a representative isolate of the type species of *Heleococcum*, *H. aurantiacum* (CBS 201.35) as well as an isolate of *H. japonense* (CBS 397.67) as references to compare with the taxonomic position of *H. alkalinum*. *Heleococcum aurantiacum* and *H. japonense* stand close together as members of the family *Bionectriaceae* within the *Hypocreales* as stated

previously (**ROSSMAN ET AL. 2001**). Surprisingly, however, all *H. alkalinum* strains clustered as a distinct clade within the highly supported (100/1.0) broader monophyletic clade comprising the family *Plectosphaerellaceae*. A novel genus name was therefore needed. We propose *Sodiomyces alkalinus* gen. & comb. nov. as the new name for this fungus. This name will be used in the remainder of this paper.

Our second, two-locus (ITS including 5.8S rDNA and 28S rDNA) phylogenetic analysis was done to further resolve the *Plectosphaerellaceae* clade and place *Sodiomyces alkalinus* strains among the other members of the family (**FIG. 2**). The data matrix consisted of 50 taxa and 1 315 characters: 289 (22 %) phylogenetically informative, 957 invariable and 69 variable non-informative. Four members of the sister family *Glomerellaceae* were used as outgroup. ML bootstrap and BI analyses had likelihoods (-Ln) of 5501.97 and 5721.683, respectively. The deviation between the Markov chain runs was around 0.004 at the end. Members of the holomorphic genus *Plectosphaerella* make up a highly supported clade (92/1.0) as does the asexual plant pathogen *Verticillium* s. str. clade (97/1.0). *Verticillium theobromae* appears closer to the root of the tree, lending some doubt to the monophyly of *Verticillium*. It has been renamed *Musicillium theobromae* by **ZARE ET AL. (2007)**. The two species of the genus *Acrostalagmus* form a long branched clade with strong 99/1.0 support values. Anamorphic *Acremonium* species known to be highly polyphyletic, occupy various separate branches in our phylogenetic tree. The overall topology is highly consistent with that seen in previous phylogenetic studies of the *Plectosphaerellaceae* (**ZARE ET AL. 2007, WEISENBORN ET AL. 2010, REBLOVA ET AL. 2011, CARLUCCI ET AL. 2012**). Here again, our *Sodiomyces alkalinus* strains group together and show a highly supported (100/1.0) clade close to *A. alcalophilum*.

Growth patterns on different substrates

Sodiomyces alkalinus is an alkaliphilic fungus clearly showing a characteristic growth pattern at different pH levels on MEA-based media (**FIG. 3**). The maximum growth rate of ex-type strain F11, 2.6 mm/d, was recorded at pH from 8.7 to 10.5, with only a small reduction in growth rate seen at pH 11.4. On the other side of the pH scale, the fungus was still able to grow at pH 6 with an almost halved growth rate (1.4 mm/d). At pH 5.2 and lower, no growth was observed. The growth rates of strain F11 on 30 carbon sources at pH 7 and 10 varied (**FIG. 4, 5**). The graphs display, from left to right, carbon sources ranging from simple to complex. Control plates with no carbon source still showed faint growth, presumably, due to the traces of nutrients in the agar. As can be seen in **FIG. 5**, simple sugars do not provide sufficient nutrients for good growth, resulting in poorly formed colonies with thin sterile hyphae. Some substrates such as D(+)-galactose inhibited growth significantly and resulted in an irregular colony shape. More complex substrates like rhamnose, maltose, cellobiose, raffinose, arabinogalactan, beechwood xylan, and oat-spelt xylan provided the nutrients for a richer morphology and facilitated initiation of conidiation. All complex media made with raw plant materials as carbon sources yielded rich colony morphology with developed aerial mycelium, abundant conidia and formation of ascomata.

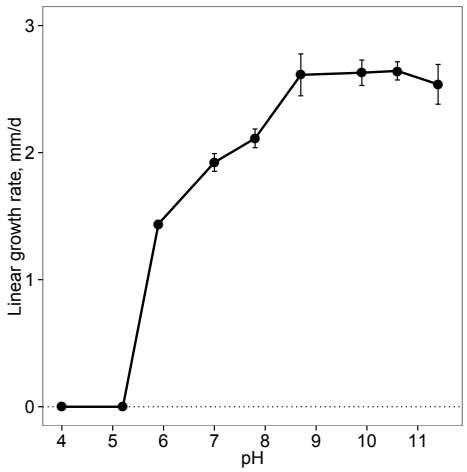

FIG. 3. Growth pattern of *Sodiomyces alkalinus* (CBS 110278^{T}) at different pH.

Apple pectin did not promote rich colony formation, but rather, initiated the formation of ascomata towards the end of the incubation period. The fungus did not produce discernible colonies on pure cellulose or chitin. In all cases, growth on media at pH 10 was better than at pH 7, again showing the adaptation of *Sodiomyces alkalinus* to alkaline environments. The species could not initiate growth on lignin and sugar beet pulp-based media at pH 7, but showed good growth at pH 10 on those substrates. On lignin at pH 10, the fungus showed morphology similar to that seen on polysaccharides, developing only asexual sporulation.

Taxonomy

Sodiomyces A.A. Grum-Grzhim., A.J.M. Debets & Bilanenko, *gen. nov.* — MycoBank **MB801368**

Etymology — From the English soda and Latin mycetes, referring to the ability of filamentous fungus grow at high ambient pH and salts.

Type species — *Sodiomyces alkalinus* (Bilanenko & M. Ivanova) A.A. Grum-Grzhim., A.J.M. Debets & Bilanenko.

A genus of the family *Plectosphaerellaceae* in *Ascomycota*. *Asexual morph*. Acremonium-like. *Sexual morph*. *Cleistothecia* dark-brown, 120–150 µm diam, peridium multi-layered, pseudoparenchymatous, with folded surface, exoperidium composed of 3–5 layers of angular cells. *Paraphyses* absent. *Asci* thin-walled, without apical apparatus, saccate, unitunicate, scattered irregularly in the ascocarp, embedded in a gelatinous matrix. *Ascospores* released by dissolution of the ascus wall before maturity, accumulating within the ascocarp, released in a slimy mass, liberated by pressure within the ascocarp. *Ascospores* ellipsoidal or ovoid, 12–15 x 5–7 µm, medially 1-septate, not constricted at the septum, thick-walled, pale brown, smooth.

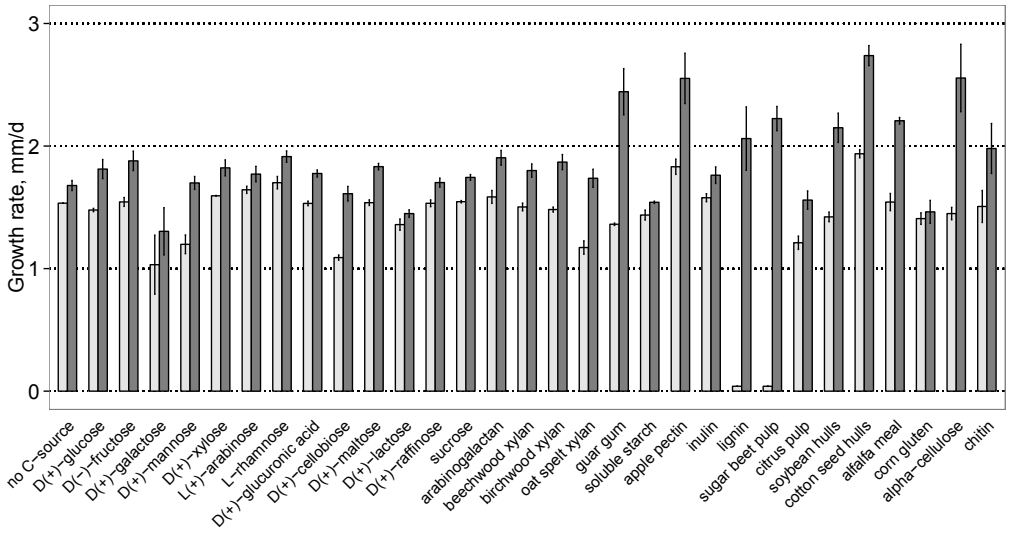

FIG. 4. Growth rate on different carbon sources of *Sodiomyces alkalinus* (CBS 110278^T) at different pH.

SODIOMYCES ALKALINUS (Bilanenko & M. Ivanova) A.A. Grum-Grzhim., A.J.M. Debets & Bilanenko, *comb. nov.* — MycoBank **MB801369**; **FIG. 6**

Basionym — *Heleococcum alkalinum* Bilanenko & M. Ivanova, **MYCOTAXON 91, 501. 2005.**

Ascomata dark brown, superficial on the substratum, globose, 120–250 µm diam, non-ostiolate, cleistothecial, not changing colour in 3 % KOH and lactic acid. *Peridium* multi-layered, pseudoparenchymatous, with folded surface; exoperidium composed of 3–5 layers of angular cells. *Paraphyses* absent. *Asci* thin-walled, without apical apparatus, saccate, unitunicate, scattered irregularly in the ascocarp, embedded in a gelatinous matrix. *Ascospores* released by dissolution of the ascus wall before maturity; ascospores accumulating within the ascocarp, released in a slimy mass, liberated by pressure within the ascocarp, ellipsoidal or ovoid, 12–15 x 5–7 µm, medially 1-septate, not constricted at the septum, thick-walled, pale brown, smooth. *Asexual morph* in *Acremonium* sect. *Nectrioidea*. *Conidiation* abundant, mostly nematogenous, partially plectonematogenous. *Conidiophores* predominantly basitonously verticillate, rarely with solitary branches. *Phialides* variable, 15–60 µm long, gradually tapering towards the apex, rather thin-walled. *Conidia* aseptate, aggregated in spherical slimy masses, rarely in short columns, lemon-shaped at first, becoming subglobose or ellipsoidal at maturity, 4.5–5.5 x 4.0–4.5 µm, smooth as observed by SEM, hyaline. *Chlamydospores* absent.

Culture characteristics — Colonies on alkaline agar (AA, pH 10–10.2) rather fast-growing, reaching 38–40 mm diam in 10 d at 25 °C. On MEA (pH 6.5) growing more slowly, reaching 5.5 mm diam in 10 d. Young colonies white; later, black concentric zones appearing as a result of formation of abundant ascomata, velvety to woolly. Reverse colourless. Odour pleasant.

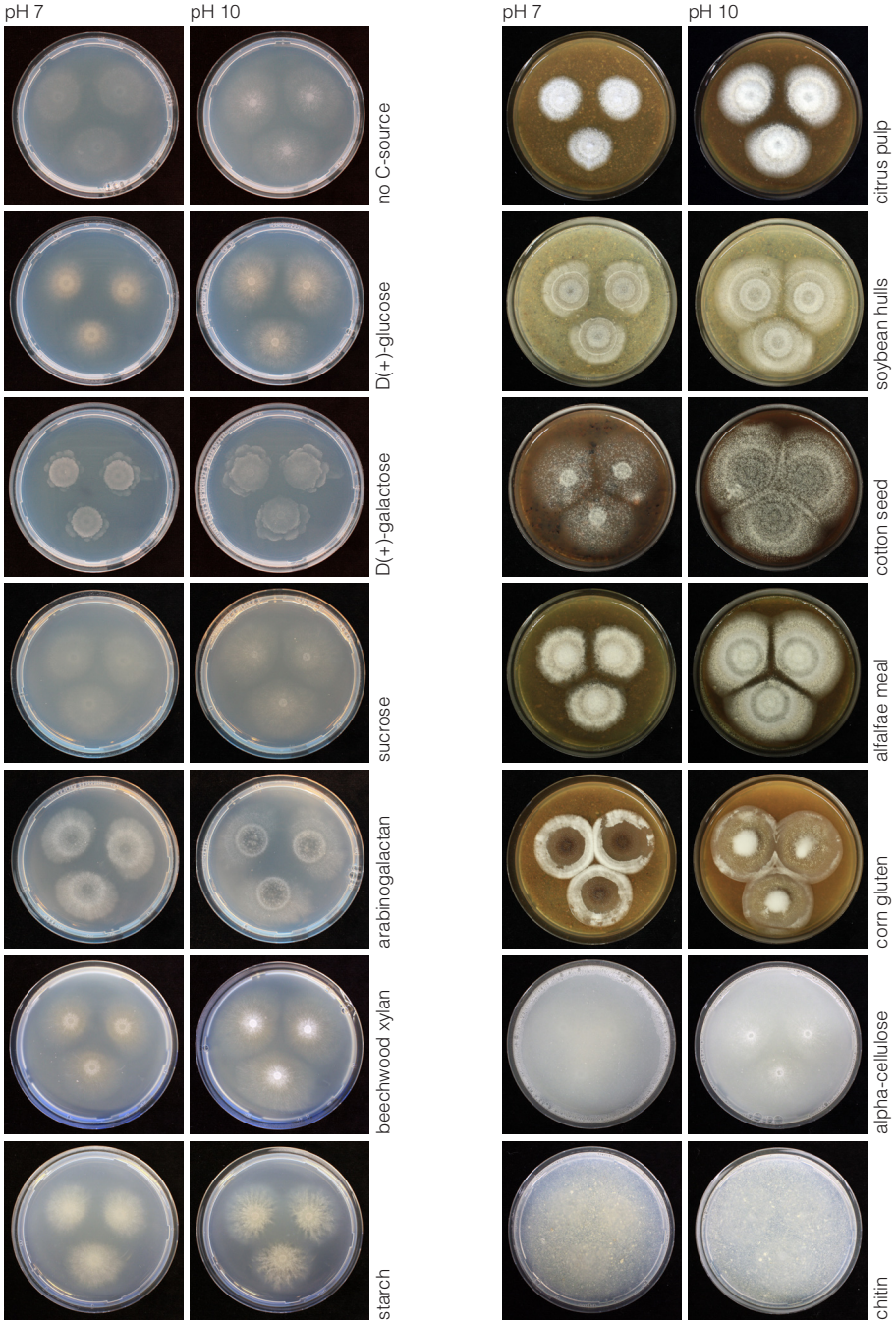

FIG. 5. Plates with *Sodiomyces alkalinus* (CBS 110278^{T}) 12 d old colonies on different carbon sources at pH 7 and 10.

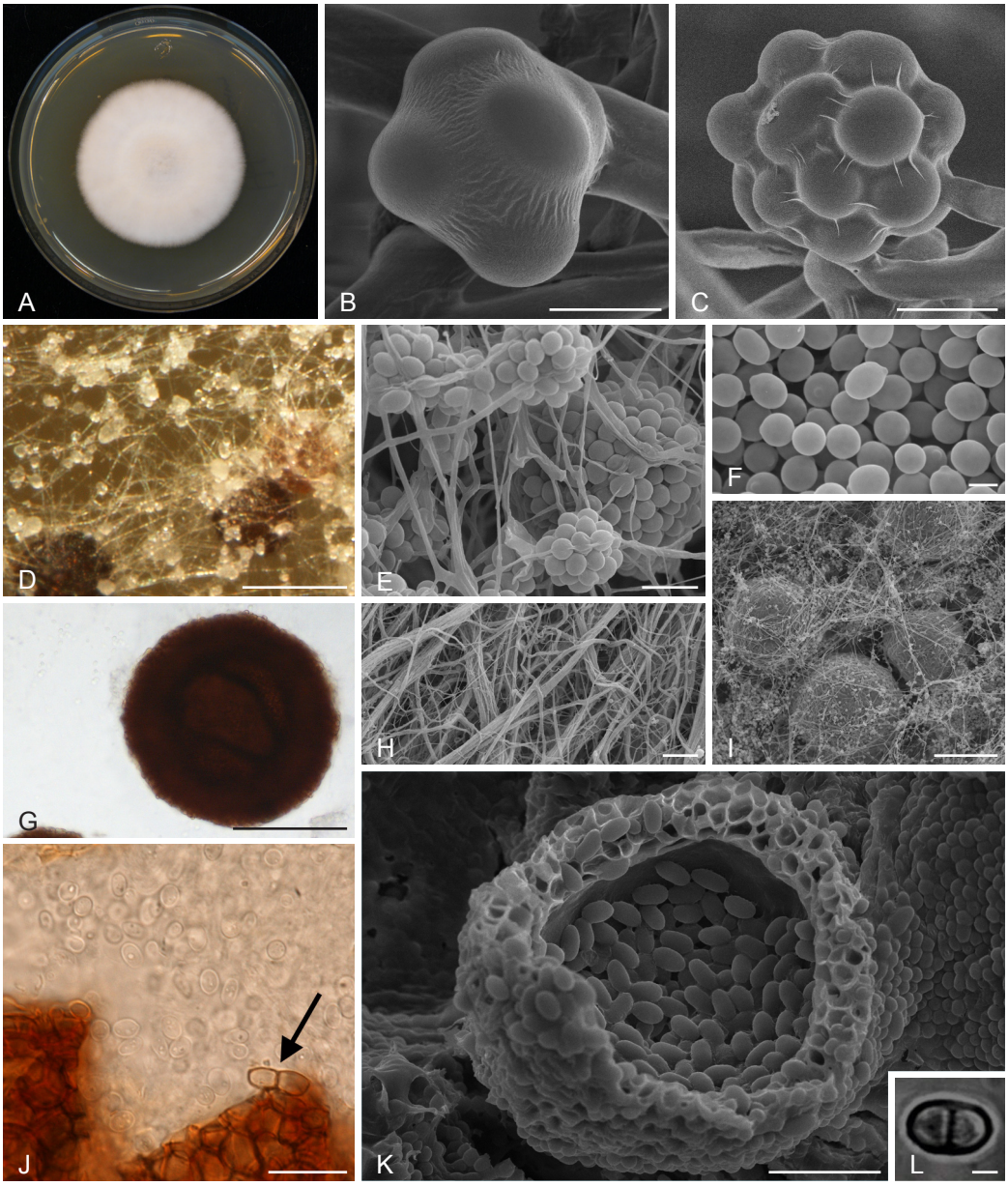

FIG. 6. *Sodiomyces alkalinus* (CBS 110278^{T}). **A.** Ten-day-old colony on alkaline agar (9 cm Petri dish); **B.** Young conidial head on monophialide (cryoSEM); **C.** Matured conidial head on (branched) monophialide (cryoSEM); **D.** Colony overview (LM); **E.** Acremonium-like conidial heads (SEM); **F.** Conidia (SEM); **G.** Cleistothecium (LM); **H.** Hyphal cords (SEM); **I.** Colony overview on alkaline agar (SEM); **J.** Open cleistothecium, surrounded by conidia, at the arrowhead a two-celled ascospore (LM); **K.** Multilayered exoperidium of ascoma (SEM); **L.** Two-celled ascospore (LM). *Scale bars*: **B** = 4 µm; **C** = 5 µm; **D, I** = 100 µm; **E** = 10 µm; **F, L** = 3 µm; **G** = 50 µm; **H, K** = 30 µm; **J** = 15 µm.

Exudate absent. Decumbent vegetative hyphae thinwalled, hyaline, 0.5–2.0 µm wide. Mycelium consisting of hyaline, smooth-walled, septate hyphae, 1–3 µm wide, often fasciculate.

Specimen examined — **MONGOLIA**, Choibalsan area, the soda soil (pH 10.7) on the edge of Shar-Burdiyn lake, 1999, *D. Sorokin*, culture ex-type F11 = CBS 110278 = VKM F-3762.

Notes — Previously described as *Heleococcum alkalinum* (*Bionectriaceae*, *Hypocreales*). Based on the results of ITS, LSU, SSU, 5.8S rDNA, RPB2, TEF1-alpha analyses, it was, however, shown to be a new genus and species in the *Plectosphaerellaceae*, with maximal support in ML and BI analyses.

Discussion

General knowledge of alkaliphilic filamentous fungi is extremely poor. Here we contribute an assessment of the taxonomic position of all 18 known isolates of *Sodiomyces alkalinus* (formerly *Heleococcum alkalinum*), a new placement for a species of holomorphic filamentous *Ascomycota* from soda soils. All isolates of *Sodiomyces alkalinus* were found, with maximal support, to belong to a new genus within the *Plectosphaerellaceae* (**ZARE ET AL. 2007**). Although the description of this family includes fungi with perithecial ascomata only, we are obliged to place the cleistothecial *Sodiomyces alkalinus* in this family. More new taxa are needed to clarify the taxonomic situation of the *Plectosphaerellaceae*.

The multi-locus phylogenies placed *Sodiomyces alkalinus* close to another alkaliphilic ascomycete – *Acremonium alcalophilum*, described by **OKADA ET AL.** in **1993**. Despite its genetic proximity, *A. alcalophilum* has significant morphological differences, leading us to decide not to transfer it into *Sodiomyces*. These differences are the following: *Acremonium alcalophilum* shows pleomorphism in conidium ontogeny among phialidic, sympodial, arthric, blastic and retrogressive modes (cladobotryum-, trichothecium- and basipetospora-like), especially on alkaline media, whereas *S. alkalinus* only has phialidic conidiogenesis. The phialoconidia and phialides of *A. alcalophilum* are widely variable in morphology, while *S. alkalinus* has uniform conidial morphology and consistently shaped phialides ranging from single to branched. *Sodiomyces alkalinus* is holomorphic unlike *A. alcalophilum*, which is only known as a mitosporic fungus. The pH growth optimum of *A. alcalophilum* is 9.0–9.2 and in *Sodiomyces* it is 9.5–10.5.

NAGAI ET AL. (1995, 1998) proposed that hypocrealean hyphomycetes show a particular tendency to develop the capacity for alkaliphilic growth. In addition, he showed that some species that we now know belong to the *Plectosphaerellaceae*, including *Stachylidium bicolor*, *Acremonium furcatum*, unidentified *Acremonium* species and some *Verticillium* species, displayed alkaliphilic abilities. *Acremonium alcalophilum* belongs to the same clade. Another study cited a *Plectosporium* species (*Plectosphaerellaceae*), isolated from mantis shrimp in seawater, that grew abundantly at pH 10 (**DUC ET AL. 2009**). Our phylogenetic data confirm that the family *Plectosphaerellaceae* constitutes an important reservoir of alkaliphilic filamentous fungi.

All 18 strains of *Sodiomyces alkalinus* display remarkable genetic similarity at the studied loci, even though the sites of isolation lay thousands of kilometres apart. This consistency might reflect the possible evolutionary constraints occurring in harsh natural environments such as

soda soils. Several relevant scenarios for the distribution of genetically similar fungi among these sites may be proposed. Firstly, the origin and worldwide dissemination of the organism might be an evolutionarily recent event. Dispersal by airborne conidia or migrant birds could be responsible. A second reason could be strong selection pressure in alkaline habitats leaving little opportunity for inhabitants to develop evolutionary variation. In other words, alkaliphiles could be evolutionary constrained by their adaption to those particular ambient conditions. The fact that non-functional or highly variable regions (ITS1, ITS2 regions and 3rd codon positions) have not accumulated mutations suggests the first scenario is more likely.

Sodiomyces alkalinus shows abundant growth and good morphological development on alkaline agar medium at pH 10; it also grows most rapidly under the same conditions. A growth pattern like the one shown in **FIG. 3** is rare among filamentous fungi. Known alkaliphilic filamentous fungi often develop only the anamorphic stage in their life cycles. *Sodiomyces alkalinus* is capable of developing teleomorphic as well as anamorphic states. This ability might be adaptive to harsh alkaline environment and may be linked to particular morphological features. As can be seen in **FIG. 6 (B, C)**, for example, conidial heads are embedded into a mucous matrix. It seems to have been altered somewhat by cryo-SEM preparation and it appears as a membranous sheath rather than as a slimy matrix. Phialidic conidiogenesis offers no mechanism through which an extra membrane can form that envelops the entire conidial head, making the presence of a mucous non-cellular substance the most likely interpretation of the structure seen. A slimy matrix might prevent conidia from suffering excessive evaporation; this would enhance their viability in the dry, saline conditions often encountered in alkaline soil areas. However, some neutrophilic fungi such as *Acremonium macroclavatum* (**WATANABE ET AL. 2001**), *Stachybotrys chartarum* and others (**SCHROERS ET AL. 2005**) are known to produce similar mucous substances in conidial heads. In *S. alkalinus*, ascospores also become embedded in a slimy matrix during early lysis of the ascus wall, a process that is complete by the time the fruiting body is mature. In addition, cleistothecia possess several layers of cells (**FIG. 6, K**) in the wall that may provide protection against pH stress.

Our growth experiments on different carbon sources might offer a clue as to the possible ecological role of *Sodiomyces alkalinus* in alkaline soils. On purified sugars only thin faint mycelium was produced even with trace metals present in the media. These results indicate that the fungus is not prototrophic and requires growth supplements for optimal growth. Fungal growth was somewhat better on di- and tri-saccharides than on mono-saccharides, and the former compounds stimulated relatively vigorous mycelial growth as well as the formation of small numbers of conidial heads. The inability to form perceptible colonies on cellulose and chitin at both pH values is unexpected, as these polymers seem to be the abundantly available substrates in soda soils and lakes. Small crustaceans with chitinous shells, such as *Artemia salina*, often are abundant in these habitats (**BROWNE & MACDONALD 1982**). The intense sporulation and well-developed morphology seen on media containing complex plant materials suggests that *S. alkalinus* is saprobic on decaying plant material in alkaline soil ecosystems. Vegetation in study sites mainly consists of halophyte grasses, *Anabasis salsa*, *Atriplex verrucifera*, *Halocnemum strobilaceum*, *Salicornia europaea*, *Suaeda acuminata*, *S. corniculata*, *S. prostrata* and *S. salsa*.

A further systematic study of fungal biodiversity in alkaline soils would help revealing the ecology and possible evolutionary trends pertinent to the alkaliphilic trait. Elucidating how alkaliphilic fungi respond to external pH and why specifically high ambient pH is needed for optimal growth might help in unravelling the adaptation hallmarks allowing access to alkaline habitats. Such studies will surely add to the general picture of signal transduction pathway mediated by ambient pH in fungi.

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SUPPLEMENTARY TABLE 1. Taxa used in the phylogenetic analyses with their GenBank accession numbers. Newly generated accessions marked in bold.

Species	Voucher	Appearance in tree*	SSU	LSU	RPB2	TEF1-alpha	ITS
Acanthonitschkea argentinensis	SMH 1395	1	_	AY695259	FJ968943	FJ969042	_
Acremonium alcalophilum	CBS 114.92	1, 2	**JX158486**	**JX158443**	**JX158465**	**JX158399**	**JX158421**
Acremonium antarcticum	CBS 987.87	1, 2	**JX158487**	**JX158444**	**JX158466**	**JX158400**	**JX158422**
Acremonium brunnescens	CBS 559.73	2		HQ231966			_
Acremonium cucurbitacearum	CBS 683.88	2		HQ231968			_
Acremonium furcatum	CBS 122.42	2		EF543831			AY378154
Acremonium murorum	CBS 154.25	1	FJ176824	FJ176880	FJ238363	_	_
Acremonium nepalense	CBS 971.72	2		HQ231970			DQ825971

Acremonium persicinum	CBS 310.59	1	FJ176822	FJ176878	FJ238361	_	_
Acremonium restrictum	CBS 178.40	2		HQ232119			_
Acremonium strictum	CBS 346.70	1	FJ176823	FJ176879	FJ238362	_	GQ376096
Acremonium stromaticum	CBS 863.73	2		HQ232143			DQ825969
Acrostalagmus annulatus	DAOM 212126	2		GU180646			GU180632
Acrostalagmus luteoalbus	CBS 194.87	2		EF543826			_
Apiognomonia errabunda	AR 2813	1	DQ862045	AF408334	DQ862014	DQ862030	DQ313525
Bionectria ochroleuca	?	1	DQ862044	DQ862027	DQ862013	DQ862029	_
Camarops microspora	CBS 649.92	1	DQ471036	AY083821	DQ470937	DQ471108	_
Camarops ustulinoides	DEH 2164	1	DQ470989	DQ470941	DQ470882	DQ471050	_
Cercophora caudata	CBS 606.72	1	DQ368659	AY999113	DQ368646	_	AY999135
Chaetosphaerella phaeostroma	SMH 4257	1	_	AY695264	FJ968940	FJ969004	_
Colletotrichum boninense	CMT74	2		JQ754138			_
Colletotrichum gloeosporioides	CMT32	2		JQ754114			_
Coniochaeta ostrea	CBS 507.70	1	DQ471007	DQ470959	DQ470909	DQ471078	_
Cordyceps cardinalis	OSC 93609	1	AY184973	AY184962	DQ522422	DQ522325	_
Cryphonectria parasitica	ATCC 38755	1	DQ862048	EU199123	DQ862017	DQ862033	AY141856
Cryptosporella hypodermia	CBS 171.69	1	DQ862049	DQ862028	DQ862018	DQ862034	EU199225
Diaporthe eres	CBS 109767	1	DQ471015	_	DQ470919	DQ479931	DQ491514
Diatrype disciformis	CBS 197.49	1	DQ471012	DQ470964	DQ470915	DQ471085	_
Didymostilbe echinofibrosa	AR 2824	1	AY489674	AY489706	_	AY489601	DQ135999
Epichloe typhina	ATCC 56429	1	U32405	_	DQ522440	AF543777	JN049832
Eutypa lata	CBS 208.87	1	DQ836896	DQ836903	DQ836889	DQ836909	DQ006927
Fracchiaea broomeana	SMH 2809	1	_	AY695268	FJ968942	FJ969039	_
Gelasinospora tetrasperma	CBS 178.33	1	DQ471032	DQ470980	DQ470932	DQ471103	AY681178
Gibellulopsis nigrescens	DAOM 226890	2		GU180648			GU180631
Gibellulopsis piscis	CBS 892.70	2		EF543835			DQ825985
Glomerella acutata	ICMP4850	2		JN939938			_
Glomerella cingulata	FAU 553	1, 2	AF543762	AF543786	_	AF543773	_
Gnomonia gnomon	CBS 199.53	1	DQ471019	AF408361	DQ470922	DQ471094	DQ491518
Graphostroma platystoma	CBS 270.87	1	DQ836900	DQ836906	DQ836893	DQ836915	_
Heleococcum aurantiacum	CBS 201.35	1	_	**JX158442**	**JX158464**	**JX158397**	**JX158419**
Heleococcum japonense	CBS 397.67	1	**JX158485**	**JX158441**	**JX158463**	**JX158398**	**JX158420**
Hydropisphaera erubescens	ATCC 36093	1	AY545722	AY545726	AY545731	DQ518174	_
Hypocrea americana	OSC 100005	1	AY544693	AY544649	_	DQ471043	DQ491488
Hypocrea lutea	ATCC 208838	1	AF543768	AF543791	DQ522446	AF543781	_
Lasiosphaeria ovina	SMH 4605	1	DQ836894	AY436413	AY600284	DQ836908	AY587923
Leucostoma niveum	AR 3413	1	DQ862050	AF362558	DQ862019	DQ862035	_
Lignincola laevis	JK 5180A	1	U46873	U46890	DQ836886	_	_
Lindra thalassiae	JK 5090A	1	DQ470994	DQ470947	DQ470897	DQ471065	DQ491508
Lulworthia grandispora	JK 4686	1	DQ522855	DQ522856	DQ518181	DQ497608	_
Melanconis alni	AR 3500	1	DQ862052	AF408371	DQ862021	DQ862037	_
Melanconis marginalis	AR 3442	1	DQ862053	AF408373	DQ862022	DQ862038	_
Melanconis stilbostoma	AR 3501	1	DQ862054	AF408374	DQ862023	DQ862039	_
Melanopsamma pomiformis	ATCC 18873	1	AY489677	AY489709	EF692511	AY489604	AF081478
Melanospora tiffanii	ATCC 15515	1	AY015619	AY015630	AY015637	_	_
Melanospora zamiae	ATCC 12340	1	AY046578	U17405	AY046580	_	_
Microascus trigonosporus	CBS 218.31	1	DQ471006	DQ470958	DQ470908	DQ471077	DQ491513
Musicillium theobromae (Verticillium theobromae) 1	CBS 968.72	2		EF543838			EF543859
Musicillium theobromae (Verticillium theobromae) 2	CBS 458.51	2		EF543837			EF543858
Peethambara spirostriata	CBS 110115	1	AY489692	AY489724	EF692516	AY489619	_
Petriella setifera	CBS 437.75	1	DQ471020	DQ470969	DQ836883	DQ836911	_
Plectosphaerella alismatis	CBS 113362	2		JF780521			JF780523
Plectosphaerella citrullae 1	Plect 189	2		HQ239050			HQ238964
Plectosphaerella citrullae 2	Plect 157	2		HQ239048			HQ238962
Plectosphaerella cucumerina	DAOM 226828	1, 2	GU180612	GU180647	GU180663	_	GU180630
Plectosphaerella melonis	Plect 148	2		HQ239007			HQ238968
Plectosphaerella pauciseptata	Plect 186	2		HQ239012			HQ238971
Plectosphaerella plurivora	Plect 329	2		HQ239017			HQ238972
Plectosphaerella ramiseptata	Plect 158	2		HQ239049			HQ238963
Pleospora herbarum	CBS 191.86	1	DQ247812	DQ247804	DQ247794	DQ471090	DQ491516

Podospora fimiseda	CBS 990.96	1	_	AY346296	AY780190	_	AY515361
Roumegueriella rufula	CBS 346.85	1	DQ522561	DQ518776	DQ522461	DQ522355	_
Selinia pulchra	AR 2812	1	_	GQ505992	_	HM484841	HM484859
***Sodiomyces alkalinus* F7**	**CBS 132729**	**1, 2**	**JX158467**	**JX158423**	**JX158445**	**JX158379**	**JX158401**
***Sodiomyces alkalinus* F8**	**CBS 133680**	**1, 2**	**JX158468**	**JX158424**	**JX158446**	**JX158380**	**JX158402**
***Sodiomyces alkalinus* F9**	**CBS 133681**	**1, 2**	**JX158469**	**JX158425**	**JX158447**	**JX158381**	**JX158403**
***Sodiomyces alkalinus* F10**	**CBS 132730**	**1, 2**	**JX158470**	**JX158426**	**JX158448**	**JX158382**	**JX158404**
***Sodiomyces alkalinus* F11 T**	**CBS 110278**	**1, 2**	**JX158471**	**JX158427**	**JX158449**	**JX158383**	**JX158405**
***Sodiomyces alkalinus* F12**	**CBS 132731**	**1, 2**	**JX158472**	**JX158428**	**JX158450**	**JX158384**	**JX158406**
***Sodiomyces alkalinus* F13**	**CBS 132732**	**1, 2**	**JX158473**	**JX158429**	**JX158451**	**JX158385**	**JX158407**
***Sodiomyces alkalinus* F14**	**CBS 133682**	**1, 2**	**JX158474**	**JX158430**	**JX158452**	**JX158386**	**JX158408**
***Sodiomyces alkalinus* F15**	**CBS 133683**	**1, 2**	**JX158475**	**JX158431**	**JX158453**	**JX158387**	**JX158409**
***Sodiomyces alkalinus* F16**	**CBS 133684**	**1, 2**	**JX158476**	**JX158432**	**JX158454**	**JX158388**	**JX158410**
***Sodiomyces alkalinus* F17**	**CBS 133685**	**1, 2**	**JX158477**	**JX158433**	**JX158455**	**JX158389**	**JX158411**
***Sodiomyces alkalinus* F18**	**CBS 132733**	**1, 2**	**JX158478**	**JX158434**	**JX158456**	**JX158390**	**JX158412**
***Sodiomyces alkalinus* F19**	**CBS 133686**	**1, 2**	**JX158479**	**JX158435**	**JX158457**	**JX158391**	**JX158413**
***Sodiomyces alkalinus* F20**	**CBS 133687**	**1, 2**	**JX158480**	**JX158436**	**JX158458**	**JX158392**	**JX158414**
***Sodiomyces alkalinus* F21**	**CBS 133688**	**1, 2**	**JX158481**	**JX158437**	**JX158459**	**JX158393**	**JX158415**
***Sodiomyces alkalinus* F22**	**CBS 133689**	**1, 2**	**JX158482**	**JX158438**	**JX158460**	**JX158394**	**JX158416**
***Sodiomyces alkalinus* F23**	**CBS 133690**	**1, 2**	**JX158483**	**JX158439**	**JX158461**	**JX158395**	**JX158417**
***Sodiomyces alkalinus* F24**	**CBS 133691**	**1, 2**	**JX158484**	**JX158440**	**JX158462**	**JX158396**	**JX158418**
Stachylidium bicolor	DAOM 226658	2		GU180651			_
Torrubiella alba (Lecanicillium alba)	CBS 726.73a	1	AF339586	AF339537	EF468934	EF468781	AJ292464
Varicosporina ramulosa	RVG 113	1	U43846	U44092	DQ836888	_	_
Verticillium albo-atrum	CBS 130.51	2		HQ231976			DQ825977
Verticillium alfalfae	PD683	2		_			JN187991
Verticillium dahliae	ATCC 16535	1, 2	AY489705	DQ470945	DQ522468	AY489632	_
Verticillium isaacii	PD752	2		_			JN188018
Verticillium zaregamsianum	PD739	2		_			JN188008
Xylaria acuta	ATCC 56487	1	AY544719	AY544676	DQ247797	DQ471048	AF163026
Xylaria hypoxylon	OSC 100004	1	AY544692	AY544648	DQ470878	DQ471042	DQ491487

* Appears in large scale tree (1), small scale tree (2) or both

Chapter III

Are alkalitolerant fungi of the *Emericellopsis* lineage (*Bionectriaceae*) of marine origin?

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Abstract

Surveying the fungi of alkaline soils in Siberia, Trans-Baikal regions (Russia), the Aral lake (Kazakhstan), and Eastern Mongolia, we report an abundance of alkalitolerant species representing the *Emericellopsis*-clade within the *Acremonium* cluster of fungi (order *Hypocreales*). On an alkaline medium (pH ca. 10), 34 acremonium-like fungal strains were obtained. One of these was able to develop a sexual morph and was shown to be a new member of the genus *Emericellopsis*, described here as *E. alkalina* sp. nov. Previous studies showed two distinct ecological clades within *Emericellopsis*, one consisting of terrestrial isolates and one predominantly marine. Remarkably, all the isolates from our study sites show high phylogenetic similarity based on six loci (LSU and SSU rDNA, RPB2, TEF1-α, β-tub and ITS region), regardless of their provenance within a broad geographical distribution. They group within the known marine-origin species, a finding that provides a possible link to the evolution of the alkaliphilic trait in the *Emericellopsis* lineage. We tested the capacities of all newly isolated strains, and the few available reference ex-type cultures, to grow over wide pH ranges. The growth performance varied among the tested isolates, which showed differences in growth rate as well as in pH preference. Whereas every newly isolated strain from soda soils was extremely alkalitolerant and displayed the ability to grow over a wide range of ambient pH (range 4–11.2), reference marine-borne and terrestrial strains showed moderate and no alkalitolerance, respectively. The growth pattern of the alkalitolerant *Emericellopsis* isolates was unlike that of the recently described and taxonomically unrelated alkaliphilic *Sodiomyces alkalinus*, obtained from the same type of soils but which showed a narrower preference towards high pH.

Key words

Acremonium, *Emericellopsis*, alkaline soils, molecular phylogeny, pH tolerance, soda soils

Introduction

Alkaline soils (or soda soils) and soda lakes represent a unique environmental niche. There are few studies available on the fungal biodiversity therein. The eye-catching characteristic of these soils is a high pH maintained mainly by the buffering capacities of soluble carbonates present. Soda accumulation is thought to be a common process associated with savannas, steppes and desert regions across the world (**JONES ET AL. 1998**). Some examples of such extreme occurrences include the Magadi Lake in Kenya and the Natron Lake in Tanzania where the pH values of water are as high as 11–12. Seventy fungi have been isolated from The Dead Sea in Israel, almost half *Eurotiales*, where the salt levels are 340–350 g salt/l (**BUCHALO ET AL. 2009**). In Russia, alkaline soils are mostly restricted to areas adjacent to saline lake basins in southwestern Siberia (**SOROKIN ET AL. 2008**). Naturally, high salts concentration and high environmental pH impose a substantial amount of stress to any living organism. Some have adapted and therefore evolved metabolic pathways in order to thrive in such harsh conditions, such as high osmotic

pressures, low water potentials, and, clearly, elevated ambient pHs (>9). The vast majority of so-called alkaliphiles, with a growth optimum at pH above 9, include prokaryotes (**DUCKWORTH ET AL. 1996**). However, some filamentous fungi have been shown to be able to grow optimally at pH values exceeding 9 (**NAGAI ET AL. 1995, 1998, GRUM-GRZHIMAYLO ET AL. 2013**). Alkaliphily in filamentous fungi is uncommon, while alkalitolerance, on the other hand, is far more widespread. Alkalitolerant fungi, i.e. fungi that can grow to some extent at an alkaline pH but with their optimum still being at neutral pH values, are not only of basic scientific interest for the molecular mechanisms of adaptation, but also in the search for potentially biotechnologically valuable enzymes. It has become more obvious that alkalitolerant fungi may be encountered in many neutral soils (**KLADWANG ET AL. 2003, ELÍADES ET AL. 2006**). The relative abundance of alkalitolerant fungi has facilitated studies on both their biodiversity and their enzymatic properties. And yet, truly alkaliphilic filamentous fungi have been isolated infrequently. The few existing descriptive studies on alkalitolerant and alkaliphilic fungi show a bias towards fungi with simple conidial morphology, commonly asexual *Acremonium* or *Verticillium* species, and typically, without the development of the any sexual morph (**OKADA ET AL. 1993, KLADWANG ET AL. 2003**). Substantial difficulties in identifying *Acremonium* species imposed by their simple morphology have stimulated the use of molecular phylogeny in their identification. The array of fungi with acremonium-like conidiation has been shown to be highly polyphyletic, occupying several lineages throughout *Ascomycota* (**SUMMERBELL ET AL. 2011**). However, most *Acremonium* species belong to *Hypocreales* (subphylum *Hypocreomycetidae*). One of the well-defined subclades within the hypocrealean acremonia is the *Emericellopsis*-clade (family *Bionectriaceae*), which includes isolates derived from various ecological niches. Notably, previous studies have shown a phylogenetic separation of marine-derived and terrestrial isolates within the *Emericellopsis*-clade (**ZUCCARO ET AL. 2004**). The marine clade also contains fungi derived from soda soils. The current study confirms the evolutionary relationships between marine-borne and soda soil fungi of the genus *Emericellopsis*. Here, we analyse acremonium-like strains isolated from soda soils in western Siberia, the Trans-Baikal area (Russia), the Aral Sea (Kazakhstan) and the Gobi Desert (Mongolia) and elucidate their phylogenetic relationships, with an emphasis on the *Emericellopsis*-clade. A new *Emericellopsis* species, *E. alkalina* sp. nov., is described. We also analysed the newly isolated strains for growth at various pH values, in comparison with reference ex-type strains, and show that the alkalitolerant strains group within the known *Emericellopsis* isolates originated from the marine habitats. We discuss a possible origin of alkalitolerance in this particular lineage of mostly sea-borne fungi.

Materials and Methods

Soil samples, strains and media

Soil samples were collected from several locations on the edge of the soda lakes (**TABLE 1**). We used alkaline agar (AA) with the antibiotic rifampicin (2 g/l) as a selective medium

for alkalitolerant species isolation. For routine subculturing on AA of the newly isolated strains, the antibiotic was not used. The AA medium was prepared as described previously (**GRUM-GRZHIMAYLO ET AL. 2013**). Several reference ex-type *Emericellopsis* strains were obtained from the CBS-KNAW Fungal Biodiversity Centre (CBS) as well as from the All-Russian Collection of Microorganisms (VKM). For the colony morphology characterization we used several types of media: WA, CZ, MYA, PDA, OA and AA (**MUELLER ET AL. 2004**). The elucidation of the pH optimum was performed in duplicate using race tubes with the media ranging in pH as described previously (**GRUM-GRZHIMAYLO ET AL. 2013**), with the following modification. Instead of using acetic buffer to generate pH 4 and 5.2, we used a citric acid buffer system. Race tubes and plates were incubated in the dark at 28 °C, and the growth rates were recorded once a week over 2 mo.

TABLE 1. Strains of used in the current study and characteristics of the sites of isolation. Newly isolated strains are given in bold.

Strain	VKM number	CBS no.	Isolation area	Isolation place	pH of the soil	Total salts (g/kg)	Saltification type
***Acremonium sclerotigenum* A101**	-	-	Trans-Baikal, Russia	near Alla River	8	-	sulfate
***Acremonium sclerotigenum* A130**	-	-	Trans-Baikal, Russia	near Alla River	8	-	sulfate
***Acremonium* sp. A104**	-	-	Kulunda steppe, Altai, Russia	-	taken from *Atriplex verrucifera* MB.		soda
***Acremonium* sp. A105**	-	-	Trans-Baikal, Russia	Orongoyskoe Lake	7.8	26	soda-sulfate
***Acremonium* sp. A106**	-	-	Trans-Baikal, Russia	Sulfatnoe Lake	8.5	3.7	sulfate-soda
***Acremonium* sp. A107**	-	-	Trans-Baikal, Russia	Chedder Lake	9.1	-	soda
***Acremonium* sp. A108**	-	-	Aral lake, Kazakhstan	Cape Aktumsyk	taken from *Sueda salsa*		chloride-sulfate
***Acremonium* sp. A109**	-	-	Trans-Baikal, Russia	Kuchiger area	9	-	sulphate
***Acremonium* sp. A110**	-	-	Trans-Baikal, Russia	Sulfatnoe Lake	10.3	139.4	sulfate-soda
***Acremonium* sp. A111**	-	-	Aral lake, Kazakhstan	Cape Aktumsyk	8	-	chloride-sulfate
***Acremonium* sp. E102**	-	-	Kulunda steppe, Altai, Russia	Bezimyannoe Lake	9.1	47	chloride
***Emericellopsis alkalina* A103**	-	-	Kulunda steppe, Altai, Russia	Mirabilit Lake	9.6	100	soda-chloride-sulfate
***Emericellopsis alkalina* A112**	-	-	North-East Mongolia	Burd Lake	10.1	33	soda
***Emericellopsis alkalina* A113**	FW-1476	-	Choibalsan area, North-East Mongolia	-	11	57	soda
***Emericellopsis alkalina* A114**	FW-1473	-	Kulunda steppe, Altai, Russia	Solyonoe Lake	10	187	chloride
***Emericellopsis alkalina* A115**	FW-1474	-	Kulunda steppe, Altai, Russia	-	9.6	225	chloride-sulfate
***Emericellopsis alkalina* A116**	-	-	Kulunda steppe, Altai, Russia	Mirabilit Lake	9.6	100	soda-chloride-sulfate
***Emericellopsis alkalina* A117**	FW-1471	-	Kulunda steppe, Altai, Russia	Shukurtuz Lake	9.9	53	chloride-sulfate
***Emericellopsis alkalina* A118**	-	-	Kulunda steppe, Altai, Russia	Zheltir' Lake	9.6	137	soda-chloride
***Emericellopsis alkalina* A119**	-	-	Kulunda steppe, Altai, Russia	Bezimyannoe Lake	10.1	38	chloride-sulfate
***Emericellopsis alkalina* A120**	-	-	Kulunda steppe, Altai, Russia	Bezimyannoe Lake	9.9	310	soda
***Emericellopsis alkalina* A121**	-	-	Kulunda steppe, Altai, Russia	Tanatar Lake	10.2	73	soda
***Emericellopsis alkalina* A122**	-	-	Kulunda steppe, Altai, Russia	-	9.5	65	chloride
***Emericellopsis alkalina* A123**	-	-	Kulunda steppe, Altai, Russia	-	taken from *Salicornia europaea* L.		soda
***Emericellopsis alkalina* A124**	-	-	Kulunda steppe, Altai, Russia	south, Berdabay	10.1	60	soda
***Emericellopsis alkalina* A125**	-	-	Trans-Baikal, Russia	Nuhe-Nur Lake	10.1	7.1	soda
***Emericellopsis alkalina* A126**	-	-	Trans-Baikal, Russia	Nuhe-Nur Lake	10.1	1.9	soda
***Emericellopsis alkalina* A127**	-	-	Trans-Baikal, Russia	Nuhe-Nur Lake	10.1	1.9	soda
***Emericellopsis alkalina* A128**	-	-	Trans-Baikal, Russia	Sulfatnoe Lake	10.3	139.4	sulfate-soda
***Emericellopsis alkalina* E101^T**	F-4108	CBS 127350	Kulunda steppe, Altai, Russia	Tanatar Lake	10.1	73	soda
***Emericellopsis alkalina* M14**	F-3905	CBS 120043	Kulunda steppe, Altai, Russia	Bezimyannoe Lake	9.9	310	soda
***Emericellopsis alkalina* M20**	FW-3040	CBS 120044	Kulunda steppe, Altai, Russia	Zheltir' Lake	9.6	137	soda-chloride
***Emericellopsis alkalina* M71**	F-3907	CBS 120049	Trans-Baikal, Russia	Sulfatnoe Lake	10.3	139	sulfate-soda
Emericellopsis maritima^T	F-1082	CBS 491.71	Black sea Sevastopol area, Crimea, Ukraine	sea water	-	-	-
Emericellopsis minima	F-1057	CBS 871.68	Germany	wheat field soil	-	-	-
Emericellopsis minima ^{T}	F-1484	CBS 190.55	Inhaca, Mozambique	mangrove soil	-	-	-
Emericellopsis pallida^T	F-925	CBS 490.71	Black sea Sevastopol area, Crimea, Ukraine	sea water	-	-	-
***Sarocladium* sp. A131**	-	-	Aral lake, Kazakhstan	Cape Aktumsyk	8.3	-	chloride-sulfate

Morphology

We used light microscopy (LM) and scanning electron microscopy (SEM) for morphological characterization of the strains, as described previously (**GRUM-GRZHIMAYLO ET AL. 2013**).

DNA extraction, PCR, and sequencing

Total genomic DNA (gDNA) was extracted from mycelium using DNeasy Plant Mini kit (Qiagen, Chatsworth, CA). We amplified and sequenced six nuclear loci (large and small subunit rDNA, internal transcribed spacers 1 and 2, including 5.8S rDNA, RPB2, TEF1-α and β-tub) from gDNA using the standard primers set. Primer sets, thermo cycling programs and sequencing procedures were performed as described previously (**GRUM-GRZHIMAYLO ET AL. 2013**). The amplification of beta-tubulin intron 3 (hereafter named as "β-tub") was as in **ZUCCARO ET AL. (2004).**

TABLE 2. Loci and substitution models used for the phylogenetic analyses.

Phylogenetic analysis	Locus	Model for each partition	Characters	Informative characters	Uninformative variable characters	Invariable characters
1	LSU	TIM1+I+G (GTR+I+G)*	962	162	62	738
2	ITS	TIM1+G (GTR+I+G)*	503	101	62	340
	β-tub	TrN+G (HKY+G)*	333	80	29	224
	RPB2	TIM3+G (GTR+G)*	1070	73	134	863
	TEF1-α	TIM3+G (GTR+G)*	904	54	58	792

* – for MrBayes

Phylogenetic analyses

We used five nuclear loci for phylogenetic analysis: large subunit rDNA (LSU), ITS region, RPB2, TEF1-α, and β-tub. The gene for small subunit rRNA (SSU), although sequenced, was not included in our phylogenetic reconstructions since it carried too little phylogenetic signal to contribute to clade differentiation. We constructed separate alignments for each of the analysed genes using the online MAFFT v. 7 service (**KATOH & STANDLEY 2013**). Ambiguous regions were removed manually from the alignments with BioEdit v. 7.1.3.0 (**HALL 1999**). Two data sets for different phylogenetic analyses were constructed in order to achieve different degrees of resolution within the studied groups. Appropriate reference sequences were obtained from GenBank. The first analysis included a single LSU gene in order to build a large-scale taxonomy for hypocrealean acremonia. The second, a four-gene (ITS, β-tub, RPB2, and TEF1-α) concatenated super-matrix, was implemented to resolve the recent evolutionary relationships in the *Emericellopsis*-clade and our newly isolated alkalitolerant strains. The four-gene concatenated data set was constructed using Mesquite v. 2.75 (**MADDISON & MADDISON 2011**) and divided into four partitions corresponding to each individual gene. The best-fit model for nucleotide substitution for each partition was chosen according to the corrected Akaike Information Criterion (AICc) as implemented in jModelTest v. 2.1.1 (**GUINDON & GASCUEL 2003, DARRIBA ET AL. 2012**) (**TABLE 2**). GARLI v. 2.0 (**ZWICKL 2006**) was used for Maximum Likelihood (ML) bootstrap analyses; for both

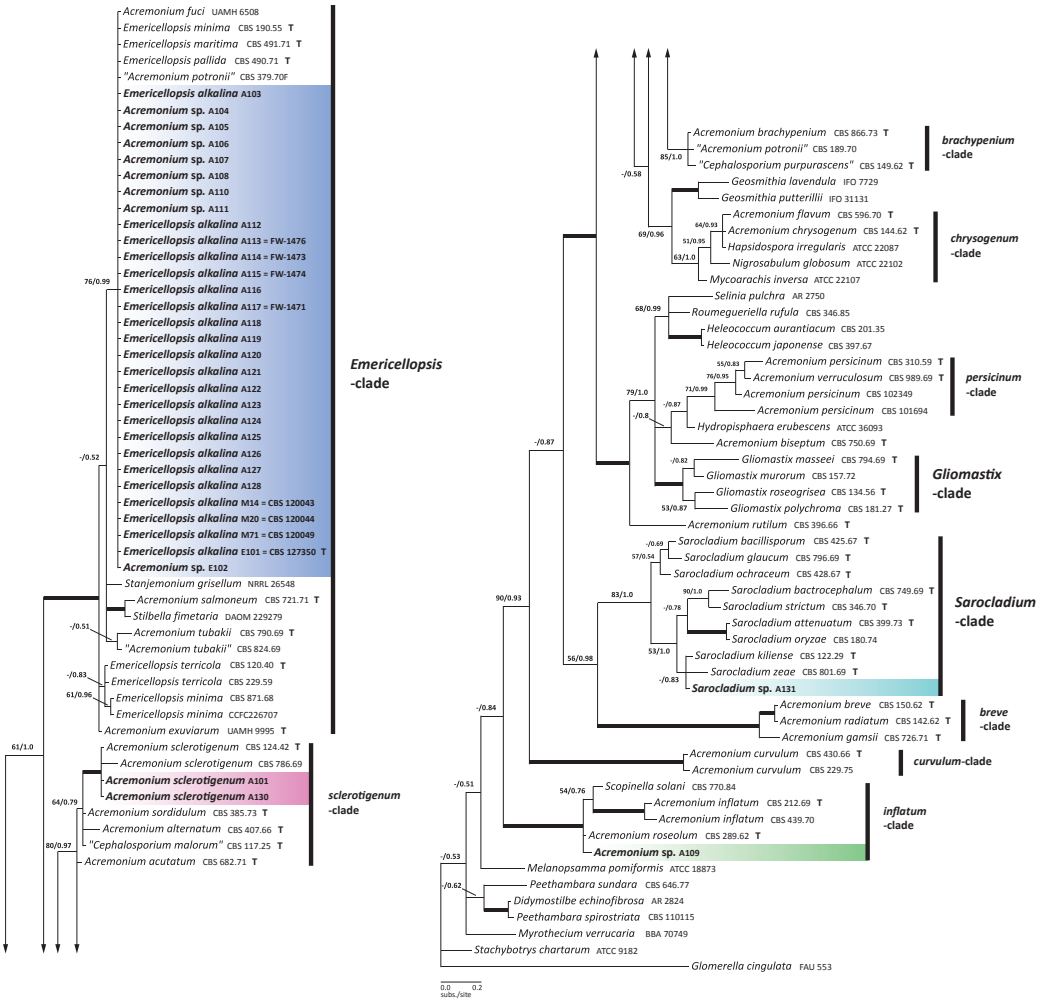

FIG. 1. Phylogenetic reconstruction of *Acremonium* species in *Bionectriaceae* as inferred from the partial LSU gene sequences. New isolates from the soda soils are marked with colour boxes. Clade delineation is from **SUMMERBELL ET AL. (2011)**. Bayesian topology with the ML/PP support values over each node is displayed. Thickened branches indicate strong combined support (ML > 90, PP > 0.94). **T** – type/ex-type strains.

phylogenetic analyses the number of searches was set to five for each of the 200 bootstrap replicates. A 50 % majority rule consensus trees were constructed using SumTrees v. 3.3.1 application within DendroPy v. 3.11.0 package (**SUKUMARAN & HOLDER 2010**) running under Python v. 2.6 platform. Bayesian analysis (BI) was performed using MrBayes v. 3.1.2 (**HUELSENBECK & RONQUIST 2001**). Two independent searches and four chains were set to run for 10 M generations for both phylogenetic analyses sampling every 100th generation. The convergence of the runs was

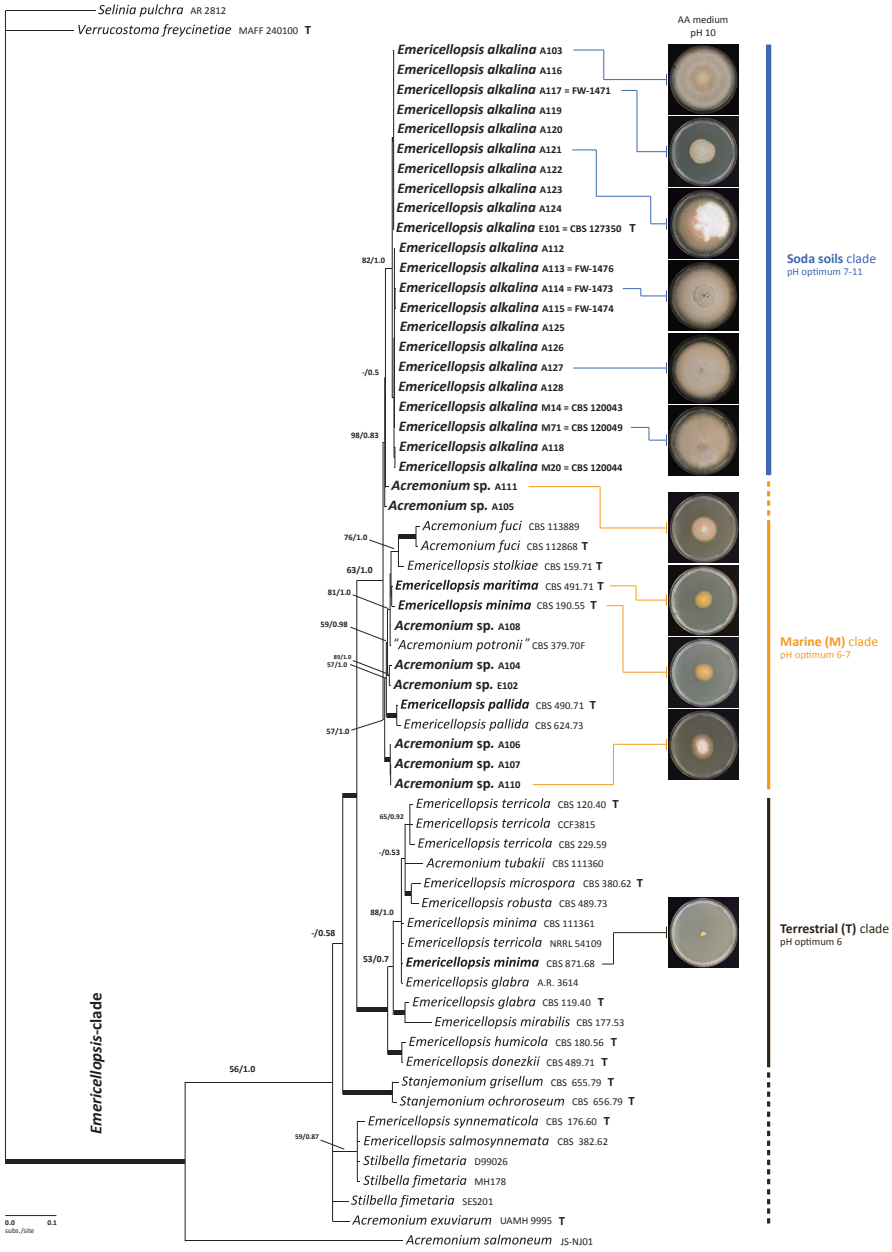

FIG. 2. Four-gene phylogeny of the new alkalitolerant isolates within the *Emericellopsis*-clade based on partial sequences for ITS (including 5.8S rDNA), β-tub, RPB2 and TEF1-α genes. All strains studied are in bold. Bayesian topology is displayed with the ML/PP support values over each node. Thickened branches indicate strong combined support (ML > 90, PP > 0.94). **T** – type/ex-type strains. Representative strains from each delineated clade are shown on AA medium plates (11-d-old).

checked in TRACER v. 1.5 (**RAMBAUT & DRUMMOND 2007**). The first 30 % (50 % for four-gene analysis) of the resulting trees was eliminated from the further analysis. The rest were used to generate a 50 % majority rule consensus tree and calculate posterior probabilities (PP). The consensus tree was visualized and edited with TreeGraph v. 2.0.47-206 beta (**STÖVER & MÜLLER 2010**) and Adobe Illustrator CS6 (Adobe Systems, San Jose, CA). The node supports were considered to be strong if they received joint scores of ML > 90 and PP > 0.94. Newly generated sequences from the studied strains were deposited in GenBank with accessions listed in **SUPPLEMENTARY TABLE 1**. Phylogenetic analyses were deposited in TreeBase (**SUBMISSION ID 14196**).

Results

Isolated strains

On the selective AA medium buffered at pH 10 and containing antibiotic, we isolated 34 strains of filamentous fungi from soda soils adjacent to the soda lake basins. Several of the isolated strains were deposited in CBS and VKM. All strains showed asexual acremonium-like sporulation and one displayed comprehensive sexual morphological features and was found to be a new species of the *Emericellopsis* lineage based on molecular, morphological and growth data (see below).

Molecular phylogenetic analyses

The alignment for the first phylogenetic analysis using the LSU gene contained 962 characters, with 162 (17 %) being phylogenetically informative (**TABLE 2**). The negative log likelihoods (-Ln) of the ML and BI consensus trees were 4696.03 and 5111.82, respectively. The phylogenetic reconstruction based on LSU sequences of our isolates from soda lakes along with the pertinent reference sequences from hypocrealean acremonia is consistent with the topology described by **SUMMERBELL ET AL. (2011)**, hence we follow the clade delineation outlined in that study. As seen in **FIG. 1**, the new isolates from the soda soils (in coloured boxes) almost exclusively fall into a strongly supported (97/1.0) *Emericellopsis*-clade (*Bionectriaceae*). This clade is known to include marine-borne fungi such as *Acremonium fuci*, *A. tubakii*, *E. maritima*, as well as terrestrial isolates like *E. terricola*, some *Stilbella* species, and the *Stanjemonium* species. The lizard-associated ex-type-strain of *A. exuviarum* (UAMH 9995^{T}), producing chains of conidia, has been shown before to have affinity to the *Emericellopsis*-clade (**SIGLER ET AL. 2004**). Thirty of our new isolates in the *Emericellopsis*-clade stand together within a weakly supported clade (76/0.99) that also includes the ex-type strains of *E. minima* (CBS 190.55^{T}), *E. maritima* (CBS 491.71^{T}), and *E. pallida* (CBS 490.71^{T}), as well as "*A. potronii*" (isolate CBS 379.70F); the latter is a single isolate of an undescribed species that has so far only been isolated from a dolphin skin lesion, apparently not as an agent of infection (**ZUCCARO ET AL. 2004**). The marine species from *Fucus*, a brown seaweed, *A. fuci* (UAMH 6508), also grouped with our isolates from soda soils. There is not enough phylogenetic signal from our LSU-based phylogenetic reconstruction to resolve the

Emericellopsis-clade further. Four new isolates from soda soils appeared to be in the sister clades, namely, two in the *sclerotigenum*-clade, one in the *Sarocladium*-clade and one in the *inflatum*-clade. They are hence identified accordingly. The second phylogenetic analysis included partial sequences of four genes (ITS, β-tub, RPB2, TEF1-α) known to have a higher mutation rate than LSU. We sampled a different set of taxa for this low-level taxonomic analysis. The sequences for the *Emericellopsis*-clade had a high degree of similarity, and were easily aligned and edited. The most variable locus in this set was the β-tub region containing introns, and this region thus contributed significantly to the reliability of the resulting tree. The alignment for this analysis had 2810 characters of which 308 (11 %) were phylogenetically informative (**TABLE 2**). The MCMC runs in Bayesian analysis reached stationary status with a deviation of 0.008 after 5M generations. The negative log likelihoods (-Ln) of the ML and BI consensus trees were 8487.81 and 8645.85, respectively. The tree that was generated for the *Emericellopsis*-clade is displayed in **FIG. 2**. Here, unlike in the first analysis, the *Emericellopsis*-clade is deeply resolved, displaying several major clades consistent with the previous study by **ZUCCARO ET AL. (2004)**. The basal group consists of a highly supported asexual *Stanjemonium* clade, asexual *Stilbella fimentaria* haplotypes, and the soil-derived ex-type isolates of *E. synnematicola*, CBS 176.60^{T}, and *E. salmosynnemata*, CBS 382.62. The ex-type isolate of *Acremonium exuviarum*, mentioned earlier, seems to be more distally basal to the rest of the core tree members. Our phylogenetic analysis confirms the presence of the two ecological groups in the *Emericellopsis* lineage, both of which were supported by the molecular studies. The clades designated as marine (M) and terrestrial (T), outlined previously by **ZUCCARO ET AL. (2004)**, also appear in our phylogenetic analysis. The T clade (98/1.0) almost exclusively contains terrestrial species of *Emericellopsis*, such as *E. robusta*, *E. terricola*, and *E. microspora*. There are a few exceptions, namely, *E. donezkii* CBS 489.71^{T}, *E. minima* CBS 111361, and *A. tubakii* CBS 111360, which were found in aquatic environments. The very weakly supported M clade (57/1.0) predominantly contains isolates from marine and soda lake habitats, with the exception of *E. pallida* CBS 624.73 and the ex-type isolate of *E. minima*, CBS 190.55^{T}. Interestingly, eight of our new isolates from the soda soils fall into the M clade while the majority (22 strains) form a well-supported sister clade (82/1.0). We name that clade the "soda soils" clade. It comprises 22 of our isolates that collectively represent a new species named *E. alkalina* sp. nov. here. Of those 22 strains, one formed ascomata, while the others only displayed asexual structures. These structures were identical to those seen in CBS 127350^{T}, the sexual strain from which we derived the type of *E. alkalina*. SSU sequences showed almost no variation among our newly isolated strains in the *Emericellopsis*-clade. We found only two variable sites among 1637 base pairs.

Growth patterns

In order to link our phylogenetic data to ecological preferences, we conducted a growth experiment testing the growth ability of all studied strains at different ambient pH values. As seen in **FIG. 3 (A)**, the pH preferences vary among the members of the different clades within the *Emericellopsis* lineage. A reference member of the T clade, *E. minima* (CBS 871.68), displayed a very narrow growth optimum at pH 6 with no ability to cope with both lower and higher pH

values. Three reference members of the M clade, the ex-type strains of *E. maritima* (CBS 491.71^{T}), *E. minima* (CBS 190.55^{T}), and *E. pallida* (CBS 490.71^{T}), had an optimum growth at pH 6–7, but were able to tolerate higher pH values. Identical growth patterns were seen in our strains *Acremonium* sp. A104, A105, A106, A107, A108, A110, A111, and E102 (data not shown) which also fall into the M clade. Two strains (A105 and A111) seem to be paraphyletic to the M clade, but based on their growth patterns they belong to the M clade (dashed line). Members of the M clade grew faster than *E. minima* (CBS 871.68) from the T clade. All new isolates of *E. alkalina* (except A117, which had very low growth rate and no pH preference) showed a higher growth rate than that seen in the members of the M and T clades. They had a broad pH optimum in the 7–11 range, and displayed a wide tolerance across the pH scale. Isolates *Acremonium* sp. A109, *A. sclerotigenum* A101, A130 and *Sarocladium* sp. A131, which fall into a sister-clade to the *Emericellopsis*-clade, had an overall slow growth rate with a slight preference for neutral pH combined with the ability to tolerate higher pH values. This pattern somewhat resembled that seen in the M clade (**FIG. 3, B**).

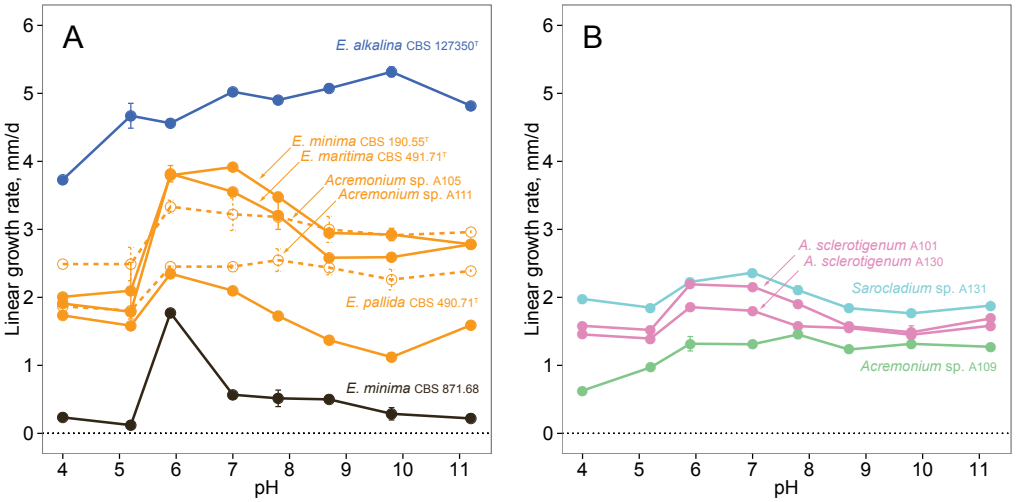

FIG. 3. Growth patterns of the representative strains at pH 4 through 11.2 based on MYA medium. **A.** Strains from the T, M and soda soils clades within the *Emericellopsis* lineage including intermediate *Acremonium* sp. isolates A105 and A111; **B.** Isolated alkalitolerant strains from the sister clade of the *Emericellopsis* lineage.

Taxonomy

EMERICELLOPSIS ALKALINA Bilanenko & Georgieva, *sp. nov.* MycoBank — **MB804572; FIG. 4–5**

Etymology — Epithet taken from the ability to grow at high ambient pH.

Diagnosis — *Asci* saccate, 12–15 µm long, unitunicate. *Ascospores* ellipsoid, pale brown,

with uneven surfaces, 4.5–5.5 x 2.5–3.0 µm, surrounded by 3, but frequently 5 longitudinal, subhyaline, smooth-edged alar appendages, width up to 1.0 µm. *Asexual* morph acremonium-like.

Type species — **RUSSIA**, Altai, Kulunda steppe, soda soil (total salts 73 g kg^{-1}, pH 10.1) on the edge of the basin of Tanatar Lake, August 2002, *D. Sorokin* (CBS H-21412 – holotype; culture ex-type E101 = CBS 127350 = VKM F-4108).

Ascomata dark brown, superficial on the substratum, globose, 50–120(–180) µm diam, nonostiolate, wall 6–10 µm thick. *Peridium* multi-layered, pseudoparenchymatous, composed of 3–5 layers of compressed cells. *Asci* saccate, 12–15 µm long, with thin deliquescent wall, soon dissolving, unitunicate, scattered irregularly in the ascocarp. *Ascospores* ellipsoid, pale brown, with uneven surfaces, 4.5–5.5 x 2.5–3.0 µm, surrounded by 3, but frequently 5 longitudinal, subhyaline, smooth-edged alar appendages, width up to 1.0 µm. *Asexual morph* acremonium-like. *Conidiation* abundant, mostly plectonematogenous, partially nematogenous. *Conidiophores* mostly simple orthotropic. *Conidiogenous cells* 20–35 µm long, tapering from 1.5–1.8 µm at the base to 0.7–0.8 µm at the apex, sometimes lateral branches form. *Conidia* narrowly ellipsoid, smooth-surfaced, 3.5–6.0 x 1.8–2.2 µm, about the same length as ascospores but narrower, hyaline, adhering in slimy heads. *Chlamydospores* absent.

Culture characteristics — Colonies on alkaline agar (AA, pH 10.0–10.2) fast-growing, reaching 70–80 mm diam in 10 d at 25 °C. On MEA (pH 6.5) growing slower, reaching 32–38 mm diam in 10 d. Colonies orange-salmon-pink, later darkening in centre due to the formation of ascomata with tufted aerial mycelium sometimes forming concentric zones upon exposure to light. Reverse colourless. Exudate absent. Decumbent vegetative hyphae thin-walled, hyaline, 0.5–2.0 µm wide. Mycelium consisting of hyaline, smooth-walled, septate hyphae, 1–3 µm wide, often fasciculate.

Additional specimens examined — A103, A112, A113 (= VKM FW-1476), A114 (= VKM FW-1473), A115 (= VKM FW-1474), A116, A117 (= VKM FW-1471), A118, A119, A120, A121, A122, A123, A124, A125, A126, A127, A128, M14 (= VKM F-3905 = CBS 120043), M20 (= VKM FW-3040 = CBS 120044), M71 (= VKM F-3907 = CBS 120049).

Notes — The current study shows a well-supported clade (82/1.0) as inferred from four phylogenetic loci (ITS, β-tub, RPB2, TEF1-α) containing 22 isolates including the type E101. Although only the type E101 strain formed a sexual morph, we assign the remaining 21 isolates to *E. alkalina* as well, based on sequence similarity and the identity of asexual morphology. All 22 isolates of *E. alkalina* showed essentially the same growth patterns with a wide pH tolerance culminating in an optimum at pH 7–11. Isolate A117 is the only exception, showing a highly reduced growth rate in general, and no obvious pH optimum.

Morphological differences from sister species — The ascomata of the type of *Emericellopsis alkalina* (CBS 127350^T), have a multilayered peridium, composed mostly of five layers of flattened cells. The peridium of *E. pallida* ex-type isolate CBS 490.71^T is thinner, 1–2 layered. The ascospore morphology of the type of *E. alkalina* (CBS 127350^T) looks similar to that of *E. pallida* and *E. minima*. However, *E. alkalina* ascospores have an uneven surface with (3–)5 alar appendages,

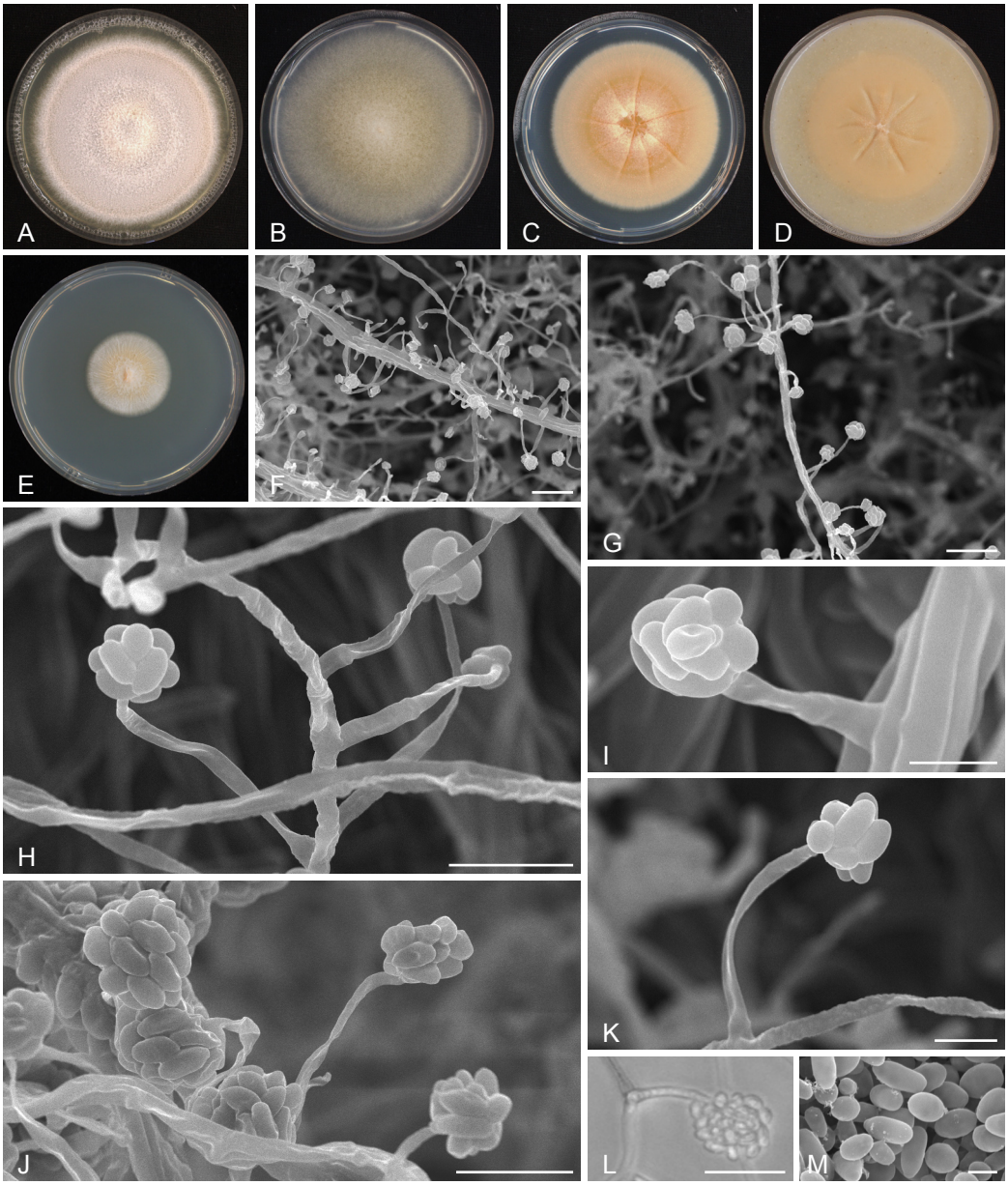

FIG. 4. *Emericellopsis alkalina* (CBS 127350^{T}) **A–E.** 11-d-old (28 °C, dark regime, 9 cm Petri dish) colony on alkaline agar (AA), Czapek agar (CZ), potato dextrose agar (PDA), oatmeal agar (OA), malt yeast extract agar (MYA). **F–G.** Hyphal bundles with acremonium-like conidiation (SEM). **H.** Conidiogeous cells emerging from single hypha (SEM). **I.** Conidial head on a single conidiogenous cell emerging from the hyphal bundle (SEM). **J.** Matured conidial heads (SEM). **K.** Single conidiogenous cell with young conidial head (SEM). **L.** Conidial head (LM). **M.** Conidia (SEM). *Scale bars:* **F–G** = 20 µm; **H, J, L** = 10 µm; **I, K** = 5 µm; **M** = 2 µm.

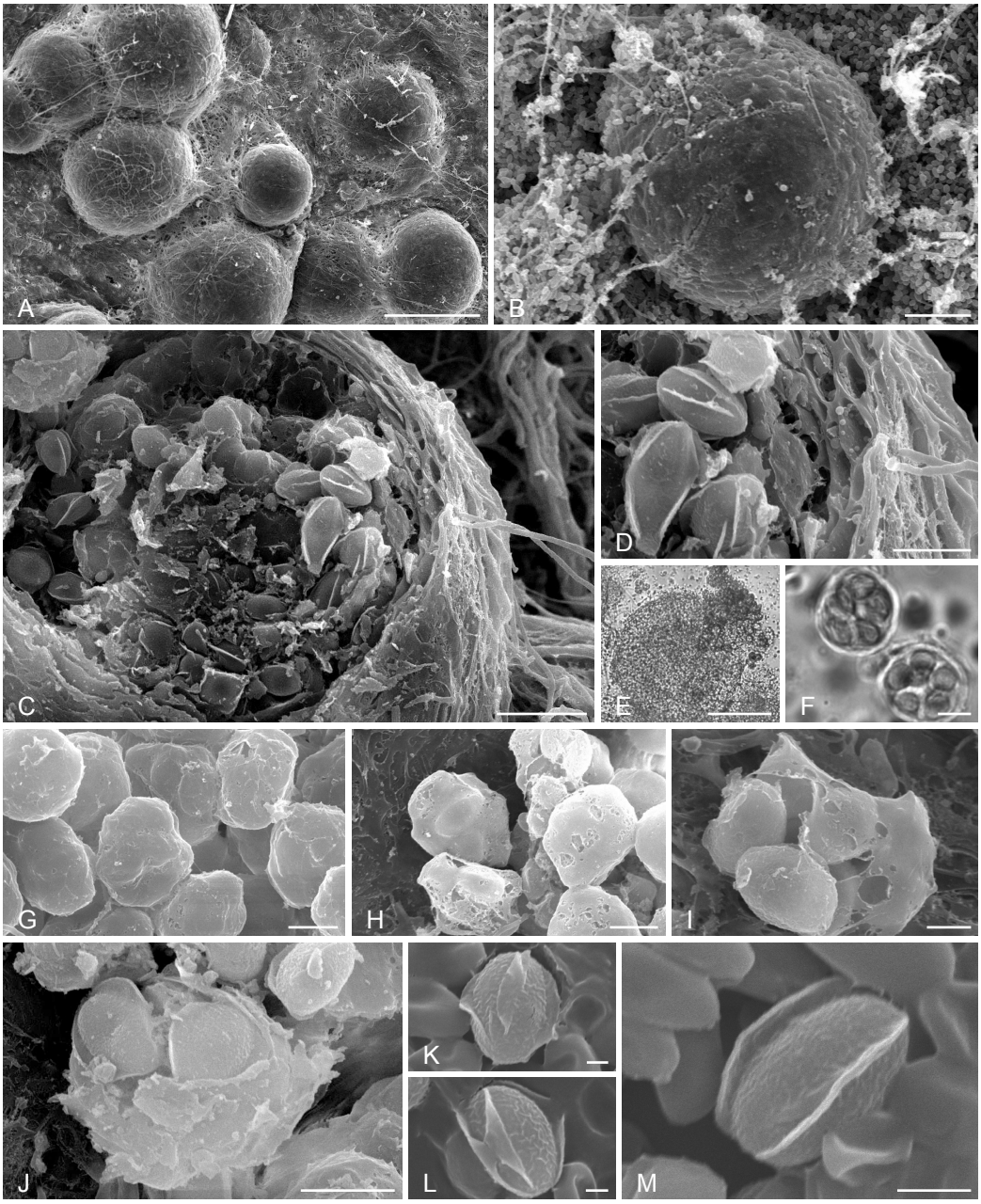

FIG. 5. *Emericellopsis alkalina* (CBS 127350^{T}) **A.** Cleistothecia (SEM). **B.** Cleistothecium surrounded by the asexual sporulation (SEM). **C.** Open cleistothecium (SEM). **D.** Magnified view on the multilayered peridium (SEM). **E.** Open cleistothecium (LM). **F.** Young asci (LM). **G.** Young asci (SEM). **H–J.** Lysing asci (SEM). **K–M.** Ascospores with alar appendages (SEM). *Scale bars:* **A, E** = 100 µm; **B** = 20 µm; **C** = 10 µm; **D, F–H, J** = 5 µm; **I, M** = 2 µm; **K–L** = 1 µm.

while *E. pallida*, as represented by ex-type CBS 490.71^{T}, has smooth ascospores often with three alar appendages. The ex-type of *E. minima* (CBS 190.55^{T}), unfortunately did not produce ascomata during our investigation. A non-type isolate of *E. minima*, CBS 871.68, has wider (2 µm) alar appendages with flexuose rims, while *E. alkalina* (CBS 127350^{T}) has narrow (1 µm) appendages with smooth rims.

Discussion

Here we provide phylogenetic evidence that our newly isolated alkalitolerant fungi from geographically diverse soda soils, are derived from marine-borne species within the genus *Emericellopsis*. Based on pH growth preference, the highly alkalitolerant strains form a "soda soils" clade distinct from the moderately alkalitolerant "marine" clade and the neutrophilic "terrestrial" clade. The genus *Emericellopsis*, previously considered to belong to *Eurotiales*, was erected in **1940**, based on the isolation of *E. terricola* and its variant *E. terricola* var. *glabra* (eventually renamed *E. glabra*; **BACKUS & ORPURT 1961**). **VAN BEYMA (1939–40)** described *E. terricola* based on an isolate from soil collected near the town of Baarn in The Netherlands. The generic name came from the close morphological resemblance of the ascospore ornamentation to that of *Emericella nidulans*, which was originally thought to be taxonomically related. Subsequent studies described additional soil-borne *Emericellopsis* species from various parts of the world (**STOLK 1955**, **GILMAN 1957, MATHUR & THIRUMALACHAR 1960, 1962, BACKUS & ORPURT 1961**). At the beginning of the **1960s**, the genus contained five species and one variety. Ascospore size and shape constituted the major criteria used to distinguish species (**DURRELL 1959**).

The beginning of the **1970s** marked a new period in the study of *Emericellopsis* with the establishment of marine mycology. New *Emericellopsis* species were discovered in the sediments of soda lakes and along the seacoasts. *Emericellopsis stolkiae*, for instance, was isolated from the soil on the edge of the soda lake in south-western Wyoming, USA (**DAVIDSON & CHRISTENSEN 1971**). That species had larger ascospores than previously known *Emericellopsis* species, and also had distinct alar appendages. **TUBAKI (1973)** suggested the conidial genus *Cephalosporium* was characteristic of aquatic sediments, and he linked *Emericellopsis* as the corresponding sexual state.

Emericellopsis was revised by the Russian mycologist **BELYAKOVA (1974)** who analysed the morphological features of the then known *Emericellopsis* species and compiled an identification key for 12 species. She also described three new aquatic species: *Emericellopsis donezkii* isolated from the basin of the North Donetz River (Ukraine), and *E. maritima* and *E. pallida* from the intertidal zone of the Black Sea in the Crimean peninsula (Ukraine) (**BELYAKOVA 1970, 1974**).

At the moment, *Emericellopsis* comprises homothallic saprobic cleistothecial species with acremonium-like conidiation; one species, *E. synnematicola*, also forms stilbella-like synnemata. However, different authors accept different numbers of species in the genus. Currently, 16 species with four varieties are listed in the MycoBank database (**CROUS ET AL. 2004**). All authors have so far supported the opinion that the main distinguishing features among species are the morphology of the ascospores and their alar appendages. Molecular studies conducted in the

late 1990s placed *Emericellopsis* in *Hypocreales* (**GLENN ET AL. 1996**). Analysis of SSU and LSU revealed it as a member of the family *Hypocreaceae* (**OGAWA ET AL. 1997**), as it was then defined, although it was subsequently assigned to *Bionectriaceae* (**ROSSMAN ET AL. 1999, 2001**). The genus appears to be monophyletic, with strong support values obtained in the analysis of the ITS and beta-tubulin sequences (**ZUCCARO ET AL. 2004**). The *Emericellopsis* lineage s. lat. also harbours the asexual genera *Stilbella* and *Stanjemonium*, along with the marine species *Acremonium tubakii* and *A. fuci* (**SUMMERBELL ET AL. 2011**).

The accumulated knowledge on the genus *Emericellopsis* suggests a wide ecological amplitude and worldwide distribution. This includes typical species of soils undergoing periodic flooding (e.g. rice paddies), as well as species found in bogs, the sediments of freshwater and seawater basins, and even the soils around subterranean wasp nests where humidity and alkalinity are elevated (**BATRA ET AL. 1973, TUBAKI 1973, DOMSCH ET AL. 2007**). Some species have a broad ecological distribution, such as *E. terricola*, which has been isolated from alkaline soils at the Mono Lake in California as well as from both acidic and saline soils in the Czech National Park (**STEIMAN ET AL. 2004, HUJSLOVÁ ET AL. 2010**). A survey of ascomycetous fungi in limestone soils in Argentina formed by mollusc shells yielded *E. minima*, with its ability to grow from pH 5 to 11 (**ELÍADES ET AL. 2006**). The pattern of marine and other salt-associated isolations has suggested that marine habitats might harbour a large number of the *Emericellopsis* species. The ability to survive in high salinity and pH does not always coincide with the ability to develop the full lifecycle in those conditions, making the salts-adapted species difficult to discriminate from "transit" species and hampering efforts to estimate their ecological contribution (**KOHLMEYER & VOLKMANN-KOHLMEYER 2003**). A study by **ZUCCARO ET AL. (2004)** revealed the presence of distinct marine and terrestrial clades within *Emericellopsis*, as noted above. The M clade contained isolates from saline habitats, including the recently described *A. fuci* from the thalli of the seaweed *Fucus serratus* and *F. distichus*. Members of the marine clade within *Emericellopsis* showed an ability to utilize sugars present in seaborne brown algae (e.g. fucoidan, fucose). The presence of marine water appeared to be necessary for conidial germination in *A. fuci*.

Involvement of additional loci in our phylogenetic analysis confirms the presence of the M and T clades (**FIG. 2**). Our new alkalitolerant isolates are exclusively linked to the M clade, with our 22 *E. alkalina* isolates displaying an extreme alkalitolerant phenotype. Both growth patterns and molecular data suggest that the *E. alkalina* group originated from the marine isolates of the M clade, linking evolutionary development in the marine habitat with that of the soda soils. Clearly, these environments share high salinity and elevated ambient pH values. As far as we know, however, such an ecological overlap has not been demonstrated for other marine fungal lineages. To address this issue, we need systematic biodiversity research on the fungi from soda lakes.

That the intron of the β-tub gene contributed extensively to the phylogenetic signal in our study suggests a relatively recent divergence of *E. alkalina* from the M clade. Our *Acremonium* sp. strains A105 and A111 seem to be intermediate isolates situated in a statistically ambiguous position between the alkaline and marine lineages. The growth pattern of these isolates

contributed significantly to our decision to include them within the M clade.

Emericellopsis alkalina grew well at pHs from 4 to 11.2, with a slight preference towards 7–11. However, a few isolates of this species, namely A113, A118, A122, A126, A127, and M20, displayed a significant dip in growth rate at neutral pH values (data not shown). This feature could be seen as a physiological trade-off that has evolved in some strains of *E. alkalina* that thrive along with alkalitolerant strains from the M clade. Interestingly, A128 from the soda soils clade, and A110, were isolated from the same soil sample at Sulfatnoe Lake. And yet, this trend does not extend to all *E. alkalina* strains that were jointly isolated with M clade strains. It is unclear what makes the majority of *E. alkalina* strains grow more vigorously than the M clade members essentially at every pH value we tested. That *E. alkalina* performs well along a large section of the pH scale makes it difficult to specify the ecology of this species in conventional terms. It is technically not correct to label it an 'alkaliphile', since it is capable of growth at low pH as well as at high pH. Nor is the term 'alkalitolerant' entirely true, since the optimal growth pH is above neutral. The term 'pH-tolerant' with the preference towards alkaline conditions might be suitable. As opposed to the soda soils clade, members of the M clade can be appropriately called 'alkalitolerant', while *E. minima* (CBS 871.68) from clade T can safely be termed a 'neutrophile'.

A link between marine and soda soil inhabitants has previously been observed in bacteria. In metabolic studies of fungi, specifically *Fusarium oxysporum*, it has been shown that the expression of the gene *ena1* encoding P-type Na^+-ATPase, which is believed to be an important player in the halotolerance adaptation cascade response, is up-regulated as the ambient pH goes up (**CARACUEL ET AL. 2003**). Therefore, halophilic or halotolerant species may hold a clue towards elucidating the mechanisms of the ability to thrive at high pH. The molecular aspects of the ability to cope with high ambient pH have not been studied in filamentous fungi. Future work aimed at revealing these molecular properties could be carried out by contrasting the genomics of neutrophiles and alkaliphiles. Such a project might provide answers to the intriguing questions inherent in the alkaliphily phenomenon.

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SUPPLEMENTARY TABLE 1. List of taxa used for phylogenetic reconstructions. Strains used in the growth experiments and newly generated accessions are in bold.

Taxon	Voucher	Appearance in phylogenetic analysis (1,2)	LSU	ITS	β-tub	RPB2	TEF1-α	SSU
Acremonium acutatum^T	CBS 682.71	1	HQ231965					
Acremonium alternatum^T	CBS 407.66	1	HQ231988					
Acremonium bisepturn^T	CBS 750.69	1	HQ231998					
Acremonium brachypenium^T	CBS 866.73	1	HQ232004					
Acremonium breve^T	CBS 150.62	1	HQ232005					
Acremonium chrysogenum^T	CBS 144.62	1	HQ232017					
Acremonium curvulum^T	CBS 430.66	1	HQ232026					
Acremonium curvulum	CBS 229.75	1	HQ232021					
Acremonium exuviarum^T	UAMH 9995	1,2	HQ232036	AY882946	AY882947	-	-	
Acremonium flavum^T	CBS 596.70	1	HQ232037					
Acremonium fuci^T	CBS 112868	2		AY632653	AY632690	-	-	
Acremonium fuci	CBS 113889	2		AY632652	-	-	-	
Acremonium fuci	UAMH 6508	1	HQ232038					
Acremonium gamsii^T	CBS 726.71	1	HQ232040					
Acremonium inflatum^T	CBS 212.69	1	HQ232050					
Acremonium inflatum	CBS 439.70	1	HQ232051					
Acremonium persicinum^T	CBS 310.59	1	HQ232077					
Acremonium persicinum	CBS 101694	1	HQ232085					
Acremonium persicinum	CBS 102349	1	HQ232086					
"*Acremonium potronii*"	CBS 189.70	1	HQ232094					
"*Acremonium potronii*"	CBS 379.70F	1,2	HQ232096	AY632655	AY632691	-	-	

Acremonium radiatum^{T}	CBS 142.62	1	HQ232104					
Acremonium roseolum^{T}	CBS 289.62	1	HQ232123					
Acremonium rutilum^{T}	CBS 396.66	1	HQ232124					
Acremonium salmoneum^{T}	CBS 721.71	1	HQ232125					
Acremonium salmoneum	JS-NJ01	2		HM747162	-	-	-	
Acremonium sclerotigenum^{T}	CBS 124.42	1	HQ232126					
***Acremonium sclerotigenum* A101**		**1**	**KC987215**	**KC987139**	**KC987101**	**KC998999**	**KC998961**	**KC987177**
***Acremonium sclerotigenum* A130**		**1**	**KC987242**	**KC987166**	**KC987128**	**KC999024**	**KC998988**	**KC987204**
Acremonium sclerotigenum	CBS 786.69	1	HQ232130					
Acremonium sordidulum^{T}	CBS 385.73	1	HQ232136					
***Acremonium* sp. A104**		**1,2**	**KC987217**	**KC987141**	**KC987103**	**KC999001**	**KC998963**	**KC987179**
***Acremonium* sp. A105**		**1,2**	**KC987218**	**KC987142**	**KC987104**	**KC999002**	**KC998964**	**KC987180**
***Acremonium* sp. A106**		**1,2**	**KC987219**	**KC987143**	**KC987105**	**KC999003**	**KC998965**	**KC987181**
***Acremonium* sp. A107**		**1,2**	**KC987220**	**KC987144**	**KC987106**	**KC999004**	**KC998966**	**KC987182**
***Acremonium* sp. A108**		**1,2**	**KC987221**	**KC987145**	**KC987107**	**KC999005**	**KC998967**	**KC987183**
***Acremonium* sp. A109**		**1**	**KC987222**	**KC987146**	**KC987108**	**KC999006**	**KC998968**	**KC987184**
***Acremonium* sp. A110**		**1,2**	**KC987223**	**KC987147**	**KC987109**	**KC999007**	**KC998969**	**KC987185**
***Acremonium* sp. A111**		**1,2**	**KC987224**	**KC987148**	**KC987110**	**KC999008**	**KC998970**	**KC987186**
***Acremonium* sp. E102**		**1,2**	**KC987248**	**KC987172**	**KC987134**	**KC999030**	**KC998994**	**KC987210**
Acremonium tubakii^{T}	CBS 790.69	1	HQ232148					
Acremonium tubakii	CBS 111360	2		AY632654	AY632689	-	-	
"*Acremonium tubakii*"	CBS 824.69	1	HQ232149					
Acremonium verruculosum^{T}	CBS 989.69	1	HQ232150					
"*Cephalosporium malorum*"^{T}	CBS 117.25	1	HQ232015					
"*Cephalosporium purpurascens*"^{T}	CBS 149.62	1	HQ232071					
Didymostilbe echinofibrosa	AR 2824	1	AY489706					
***Emericellopsis alkalina* A103**		**1,2**	**KC987216**	**KC987140**	**KC987102**	**KC999000**	**KC998962**	**KC987178**
***Emericellopsis alkalina* A112**		**1,2**	**KC987225**	**KC987149**	**KC987111**	**KC999009**	**KC998971**	**KC987187**
***Emericellopsis alkalina* A113**	**FW-1476**	**1,2**	**KC987226**	**KC987150**	**KC987112**	**KC999010**	**KC998972**	**KC987188**
***Emericellopsis alkalina* A114**	**FW-1473**	**1,2**	**KC987227**	**KC987151**	**KC987113**	**KC999011**	**KC998973**	**KC987189**
***Emericellopsis alkalina* A115**	**FW-1474**	**1,2**	**KC987228**	**KC987152**	**KC987114**	**KC999012**	**KC998974**	**KC987190**
***Emericellopsis alkalina* A116**		**1,2**	**KC987229**	**KC987153**	**KC987115**	-	**KC998975**	**KC987191**
***Emericellopsis alkalina* A117**	**FW-1471**	**1,2**	**KC987230**	**KC987154**	**KC987116**	**KC999013**	**KC998976**	**KC987192**
***Emericellopsis alkalina* A118**		**1,2**	**KC987231**	**KC987155**	**KC987117**	**KC999014**	**KC998977**	**KC987193**
***Emericellopsis alkalina* A119**		**1,2**	**KC987232**	**KC987156**	**KC987118**	**KC999015**	**KC998978**	**KC987194**
***Emericellopsis alkalina* A120**		**1,2**	**KC987233**	**KC987157**	**KC987119**	**KC999016**	**KC998979**	**KC987195**
***Emericellopsis alkalina* A121**		**1,2**	**KC987234**	**KC987158**	**KC987120**	**KC999017**	**KC998980**	**KC987196**
***Emericellopsis alkalina* A122**		**1,2**	**KC987235**	**KC987159**	**KC987121**	**KC999018**	**KC998981**	**KC987197**
***Emericellopsis alkalina* A123**		**1,2**	**KC987236**	**KC987160**	**KC987122**	**KC999019**	**KC998982**	**KC987198**
***Emericellopsis alkalina* A124**		**1,2**	**KC987237**	**KC987161**	**KC987123**	**KC999020**	**KC998983**	**KC987199**
***Emericellopsis alkalina* A125**		**1,2**	**KC987238**	**KC987162**	**KC987124**	**KC999021**	**KC998984**	**KC987200**
***Emericellopsis alkalina* A126**		**1,2**	**KC987239**	**KC987163**	**KC987125**	**KC999022**	**KC998985**	**KC987201**
***Emericellopsis alkalina* A127**		**1,2**	**KC987240**	**KC987164**	**KC987126**	-	**KC998986**	**KC987202**
***Emericellopsis alkalina* A128**		**1,2**	**KC987241**	**KC987165**	**KC987127**	**KC999023**	**KC998987**	**KC987203**
***Emericellopsis alkalina* E101**^{T}	**CBS 127350 (=VKM F-4108)**	**1,2**	**KC987247**	**KC987171**	**KC987133**	**KC999029**	**KC998993**	**KC987209**
***Emericellopsis alkalina* M14**	**CBS 120043 (=VKM F-3905)**	**1,2**	**KC987244**	**KC987168**	**KC987130**	**KC999026**	**KC998990**	**KC987206**
***Emericellopsis alkalina* M20**	**CBS 120044 (=VKM F-3040)**	**1,2**	**KC987245**	**KC987169**	**KC987131**	**KC999027**	**KC998991**	**KC987207**
***Emericellopsis alkalina* M71**	**CBS 120049 (=VKM F-3907)**	**1,2**	**KC987246**	**KC987170**	**KC987132**	**KC999028**	**KC998992**	**KC987208**
Emericellopsis donezkii^{T}	CBS 489.71	2		AY632658	AY632674	-	-	
Emericellopsis glabra^{T}	CBS 119.40	2		AY632657	AY632673	-	-	
Emericellopsis glabra	A.R. 3614	2		HM484860	HM484879	-	HM484843	
Emericellopsis humicola^{T}	CBS 180.56	2		AY632659	AY632675	-	-	
***Emericellopsis maritima*^{T}**	CBS 491.71 (=VKM F-1082)	1,2	**KC987251**	**KC987175**	**KC987137**	**KC999033**	**KC998997**	**KC987213**
Emericellopsis microspora^{T}	CBS 380.62	2		AY632663	AY632679	-	-	
***Emericellopsis minima*^{T}**	CBS 190.55 (=VKM F-1484)	1,2	**KC987249**	**KC987173**	**KC987135**	**KC999031**	**KC998995**	**KC987211**
Emericellopsis minima	CBS 111361	2		AY632661	AY632677	-	-	
Emericellopsis minima	CBS 871.68 (=VKM F-1057)	1,2	**KC987250**	**KC987174**	**KC987136**	**KC999032**	**KC998996**	**KC987212**
Emericellopsis minima	CCFC226707	1	AY283560					
Emericellopsis mirabilis	CBS 177.53	2		AY632656	-	-	-	
***Emericellopsis pallida*^{T}**	CBS 490.71 (=VKM F-925)	1,2	**KC987252**	**KC987176**	**KC987138**	**KC999034**	**KC998998**	**KC987214**
Emericellopsis pallida	CBS 624.73	2		AY632667	AY632683	-	-	
Emericellopsis robusta	CBS 489.73	2		AY632664	AY632680	-	-	
Emericellopsis salmosynnemata	CBS 382.62	2		AY632666	AY632682	-	-	
Emericellopsis stolkiae^{T}	CBS 159.71	2		AY632668	AY632684	-	-	

Emericellopsis synnematicola^{T}	CBS 176.60	2		AY632665	AY632681	-	-	
Emericellopsis terricola^{T}	CBS 120.40	1,2	U57082	U57676	-	-	-	
Emericellopsis terricola	CBS 229.59	1,2	AY305034	AY632662	AY632678	-	-	
Emericellopsis terricola	CCF3815	2		FJ430737	-	-	-	
Emericellopsis terricola	NRRL 54109	2		HQ698592	-	-	-	
Geosmithia lavendula	IFO 7729	1	D88325					
Geosmithia putterillii	IFO 31131	1	AB047215					
Gliomastix masseei^{T}	CBS 794.69	1	HQ232060					
Gliomastix murorum	CBS 157.72	1	HQ232067					
Gliomastix polychroma^{T}	CBS 181.27	1	HQ232091					
Gliomastix roseogrisea^{T}	CBS 134.56	1	HQ232121					
Glomerella cingulata	FAU 553	1	AF543786					
Hapsidospora irregularis	ATCC 22087	1	AF096192					
Heleococcum aurantiacum	CBS 201.35	1	JX158442					
Heleococcum japonense	CBS 397.67	1	JX158441					
Hydropisphaera erubescens	ATCC 36093	1	AY545726					
Melanopsamma pomiformis	ATCC 18873	1	AY489709					
Mycoarachis inversa	ATCC 22107	1	GQ505991					
Mycopepon smithii	SMH 1609	1	AF279400					
Myrothecium verrucaria	BBA 70749	1	AJ301999					
Nigrosabulum globosum	ATCC 22102	1	AF096195					
Peethambara spirostriata	CBS 110115	1	AY489724					
Peethambara sundara	CBS 646.77	1	AF193245					
Roumegueriella rufula	CBS 346.85	1	DQ518776					
Sarocladium attenuatum^{T}	CBS 399.73	1	HQ232165					
Sarocladium bacillisporum^{T}	CBS 425.67	1	HQ231992					
Sarocladium bactrocephalum^{T}	CBS 749.69	1	HQ231994					
Sarocladium glaucum^{T}	CBS 796.69	1	HQ232041					
Sarocladium kiliense^{T}	CBS 122.29	1	HQ232052					
Sarocladium ochraceum^{T}	CBS 428.67	1	HQ232070					
Sarocladium oryzae	CBS 180.74	1	HQ232166					
***Sarocladium* sp. A131**		**1**	**KC987243**	**KC987167**	**KC987129**	**KC999025**	**KC998989**	**KC987205**
Sarocladium strictum^{T}	CBS 346.70	1	HQ232141					
Sarocladium zeae^{T}	CBS 801.69	1	HQ232152					
Scopinella solani	CBS 770.84	1	AY015632					
Selinia pulchra	AR 2750	1	AF193246					
Selinia pulchra	AR 2812	2		HM484859	HM484884	-	HM484841	
Stachybotrys chartarum	ATCC 9182	1	AY489714					
Stanjemonium grisellum	NRRL 26548	1	AF049171					
Stanjemonium grisellum^{T}	CBS 655.79	2		AY632671	AY632687	-	-	
Stanjemonium ochroroseum^{T}	CBS 656.79	2		AY632672	AY632688	-	-	
Stilbella fimetaria	D99026	2		AY952467	-	-	-	
Stilbella fimetaria	DAOM 229279	1	HQ232176					
Stilbella fimetaria	MH178	2		FJ430712	-	-	-	
Stilbella fimetaria	SES201	2		FJ939394	-	-	-	
Verrucostoma freycinetiae^{T}	MAFF 240100	2		HM484866	HM484885	-	HM484853	

Chapter IV

On the diversity of fungi from soda soils

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Abstract

The diversity of filamentous fungi that can grow at high ambient pH values (i.e., 8–11) remains largely understudied. Here we study 100 alkalitolerant and alkaliphilic isolates from the soils around the basin of soda lakes in Asia and Africa to assess the major evolutionary lineages and morphologies pertinent to the alkaliphilic trait in filamentous fungi. The *Emericellopsis* lineage (*Hypocreales, Hypocreomycetidae*), along with *Plectosphaerellaceae* (*Hypocreomycetidae*), *Pleosporaceae* (*Dothideomycetes*), *Chaetomiaceae* (*Sordariomycetidae*) families appeared to be overrepresented with strong alkalitolerants and effective alkaliphiles. In particular, *Sodiomyces* species (*Plectosphaerellaceae*), *Acrostalagmus luteoalbus* (*Plectosphaerellaceae*), *Emericellopsis alkalina* (*Hypocreales*), *Thielavia* sp. (*Chaetomiaceae*), and *Alternaria* sect. *Soda* (*Pleosporaceae*) grew best at high ambient pH. The pH tolerance of *Chordomyces antarcticum*, *Acrostalagmus luteoalbus* and some other species was largely affected by the presence of extra Na^+ in the growth medium. Moderate alkalitolerants included *Scopulariopsis* members (*Microascales*), *Fusarium*, *Cladosporium*, and many asexual acremonium-like species from *Bionectriaceae*. Weak alkalitolerants were represented by sporadic isolates of *Penicillium*, *Purpureocillium lilacinum*, and *Alternaria alternata* species, with the growth optimum at neutral or acidic pH.Weak alkalitolerants develop loose dry chains of spores easily dispersed by air. Their presence at low frequency with the growth optimum at neutral or acidic pH leads us to treat them as transient species in the alkaline soils, as those are also ubiquitous saprobes in normal soils. Our phylogenetic analyses show that the alkaliphilic trait in filamentous fungi has evolved several times. Several lineages harboring strong alkalitolerants derived from the known marine-borne fungi (*Emericellopsis, Alternaria* sect. *Phragmosporae*), or fall within the fungi associated with halophytic grasses (*Pleosporaceae*). Soda soils contain a diversity of fungi that range from weak alkalitolerant to alkaliphilic, which in few cases is associated with darkly pigmented mycelium and formation of microsclerotia. The alkaliphilic trait is spread throughout the *Ascomycota*, and usually juxtaposes with slime-covered polyphyletic acremonium-, verticillium-, gliocladium-types of asexual morphology, hyphae aggregating in chords, and enclosed fruit bodies.

Key words

alkaliphilic fungi, pH, soda soils, extremophile, *Sodiomyces*, *Acrostalagmus*, *Chordomyces*, *Verticillium*, *Emericellopsis*, *Acremonium*, *Scopulariopsis*, *Alternaria*, *Thielavia*, *Plectosphaerellaceae*, *Pleosporaceae*

Introduction

Nature possesses ample variation in environmental niches with diverse abiotic conditions. In some locations, abiotic factors (such as temperature, ion content and pH) may deviate from those of most habitats, resulting in the formation of specific zones termed ‘extreme habitats’ restricting growth of most organisms. Soda soils (or alkaline soils) with high pH values (>8)

represent an example of such extreme habitats. They usually develop in arid and semi-arid lands throughout the world, and may vary in salt concentrations from low to saturation. The driving force for the soda accumulation is the depletion of Ca^{2+} trapped by CO_3^{-2} éion, leaving Na^+ as the dominant cation (**JONES ET AL. 1998**). Soda soils formas a result of carbonate accumulation under poor Ca^{2+} and Mg^{2+} conditions. Water evaporation reinforces the process of soda accumulation. With few exceptions, soda soils usually are highly saline as both abiotic factors coincide.

Naturally, as high ambient pH and salts impose significant stress on a living organism, the overall biodiversity is expected to be compromised. Surprisingly, soda lakes, water basins which are often formed in the area of soda soils, have been shown to be highly productive, in fact, more productive than their freshwater counterparts. As pointed out by **ZAVARZIN (1993)**, soda lakes should exhibit complete recycling of the nutrients. Studies on the biodiversity of microorganisms in soda lakes have revealed abundant bacterial communities, which also act as primary producers, usually dominated by cyanobacteria species (**ANTONY ET AL. 2013**). High densities of bacterial and archaeal blooms may even cause the coloration of water basins associated with the soda soils, for example, as in Magadi Lake (Kenya) due to massive expansion of red haloalkaliphilic *Archaea* (**JONES ET AL. 1998**). Studies of 16S rDNA gene sequences revealed a complex phylogenetically heterogeneous structure of the bacterial communities inhabiting soda lakes (**DUCKWORTH ET AL. 1996, REES ET AL. 2004, DONG ET AL. 2006, WANI ET AL. 2006**) often with the recovery of many new bacterial species (**SOROKIN & MUYZER 2010, SOROKIN ET AL. 2011, KOMPANTSEVA ET AL. 2012, ANTONY ET AL. 2012**). The accumulated data suggest that soda lakes in diverse geographical regions harbor alkaliphilic bacteria and *Archaea* from all major trophic groups (**REES ET AL. 2004**). Biodiversity surveys showed the presence of eukaryotic organisms thriving in alkaline lakes too, like plankton diatoms, green algae, cryptophytes and haptophytes (**NAGY ET AL. 2006, KERESZTES ET AL. 2012**). The vegetation in the soda soils area is usually dominated by halophytic grasses from *Chenopodiaceae* (**GRUM-GRZHIMAYLO ET AL. 2013A**). Often few dominant eukaryotic species may be encountered in soda lakes. For example, diverse crustaceans, like brine shrimp *Artemia salina*, as well as many species of copepods, are characteristic of hyper-saline alkaline water environments (**HAMMER 1986, SCHNEIDER ET AL. 2012**). Intriguingly, eukaryotic 18S rDNA sequences pool analysis seems to have recovered signatures of alveolata, stramenopiles, choanoflagellates, amoebozoans and cercozoans present in Lonar Lake (India) sediments (**ANTONY ET AL. 2013**). Recent studies by **XIONG ET AL. (2012)** and **LANZÉN ET AL. (2013)** confirmed both prokaryotic and eukaryotic biodiversity richness and heterogeneity by investigating several soda lakes employing high-throughput sequencing techniques.

Despite the general preference of fungi to grow at neutral or slightly acidic pH, some have been shown to grow at high pH too (**DI MENNA 1959, GOTO ET AL. 1981, AONO 1990, STEIMAN ET AL. 2004**). In many cases, alkaline soils were surveyed using neutral or acidic isolation media, thereby lowering the chances of recovering alkaliphilic strains. **LISICHKINA ET AL. (2003)** reported a first recovery of alkalitolerant yeast species from soda soils using alkaline selective medium. In **1993**, Okada and collaborators isolated the first alkaliphilic acremonium-like hyphomycete from manure, and named it *Acremonium alcalophilum*, as there was no other known alkaliphilic

Acremonium species at that time (**OKADA ET AL. 1993**).

Later, extensive studies by **NAGAI ET AL. (1995, 1998)** focused on the taxonomic distribution of fungi in alkaline limestone caves in Japan as well as from Indonesian grassland. Using an alkaline medium, members of *Acremonium*, *Fusarium*, *Gliocladium*, *Mucor* and *Plectosporium* genera were isolated frequently. Many other species were termed alkalitolerant as they are capable of growing to some extent at elevated pH, but with the growth optimum still lying at neutral or acidic pH values. In particular, *Acremonium* species had shown wide alkalitolerance and this genus sensu lato was proposed to have properties to tolerate high ambient pH values. The abundance of alkalitolerant strains from alkaline habitats led to assumption that these strains are physiologically active in this environment. Interestingly, however, according to **NAGAI ET AL. (1995)**, acidic soils harbored few alkaliphiles as well, which is in line with similar observations in the studies of alkaliphilic bacterial communities (**HORIKOSHI & AKIBA 1982, HORIKOSHI 1999**). Generally speaking, the frequency of alkaliphilic fungi is low, while alkalitolerants seem to be far more widespread, as alkalitolerants may often be encountered in many neutral types of soil.

The vast majority of studies on the alkaliphilic and alkalitolerant fungi conducted so far lack DNA sequence data to robustly identify the strains. As mentioned above, many alkalitolerant fungi possess acremonium-type asexual development as one of the simplest structures found in asexual fungi known to be highly polyphyletic throughout *Ascomycota* (**SUMMERBELL ET AL. 2011**), imposing substantial difficulties for proper identification. It is therefore important to use DNA-based information to be able to pinpoint the taxonomy of a studied isolate. The sequencing data are also needed to understand whether the alkaliphilic trait in fungi has evolved once or several times throughout evolutionary history. Evidence for the latter scenario has been provided by our recent studies, in which we described few new taxonomically diverse alkaliphilic and alkalitolerant filamentous fungi inhabiting soda soils (**GRUM-GRZHIMAYLO ET AL. 2013A, B**). Being polyphyletic, halophilic filamentous fungi have been shown to utilize different strategies of dealing with extreme ambient salt stress (**KIS-PAPO ET AL. 2014**).

In the present study, we undertook a comprehensive approach to better understand the evolution of alkaliphily in fungi. To address this, on a buffered selective medium (pH 10) with an antibiotic, as well as on neutral common media, we isolated over 100 strains of alkalitolerant and alkaliphilic fungi sampled from the alkaline soils with different degree of salinity collected in Russia, Mongolia, Kazakhstan, Kenya, Tanzania, and Armenia. Essentially all isolated strains were tested for their pH preference to provide insight into the frequency and distribution of alkaliphiles in natural populations. The pH effect on the growth of the recovered isolates could discriminate between the physiologically active species and accidental transient species in alkaline habitats. Next, we sequenced several loci and reconstructed phylogenies to determine the positions of the alkaliphilic fungal lineages. Our strains were genetically heterogeneous and displayed various taxonomic affinities. We hence selected phylogenetically informative loci, which would better resolve the taxonomic group of interest according to the recent published studies. We studied representative strains using light microscopy as well as with a scanning electron microscope, and made photographic plates highlighting morphological features along

with some drawings to better depict the key morphologies.

To make the present work more complete, we combined our new data with selected data from our previous studies (**GRUM-GRZHIMAYLO ET AL. 2013A, B**). Noteworthy, our isolation method recovered only ascomycetous fungi. Overall, we found the alkaliphilic/strong alkalitolerant phenotype in about 2/3 of our recovered strains from soda soils. In addition, our data show a strong bias towards fungi having simple reproductive structures, which is in line with the previous studies. The results indicate that asexual sporulation with conidia likely glued together by slime (for example, in *Sodiomyces*, *Acremonium*, *Acrostalagmus*, *Verticillium*) forming compact heads correlates with the ability to tolerate high ambient pH. The systematic approach involving growth, microscopy and phylogenetic multi-gene analyses helped us to evaluate characteristic morphological characters of alkalitolerant and alkaliphilic fungi that evolved independently in several lineages (genus *Emericellopsis* and families *Plectosphaerellaceae*, *Pleosporaceae*, *Chaetomiaceae*) across *Ascomycota*.

Materials and methods

Locations and soda soil samples

We collected samples of the soda soils at the locations shown on the map in **FIG. 1**. The pH of the samples varied from 7.6 to 11 with the range of total soluble salts of 1.4–310 g/kg. The details on the isolation places are provided in **SUPPLEMENTARY TABLE 1**.

Strains and media

For initial isolation of alkalitolerant/alkaliphilic strains we used alkaline agar (AA) selective medium (ca. pH 10) supplemented with antibiotic rifampicin (2 g/l), as in **GRUM-GRZHIMAYLO ET AL. (2013A)**. Rifampicin was selected as the most effective antibiotic for the suppression of bacterial growth at alkaline conditions without hindering the fungal growth, among tested: ampicillin, amoxicillin (clavulanic acid), amikacin, bacitracin, chloramphenicol, clindamycin, doxycycline, erythromycin, gentamicin, kanamycin, lincomycin, levofloxacin, neomycin, novobiocin, ofloxacin, oxytetracycline, penicillin, tetracycline, tobramycin, vancomycin and rifampicin. In parallel, we implemented MYA, CZ and WA media (**MUELLER ET AL. 2004**) for the possible recovery of weak alkalitolerant fungi. Emerged fungal isolates were purified and pre-identified by morphological characters. To avoid redundancy, in few cases of joint isolations from both media, we discarded the co-isolated strains down to a minimum number of morphotypes from a particular soil sample. The recovered fungal isolates were kept at 6 °C for further characterization. Newly acquired strains were deposited at the CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands) and partly in the All-Russian Collection of Microorganisms (Pushchino, Russia). Colony morphology was characterized by growing the strains on several types of media: WA, CZ, MYA, PDA, OA, and AA (malt/yeast extract based) (**MUELLER ET AL. 2004**, **GRUM-GRZHIMAYLO ET AL. 2013A**) at 28 °C in the dark. Additionally, newly acquired *Alternaria*

strains were grown on SNA (synthetic nutrient-poor agar) medium (**NIRENBERG 1976**) at room temperature to induce sporulation as described in **WOUDENBERG ET AL. (2013)**. For pH-optimum elucidation, we implemented race tubes (**PERKINS AND POLLARD 1986**) with MYA-based medium buffered at pH from 4 to 11.2, as follows. The complete medium consisted of two components: the salt/buffer component **(1)** and the nutrient component **(2)**. 500 ml of **(1)** had 0.2 M concentration of buffer supplemented with 5 g NaCl (Merck), 1 g KNO_3 (Merck), 1 g K_2HPO_4 (Merck). 500 ml of **(2)** contained 17 g malt extract (Merck), 1 g yeast extract (BBL), 20 g agar (Duchefa Biochemie). **(1)** and **(2)** were autoclaved separately at 120 °C for 20 min, allowed to cool down to 55 °C and then mixed in 1:1 ratio, yielding final concentrations of the complete medium: 0.1 M buffer, 5 g/l NaCl, 1 g/l KNO_3, 1 g/l K_2HPO_4, 17 g/l malt extract, 1 g/l yeast extract, 20 g/l agar. Buffer choices for generating different final pH values were: $Na_3C_6H_5O_7$/citric acid system for pH 4, 5.2, Na_2HPO_4/NaH_2PO_4 system for pH 5.9, 7, 7.8, Na_2CO_3/$NaHCO_3$ carbonate system for pH 8.7, 9.8, Na_2HPO_4/NaOH system for pH 11.2. The above listed pH values are measured final pH of the complete media, which in few cases differed from the initial pH of the **(1)** component alone. Race tubes were inoculated in duplicate with agar plugs (1 x 1 mm) from the parental strain grown on Petri dish. Race tubes were put at 28 °C in the dark, and depending on the strain, the growth expansion was followed for 1–2 months.

Morphology

We used light microscopy (LM), scanning electron microcopy (SEM), and low-temperature scanning electron microscopy (cryoSEM) to morphologically characterize the isolated strains. For microscopy studies, fungal strains were taken from the medium, where the most developed morphology was observed. Specimens for SEM and cryoSEM were prepared as described previously (**GRUM-GRZHIMAYLO ET AL. 2013A**). *Alternaria* specimens for LM were prepared as in **WOUDENBERG ET AL. (2013)**. Characteristic structures were recorded digitally. However, we drew the reproductive structures of a few representative strains to clarify the morphology seen in the photomicrographs. Taxonomic novelties were deposited in MycoBank (**WWW.MYCOBANK.ORG; CROUS ET AL. 2004**).

DNA extraction, PCR, and sequencing

For total genomic DNA (gDNA) extraction, mycelium was grown on a cellophane membrane placed on top of the AA medium. The collected mycelium was stored in Eppendorf tubes at -80 °C until used. For gDNA extraction, we used approximately 0.1 g of wet mycelium for each sample preparation with DNeasy PlantMini kit (Qiagen, Chatsworth, CA), according to the manufacturer's instructions. The quality and quantity of the isolated gDNA was verified on a NanoDrop 2000.We amplified various loci of our newly isolated strains, however, not all of them were used for phylogenetic reconstructions. A total set of the studied genes include the following: large and small subunit rDNA (LSU and SSU), internal transcribed spacers 1 and 2 including 5.8S rDNA (ITS), second largest subunit of RNA polymerase II (RPB2), transcriptional elongation factor 1 subunit α (TEF1-α), actin (Act), glyceraldehyde-3-phosphate dehydrogenase

(GAPDH), and tryptophan synthase (TS). PCR primer sets for above listed loci were the following: LR0R/LR9 (LSU), NS1/NS8 (SSU), ITS1f/ITS4r (ITS), fRPB2-5F/fRPB2-7cR (RPB2), EF1α-983f/EF1α-2218r (TEF1-α). For verticillia strains, we used VActf/VActr (Act), VGPDf2/VGPDr (GAPDH), and VTs3f/VTs3r (TS) as in (**INDERBITZIN ET AL. 2011A**). For *Alternaria* isolates in section *Soda*, we additionally amplified and sequenced the GAPDH marker with gpd1/gpd2 primer pair, as in (**BERBEE ET AL. 1999**). PCR mixes, PCR products purification, and sequencing procedures of the remainder loci were performed as described previously (**GRUM-GRZHIMAYLO ET AL. 2013A**). For the details on the selection of the annealing temperatures in PCR, see **TABLE 1**. Raw chromatograms of the newly generated sequences were viewed, edited and assembled with CodonCodeAligner v. 3.7.1 (CodonCode Corporation, Dedham, MA) and DNAStar Lasergene EditSeq v. 7.1.0 (DNASTAR Inc., Madison, WI). The resultant sequences were deposited in GenBank (**SUPPLEMENTARY TABLES 2–9**).

TABLE 1. Detais of the PCRs, loci, DNA substitution models, and phylogenetic matrices used in the study.

Taxonomic group treated	Loci used	Primer pair	PCR T_a	DNA substitution model	Characters	Informative characters	Uninformative variable characters	Invariable characters	BI PP mean -LnL	ML BP mean -LnL
Ascomycota	SSU	NS1/NS8	Touchdown PCR (62 °C to 52 °C)	GTR+I+G	1629	428	205	996	12261.38	12076.38
Plectosphaerellaceae	LSU	LR0R/LR9	Touchdown PCR (59 °C to 49 °C)	GTR+I+G	814	144	65	605	6576.06	6326.72
	ITS	ITS1f/ITS4r	51 °C	GTR+I+G	472	139	26	307		
Verticillium s. str.	Act	VActf/VActr	51 °C	HKY+G	452	219	18	215	7293.48	7229.27
	GAPDH	VGPDf2/VGPDr	Touchdown PCR (67 °C to 57 °C)	TIM1+I+G (GTR+I+G)*	559	140	14	405		
	TS	VTs3f/VTs3r	59 °C	TIM2+I+G (GTR+I+G)*	411	151	9	251		
	ITS	ITS1f/ITS4r	51 °C	TIM3ef+G (GTR+G)*	418	62	5	351		
Sodiomyces	ITS	ITS1f/ITS4r	51 °C	TIM3+I (HKY+I)*	509	13	13	483	929.66	878.19
Scopulariopsis	LSU	LR0R/LR9	Touchdown PCR (59 °C to 49 °C)	TIM3+I (GTR+G)*	498	51	29	418	5438.52	5360.91
	TEF1-α	EF1α-983f/EF1α-2218r	Touchdown PCR (66 °C to 56 °C)	TIM3+I+G (GTR+I+G)*	931	136	123	672		
Lasiosphaeriaceae/ Chaetomiaceae	LSU	LR0R/LR9	Touchdown PCR (59 °C to 49 °C)	GTR+I+G	810	103	70	637	7590.02	7387.85
	ITS	ITS1f/ITS4r	51 °C	TVM+I+G (GTR+I+G)*	456	136	30	290		
Pleosporaceae	SSU	NS1/NS8	Touchdown PCR (62 °C to 52 °C)	TIM2+I+G (GTR+I+G)*	1020	74	31	915	19040.51	18751.68
	LSU	LR0R/LR9	Touchdown PCR (59 °C to 49 °C)	GTR+I+G	849	70	33	746		
	RPB2	fRPB5F/fRPB7cR	Touchdown PCR (60 °C to 50 °C)	TIM2+I+G (GTR+I+G)*	865	359	49	457		
	ITS	ITS1f/ITS4r	51 °C	SYM+I+G	480	123	29	328		
Alternaria sect. *Soda*	GAPDH	gpd1/gpd2	Touchdown PCR (65 °C to 55 °C)	TrN+I+G (GTR+I+G)*	484	113	36	335	2578.57	2516.9

* — for MrBayes

Phylogenetic analyses

To analyze the taxonomy of our strains, we performed several phylogenetic reconstructions with the various sets of loci resulting in a different degree of resolution. Appropriate reference sequences were obtained from GenBank. Multiple sequence alignments were created with online MAFFT v. 7 service (**KATOH AND STANDLEY 2013**) using automatic alignment strategy. The resulted alignments were reviewed and corrected manually. Ambiguously aligned regions, long gaps and introns were removed from the alignments using BioEdit v. 7.1.3.0 (**HALL 1999**). The first dataset focused on the class-wide taxonomy of *Ascomycota* using single SSU locus to accommodate our all studied strains from soda soils. Next, for most of the strains, we produced separate trees

FIG. 1. Sampling locations. **A.** Map of the soda soil sampling sites. **B.** Zhivopisnoe Lake, Kulunda Steppe (Russia). **C.** Nuhe-Nur Lake, Trans-Baikal area (Russia). **D.** Petuchovskoe Lake, Kulunda Steppe (Russia). **E.** Aktumsyk Cape, Aral Lake (Kazakhstan). **F.** Ulan-Nur Lake, Gobi Desert (Mongolia). **G.** Magadi Lake (Kenya).

on levels of families or genera. The set of trees include the taxonomic reconstructions of the studied strains within: *Plectosphaerellaceae* (LSU, ITS), *Verticillium* s. str. (Act, GAPDH, TS, ITS), *Sodiomyces* (ITS), *Scopulariopsis* (LSU, TEF1-α), *Lasiosphaeriaceae/Chaetomiaceae* (LSU, ITS), *Pleosporaceae* (SSU, LSU, RPB2, ITS), and *Alternaria* sect. *Soda* (GAPDH). For the details on the above mentioned phylogenetic analyses, see **TABLE 1**. In the phylogenetic analyses, which involved more than one gene, matrixes were concatenated using Mesquite v. 2.75 (**MADDISON AND MADDISON 2011**). All multi-gene matrixes were divided into unlinked partitions corresponding to each gene. The best fit model for DNA substitution was estimated with jModelTest v. 2.1.1 (**GUINDON AND GASCUEL 2003, DARRIBA ET AL. 2012**). Phylogenetic reconstructions were made with both Bayesian and maximum likelihood analyses. For Bayesian estimation of phylogeny, we used MrBayes v. 3.1.2 (**HUELSENBECK AND RONQUIST 2001**) with 4 Metropolis Coupled Markov Chain Monte Carlo (MCMCMC) chains running for 10 M generations starting with a random tree topology. Each 100th tree was sampled and their likelihood scores were checked in TRACER v. 1.5 (**RAMBAUT AND DRUMMOND 2007**) to verify the convergence of the runs. The burn-in factor was set to 50 % in all our phylogenetic inferences, which accepted stationary stable set of trees. The consensus tree was generated employing the 50 % majority rule with the recovery of posterior probabilities (BI PP) at each node. Maximum likelihood bootstrap analysis (ML BP) was performed with GARLI v. 2.0 (**ZWICKL 2006**) with 200 bootstrap replicates including five searches for each. A 50 % majority rule consensus tree was constructed with SumTrees v. 3.3.1 script within DendroPy v. 3.11.0 library (**SUKUMARAN AND HOLDER 2010**) running in Python v. 2.6 environment. For visualization of the consensus trees, we used TreeGraph v. 2.0.47-206 beta (**STÖVER AND MÜLLER 2010**) and Adobe Illustrator CS6 (Adobe Systems, San Jose, CA). The node supports were considered strong and displayed as thickened lines, if they gained joint supports of ML > 90 and PP > 0.94. Phylogenetic analyses and matrixes were deposited in TreeBase (**SUBMISSION ID 15631**).

Results

Soda soil samples

Initially we screened our soda soil samples using soil suspensions diluted in water, which did not recover any fungal isolates, but rather revealed massive bacterial expansion. This observation led us to use soil clumps put directly on the selective medium for the recovery of fungi. Additionally, we boosted up the concentration of antibiotic rifampicin to a final concentration of 2 g/l.

Strains

The frequency of fungi recovery from soda soils was substantially lower in comparison to neutral habitats even with salinification. Nonetheless, we recovered 48 new strains within the current framework, however, we shall take into account a total set of strains derived from our three studies (**GRUM-GRZHIMAYLO ET AL. 2013A, B**) to draw a broader picture of the distribution and

features of the fungi fromsoda soils. All combined, from soda soils at the edge of the lake basins, we isolated 100 strains of fungi with various degrees of alkalitolerance and taxonomic affinity within *Ascomycota*. The majority of the recovered strains (>90) readily produced asexual spores, whereas only members of *Sodiomyces* and two strains of *Emericellopsis* developed the sexual stage. The rest of the strains remained sterile during our investigation, although they often formed resting structures, like dark thickwalled mycelium or, sometimes, chlamydospores and microsclerotia (observed in *Pleosporaceae*). Our isolation method employing the MYA- and CZ-based medium buffered at pH 10 did not yield yeasts or basidiomycetous, zygomycetous, chytridiomycetous fungi as well as lower fungi-like organisms, like *Oomycota*.

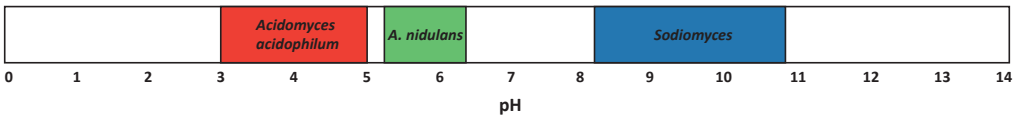


FIG. 2. Representatives of extreme physiologies with respect to pH effect. pH growth optima are outlinedwith colour boxes for each species. *Acidomyces acidophilum* CBS 335.97 – an acidophile, *Aspergillus nidulans* W6 – a neutrophile, *Sodiomyces* species – alkaliphiles.

Growth experiments

The growth experiments using plates with various media (WA, CZ, MYA, PDA, OA, AA) and race tubes with MYA-based medium (containing 5 g/l NaCl) ranging in pH values, showed different effect on growth of the studied isolates. The interpretation of growth experiments is often an ambiguous task, resulting in a high degree of subjectivity. Therefore we first shall set a few anchoring definitions, to orient across the growth pattern data we obtained. In **FIG. 2** we plotted three distinct growth classes with relation to pH: acidophiles, neutrophiles, and alkaliphiles. *Acidomyces acidophilum* species grows best at acidic pH (from 3 to 5), while *Sodiomyces* species display the opposite – their growth optimum lie at pH between 8.5 and 11 with no ability to grow at pH 4. The neutrophilic physiology is the most abundant, and here is represented by *Aspergillus nidulans*, which growth optimum is at pH around 5.5–6. These clearly distinct physiological boundaries are rarely observed, as there are many intermediate growth types.

In the current framework, we accept several categories of growth types with relation to pH, adopted from the existing bacterial studies. The alkaliphiles, with the growth optimum at pH above 8 are subdivided into obligate and facultative. Obligate alkaliphiles cannot grow at pH below 4–5, while facultative can. The next group is alkalitolerants, which can cope with high pH to different extent, however, the growth optimumlies at pH below 8 (at neutral, or even acidic pH). We further divide alkalitolerants into three classes by the severity at which the growth is hindered as the pH increases. These classes are: strong, moderate, and weak alkalitolerants. Weak alkalitolerants are often neutrophiles, which can barely cope with high ambient pH showing highly reduced growth. Moderate alkalitolerants are the isolates which

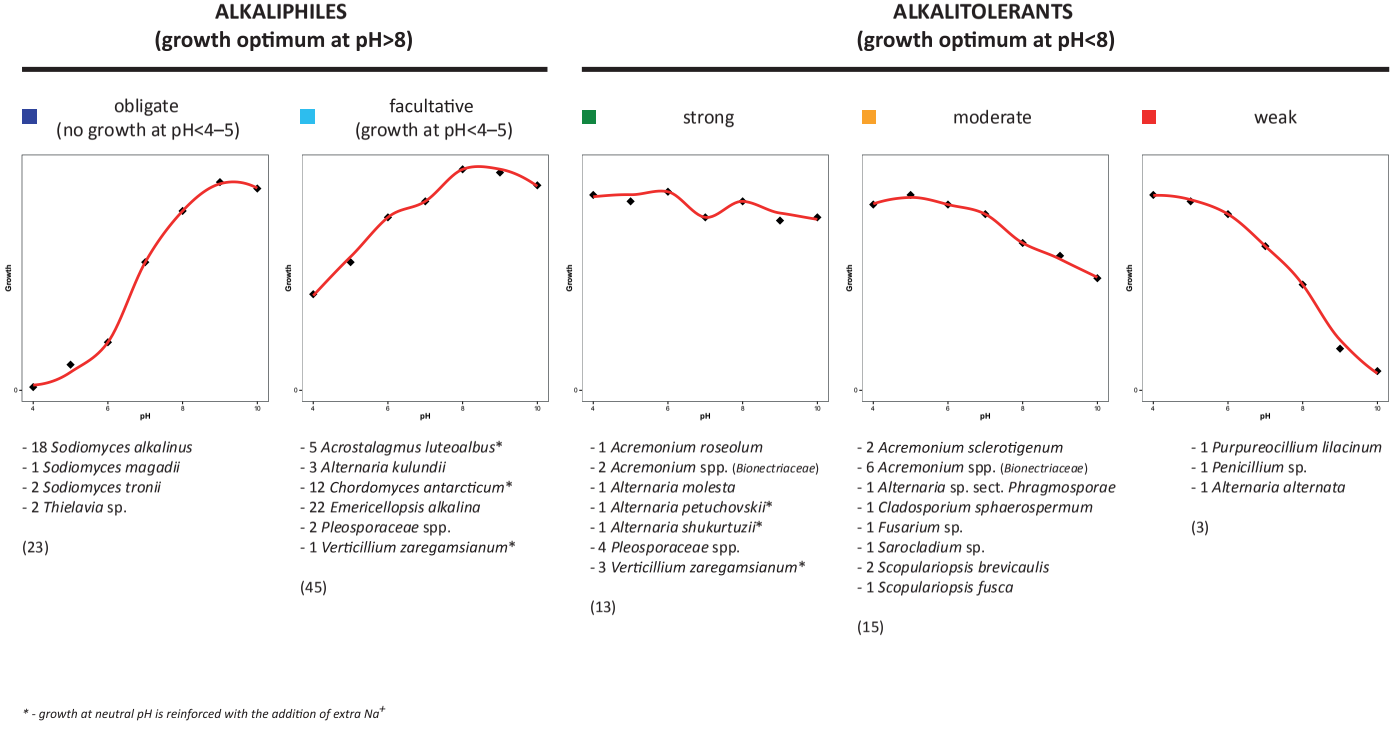


FIG. 3. Classification of the pH preferences of the fungi adopted in the current study. Number of the isolated strains are indicated. Note the colour codes, which are used in the subsequent phylogenetic tree.

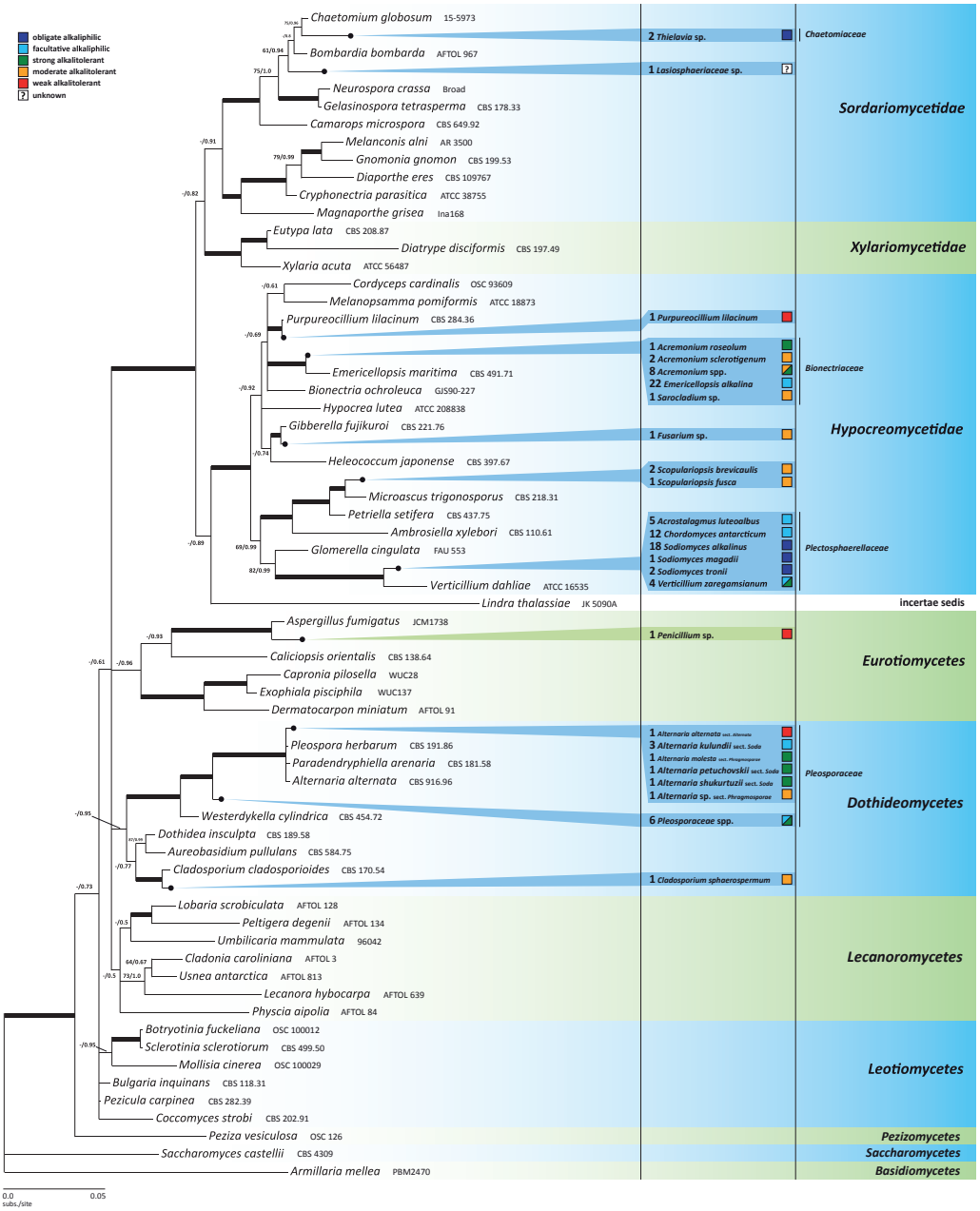


FIG. 4. Overview of the wide scale *Ascomycota* tree indicating the phylogenetic positions of the alkalitolerant and alkaliphilic isolates derived from the current study as well as from **GRUM-GRZHIMAYLO ET AL. 2013A, 2013B**. Bayesian 50 % majority rule consensus tree as inferred from the phylogenetic analysis of the single locus (SSU). Thickened branches indicate strong combined support (ML > 90, PP > 0.94). The colour-coded pH growth preference is indicated.

grow at about half the growth rate at high pH, as compared to neutral pH. Strong alkalitolerants show little or no reduction in growth at neutral pH with often wide pH growth optimum. In some cases, our growth patterns in Petri dishes on commonly used media at neutral pH and MYA-based media with various pHs are inconsistent. In such cases, the pH tolerance is greatly affected by the presence of extra Na^+ (in the form of NaCl) in the media. The terminology used in the current study is summarized in **FIG. 3**. It should be pointed out, that studying the pH effect alone is challenging, as this factor interweaves with osmotic pressure, water potential and ion concentrations effect, making the pH factor hard to disentangle.

In general, our results show that the *Plectosphaerellaceae* (**ZARE ET AL. 2007**) harbours a large number of strong alkalitolerants and alkaliphilic isolates (**FIG. 4, 5**). Genera *Chordomyces*, *Acrostalagmus*, and *Sodiomyces* of the *Plectosphaerellaceae* showed alkaliphilic phenotype, as seen in **FIG. 6, 7, 8** and **9**. For *Sodiomyces*, comparisons of growth in race tubes and on plates suggest that the presence of extra Na^+ does not have a large impact on the growth pattern. From the graphs and plate images, it is seen that the neutral pH media show reduced growth, while at high pH the growth substantially improved. These results led us to assume that *Sodiomyces* is more affected by the pH factor than the extra Na^+ supplementation, displaying truly alkaliphilic phenotype. *Chordomyces antarcticum* and *Verticillium zaregamsianum* isolates, on the other hand, seem to be more affected by the presence of extra Na^+. As seen in **FIG. 6** and **11**, the growth in tubes at neutral pH supplemented with NaCl was almost as good as at high pH. However, on plates with commonly used neutral media like MYA, PDA, and to a lesser extent on OA, the growth was greatly inhibited. The growth on AA medium was best for these species. Interestingly, CBS 987.87 isolate of *Chordomyces antarcticum* (identified as *Acremonium antarcticum* before) seems to possess less pH tolerance capacity compared to our isolates of *Chordomyces* from soda soils, as the growth was best at pH 6–7 with extra Na^+ in the medium. Facultative alkaliphile *Acrostalagmus luteoalbus* displays intermediate effect of extra Na^+êts growth was indeed improved with the addition of NaCl, but not as dramatic as for *Chordomyces antarcticum* or *Verticillium zaregamsianum* (see **FIG. 7**).

Two *Thielavia* sp. isolates M316 and M317, *Chaetomiaceae* (**DE HOOG ET AL. 2013, KRUYS ET AL. 2014, WANG ET AL. 2014**), displayed an obligate alkaliphilic phenotype, as seen in **FIG. 16**. Isolate *Lasiosphaeriaceae* sp. M314 showed different results on plate and in race tubes. In tubes, it displayed alkaliphily, while on plates the growth was better at neutral pH values; these observations did not allow us to establish the relation to pH for this strain in our study. Three *Scopulariopsis* isolates (two *S. brevicaulis* and one *S. fusca*) showed moderate alkalitolerant abilities confirmed by the growth experiments both in tubes and on plates. As seen in **FIG. 10**, the growth optima for *Scopulariopsis* isolates are at neutral pH, and OA medium in particular supported a better growth over the rest of neutral pH media (MYA, PDA). Growth on CZ medium was the fastest, but the sporulation was reduced. *Scopulariopsis fusca* displayed somewhat better alkalitolerance as compared to *S. brevicaulis* isolates. Single case isolations of *Cladosporium sphaerospermum*, *Purpureocillium lilacinum*, *Fusarium* sp., and *Penicillium* sp. show weak to moderate alkalitolerance, as seen in **FIG. 22**.

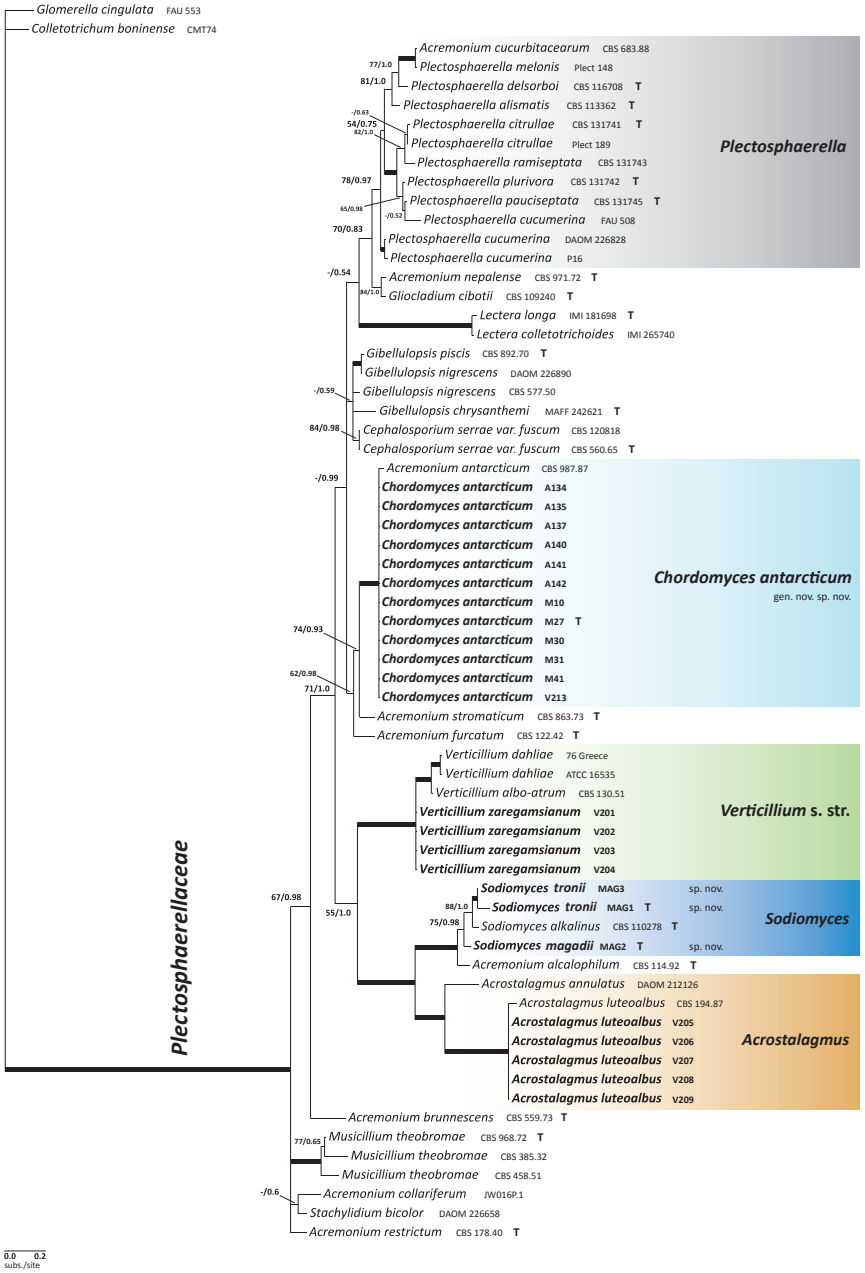


FIG. 5. Position of the alkalitolerant and alkaliphilic isolates within *Plectosphaerellaceae*. Bayesian 50 % majority rule consensus tree as inferred from the phylogenetic analysis of two loci (LSU, ITS). Thickened branches indicate strong combined support (ML > 90, PP > 0.94). **T** – type/ex-type strains; designation holds for the remainder of the figures throughout the study.

Isolates falling in the *Pleosporaceae* (**HYDE ET AL. 2013**) showed different capabilities for the pH tolerance (**FIG. 18, 19** and **21**). *Alternaria* isolates displayed various degrees of alkalitolerance: weak alkalitolerant (single *A. alternata* sect. *Alternata* isolate G408),moderate alkalitolerant *Alternaria* sp. sect. *Phragmosporae* M301 and strong alkalitolerant *A. molesta* sect. *Phragmosporae* M312, and five strong alkalitolerant/facultative alkaliphilic isolates (M304^T, M307^T, M309, M310, M313^T) of new *Alternaria* species fall in a new section *Soda* described below. Outside *Alternaria*, allied to

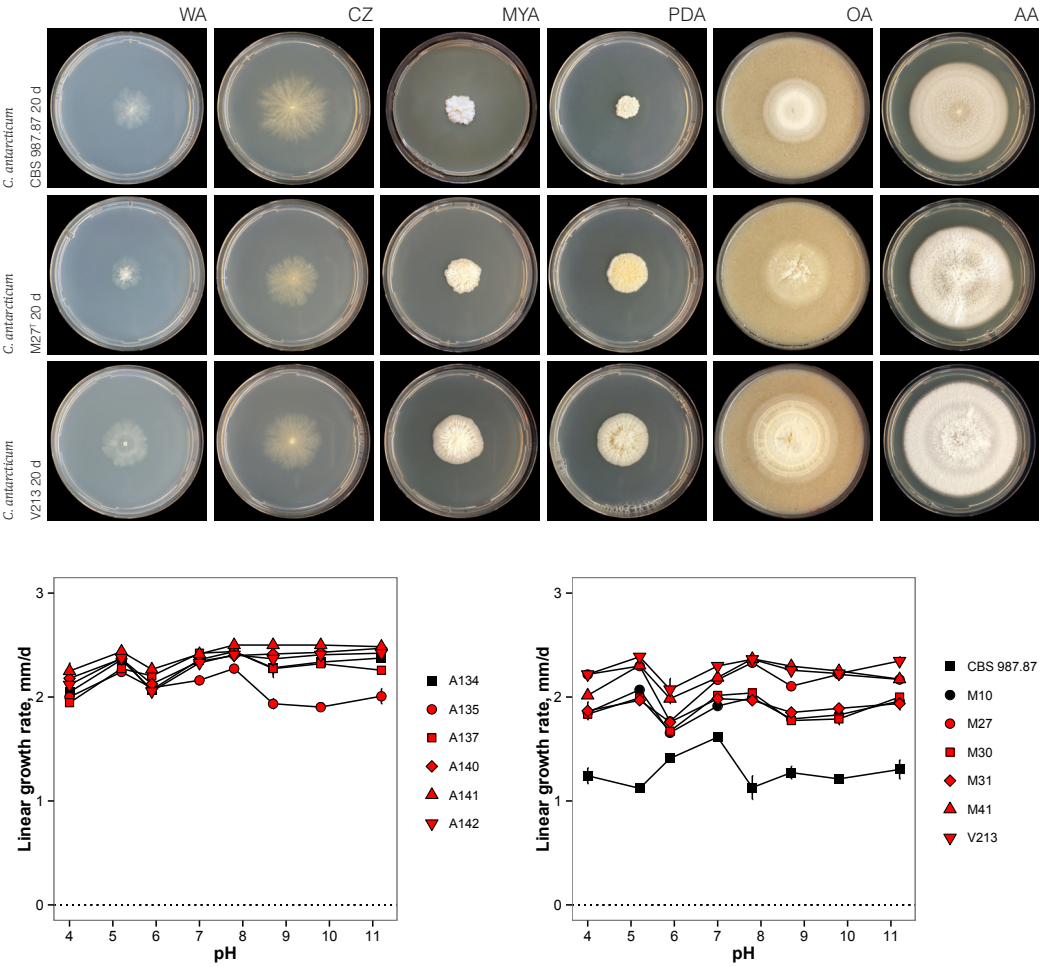


FIG. 6. Facultative alkaliphilic *Chordomyces antarcticum* (*Plectosphaerellaceae*) colony morphology on various around-neutral pH (except for AA with pH 10) media (top) and linear growth rate patterns at different pH values in race tubes (below). Media in race tubes contained extra Na^+ as compared to neutral WA, CZ, MYA, PDA, and OA media used for growth on plates. Extra Na^+ boosts growth at neutral pH for *Chordomyces*.

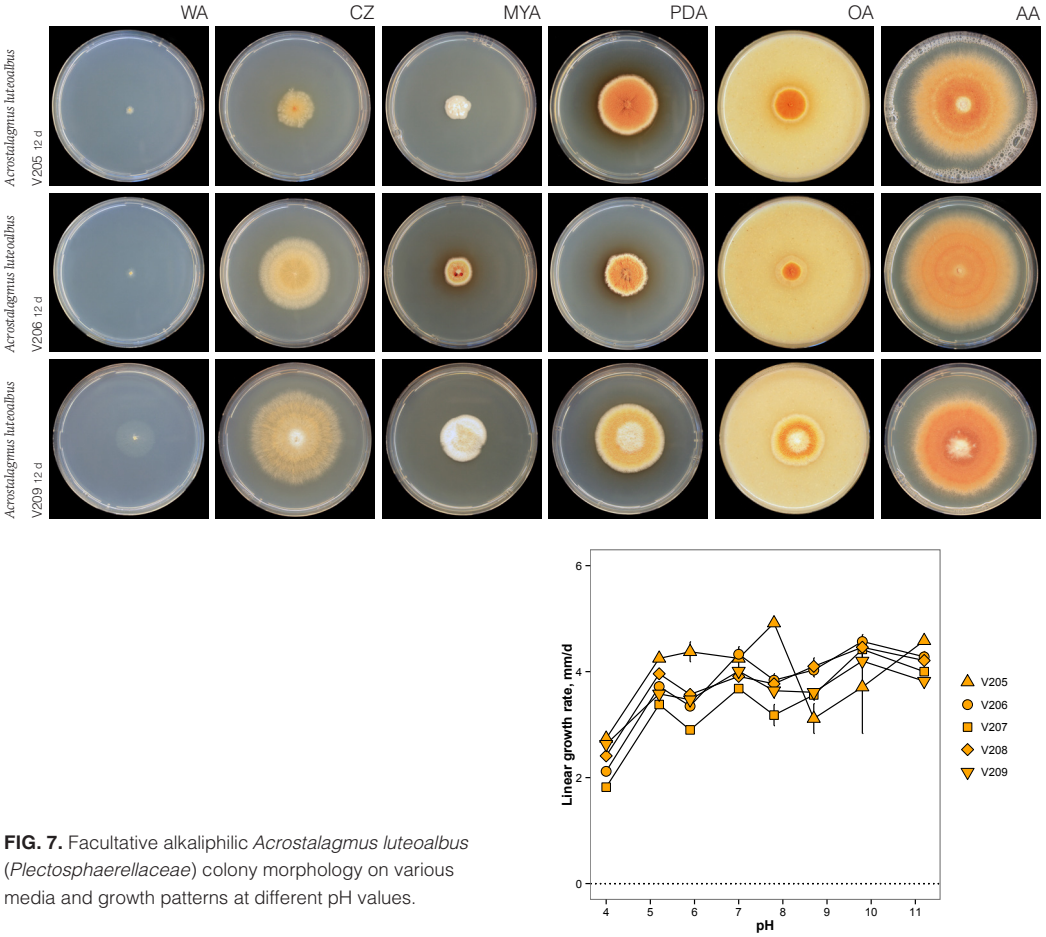


FIG. 7. Facultative alkaliphilic *Acrostalagmus luteoalbus* (*Plectosphaerellaceae*) colony morphology on various media and growth patterns at different pH values.

Pleospora, we obtained six isolates, which have been sterile. Four of them (M302, M303, M306, and M315) show strong alkalitolerance. Finally, sterile *Pleosporaceae* spp. M305 and M311 isolates display facultative alkaliphilic phenotype.

Phylogenetic analyses

Ascomycota (SSU) We reconstructed a high-level *Ascomycota* tree to map all our newly isolated strains from soda soils onto the ascomycetous lineages (**FIG. 4**). We used the SSU locus as a single partition with 66 taxa sampled throughout several phyla within *Ascomycota*. The basidiomycetous *Armillaria mellea* PBM2470 and *Saccharomyces castellii* CBS 4309 yeast served as an outgroup for this phylogenetic analysis. The dataset contained 1629 characters of which 428 (26.3 %) were phylogenetically informative. The Bayesian MCMC runs converged after

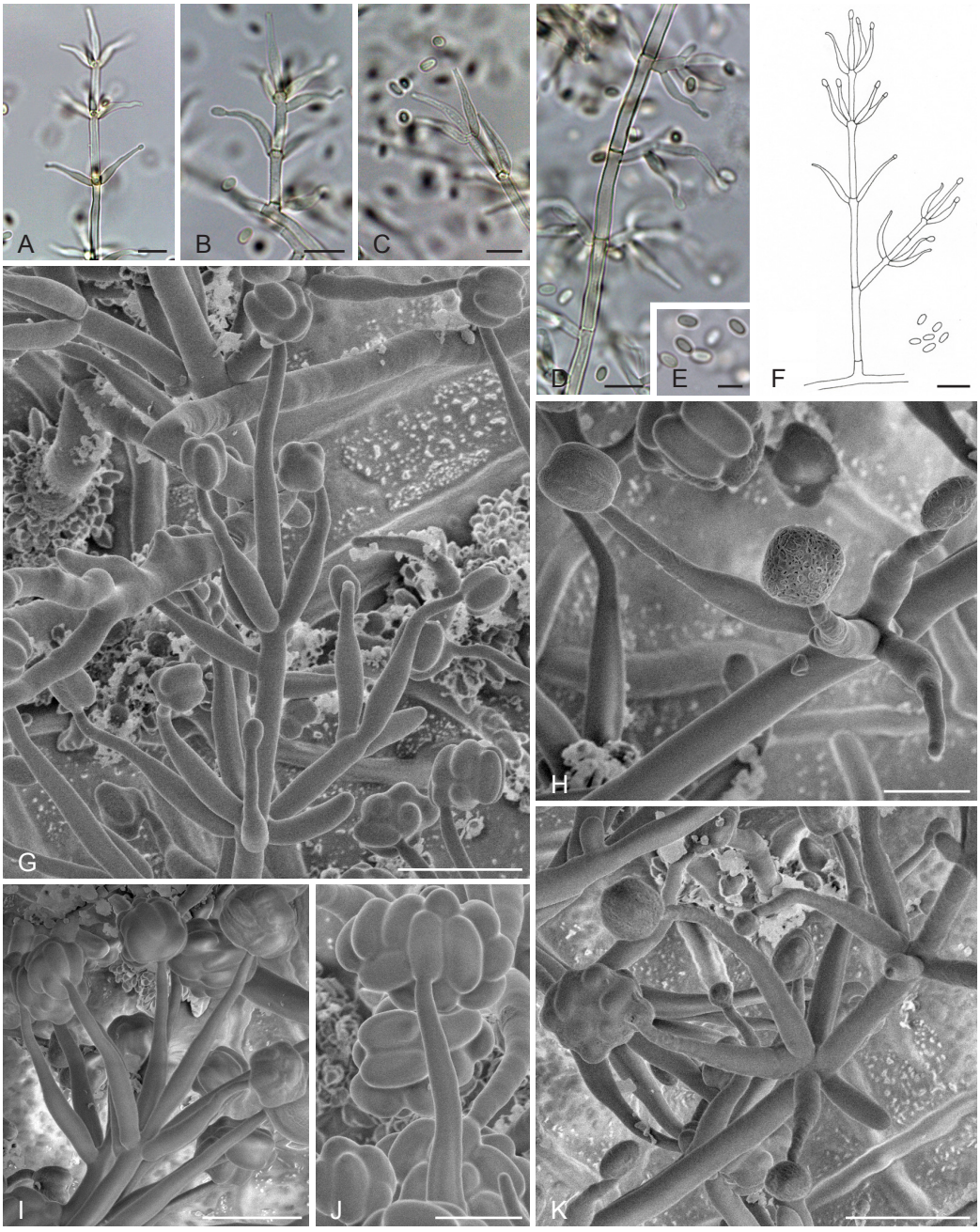


FIG. 8. *Acrostalagmus luteoalbus* V205 (= CBS 137625): **A–D.** Conidiophores and conidia (LM). **E.** Conidia (LM). **F.** Conidiophore with conidia. **G.** Conidiophore tip (cryoSEM). **H, K.** Phialide nodes (cryoSEM). **I, J.** Conidial heads (cryoSEM). *Scale bars:* **A–D, F, G, I, K** = 10 µm; **E, H, J** = 5 µm.

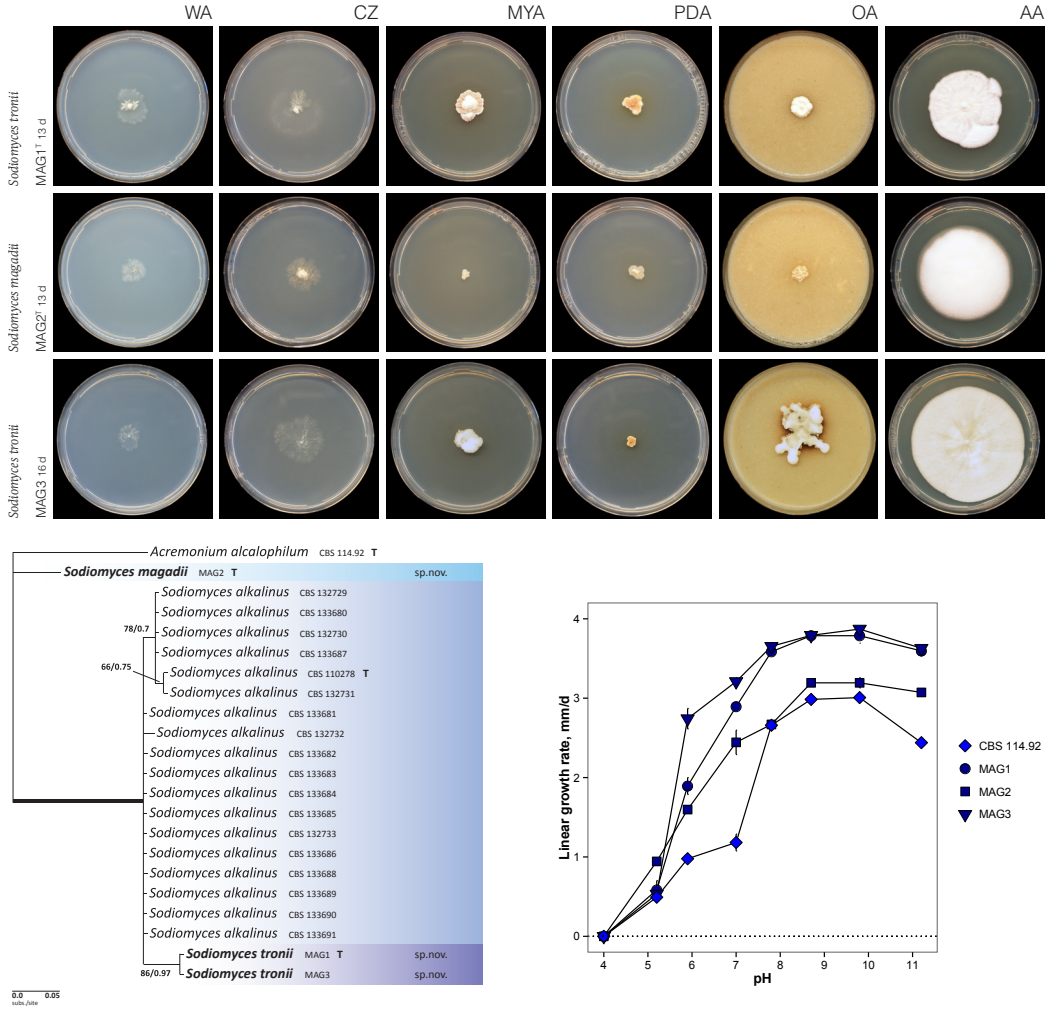


FIG. 9. Obligate alkaliphilic *Sodiomyces tronii* and *Sodiomyces magadii* (*Plectosphaerellaceae*) species colony morphology on various media. The phylogenetic position of *Sodiomyces* species is shown on the 50 % majority rule Bayesian consensus tree, which is constructed based on single ITS locus. Thickened branch indicate strong combined support (ML > 90, PP > 0.94). The growth pattern at various pH values is given also for *Acremonium alcalophilum* CBS 114.92^{T} ex-type isolate. Note that for *Sodiomyces*, extra Na^{+} present in linear growth experiment has little effect on alkaliphilic phenotype, compared to *Chordomyces antarcticum*.

5 M generations and remained stationary afterwards with the deviation of approximately 0.006 between the runs. Our single-locus tree provided enough resolution to identify the major lineages of *Ascomycota*. The clade delineation followed from the **SPATAFORA ET AL. (2006)**. On this tree, we often used a single sequence reference to serve as a point for depicting the position of

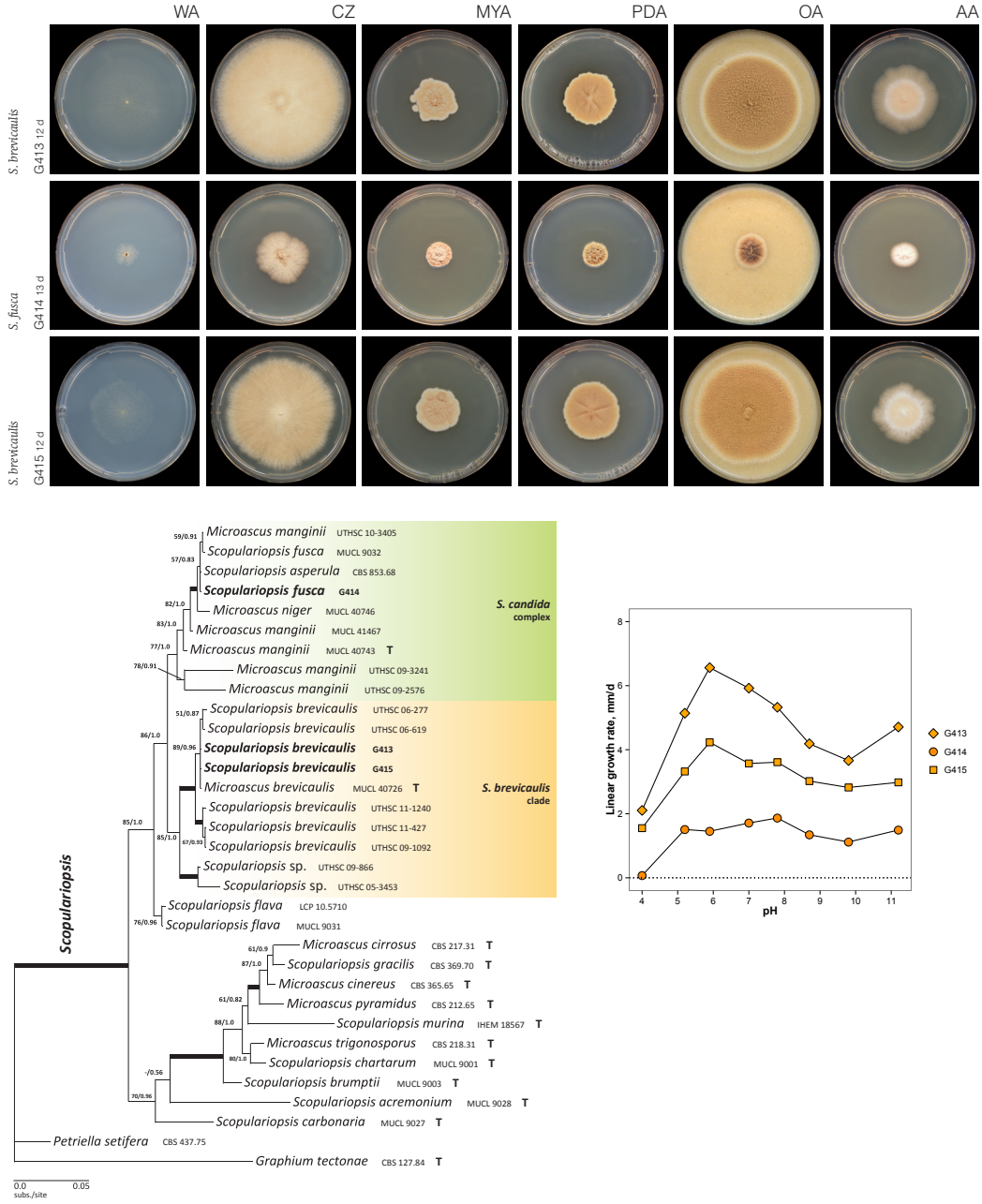


FIG. 10. Moderate alkalitolerant isolates of *Scopulariopsis* (*Microascales*) colony morphology on various media. The tree is a 50 % majority rule Bayesian consensus as inferred from the analysis of two loci (LSU, TEF1-α). Thickened branches indicate strong combined support (ML > 90, PP > 0.94). Growth patterns at different pH values are displayed.

our multiple strains isolated from soda soils. To show the distribution of the alkalitolerant trait across our isolates, we included the colour-coded pH growth preference, as inferred from our growth experiments. Our alkalitolerant and alkaliphilic isolates fall within many taxonomic lineages across *Ascomycota*. However, our results indicate that subphylum *Hypocreomycetidae* seems to be particularly enriched with alkaliphilic and alkalitolerant fungi, as 80 % of our isolates belong there. The presented phylogeny based on the single SSU gene analysis approach is not meant for high phylogenetic accuracy, as it is used only to illustrate the approximate positions of our alkalitolerant fungi throughout the *Ascomycota*. Later in this study, we will treat most of the alkalitolerant and alkaliphilic fungal lineages in a greater detail. Noteworthy, for drawing a broader picture of the biodiversity of fungi and their frequencies in soda lakes, we include strains from our recent studies (**GRUM-GRZHIMAYLO ET AL. 2013A, B**).

Plectosphaerellaceae (LSU, ITS) The dataset for the *Plectosphaerellaceae* (*Hypocreomycetidae*) contained 65 taxa and included two loci concatenated in a single alignment (LSU and ITS). The sequences for the alignment varied little and thus were easily aligned. The alignment comprised 1286 characters with 283 (22 %) of phylogenetically informative characters, which resolved major genera within the *Plectosphaerellaceae* (**FIG. 5**). Sister members of *Glomerellaceae*, *Glomerella cingulata* FAU 553 and *Colletotrichum boninense* CMT74 were used to root the tree. Members of *Plectosphaerella* fall into 78/0.97 supported clade. Recently described isolates of *P. oligotrophica* (**LIU ET AL. 2013**) sister to *P. ramiseptata* and *P. citrullae* ex-types were not included in our analysis. Another recently introduced *Plectosphaerellaceae* genus *Lectera*, harboring plantassociated species (**CANNON ET AL. 2012**) clearly stand out in a long-branched clade with maximal support values. Our new twelve facultative alkaliphilic isolates robustly (100/1.0) group with the known isolate identified as *Acremonium antarcticum* CBS 987.87, which currently has no nomenclatural status. We designated a type isolate (strain M27^{T}) for this clade and accommodated a new genus, *Chordomyces*, with a single species – *Chordomyces antarcticum*. Morphology of the ex-type culture is provided in **FIG. 30**. Several other isolates of *Chordomyces antarcticum* were included in morphological analysis with low-temperature SEM (see **FIG. 31**). Also, we obtained five facultative alkaliphilic isolates, which were identified as *Acrostalagmus luteoalbus* (**FIG. 8**), as they unambiguously (100/1.0) group within known CBS 194.87 isolate, a combination introduced by **ZARE ET AL. (2004)**. Obligate alkaliphilic *Sodiomyces* isolates group in a well-supported clade (75/0.98), few of them display different morphological features and diverge molecularly, therefore, they were described here as new species, *S. tronii* and *S. magadii*. The morphology and ITS-based phylogeny of the *Sodiomyces* species is treated below.

Sodiomyces (ITS) We isolated two new obligate alkaliphilic species of *Sodiomyces* from Magadi Lake (Kenya), which is supported by both morphological and phylogenetic analyses. The ITS phylogeny of *Sodiomyces* (*Plectosphaerellaceae*) isolates is depicted in **FIG. 9**, which is rooted to a close alkaliphilic asexual *Acremonium alcalophilum* CBS 114.92^{T} ex-type strain. The matrix for this analysis had 509 characters, only 13 of which were phylogenetically informative (2.5 %). Such a small variation within the ITS locus was enough to reconstruct the phylogenetic relationships within the *Sodiomyces*. Two isolates of *S. tronii* (MAG1^{T}, MAG3) form a well-supported clade

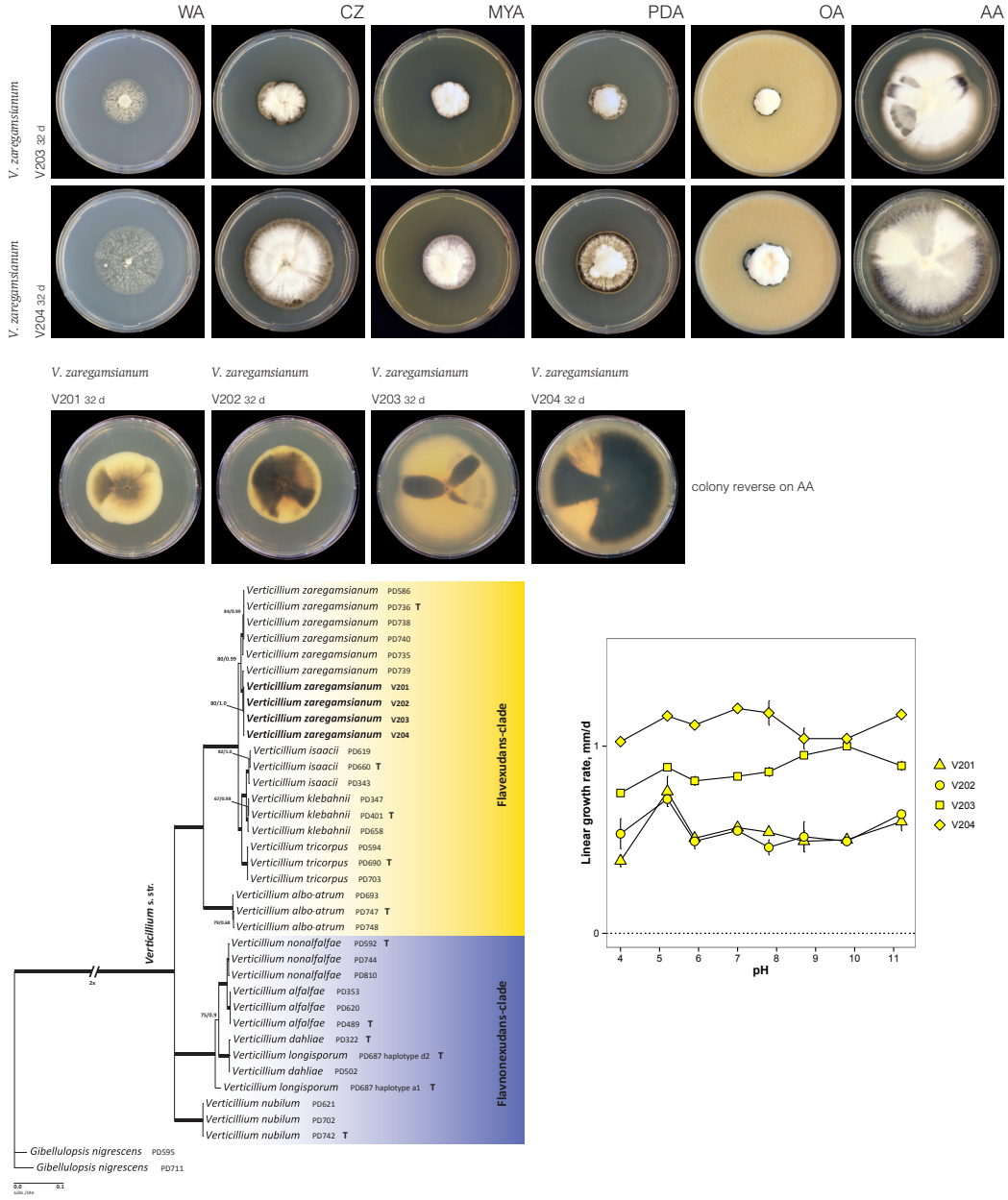


FIG. 11. *Verticillium zaregamsianum* colony morphology on various media. The position of the strong alkalitolerant (strains V201, V202, V204) and facultative alkaliphilic (strain V203) *Verticillium zaregamsianum* isolates are displayed on the tree. The tree is a 50 % majority rule Bayesian consensus as inferred from the phylogenetic analysis of the four loci (Act, GAPDH, TS, ITS). Thickened branches indicate strong combined support (ML > 90, PP > 0.94). The growth patterns at different pH values are given.

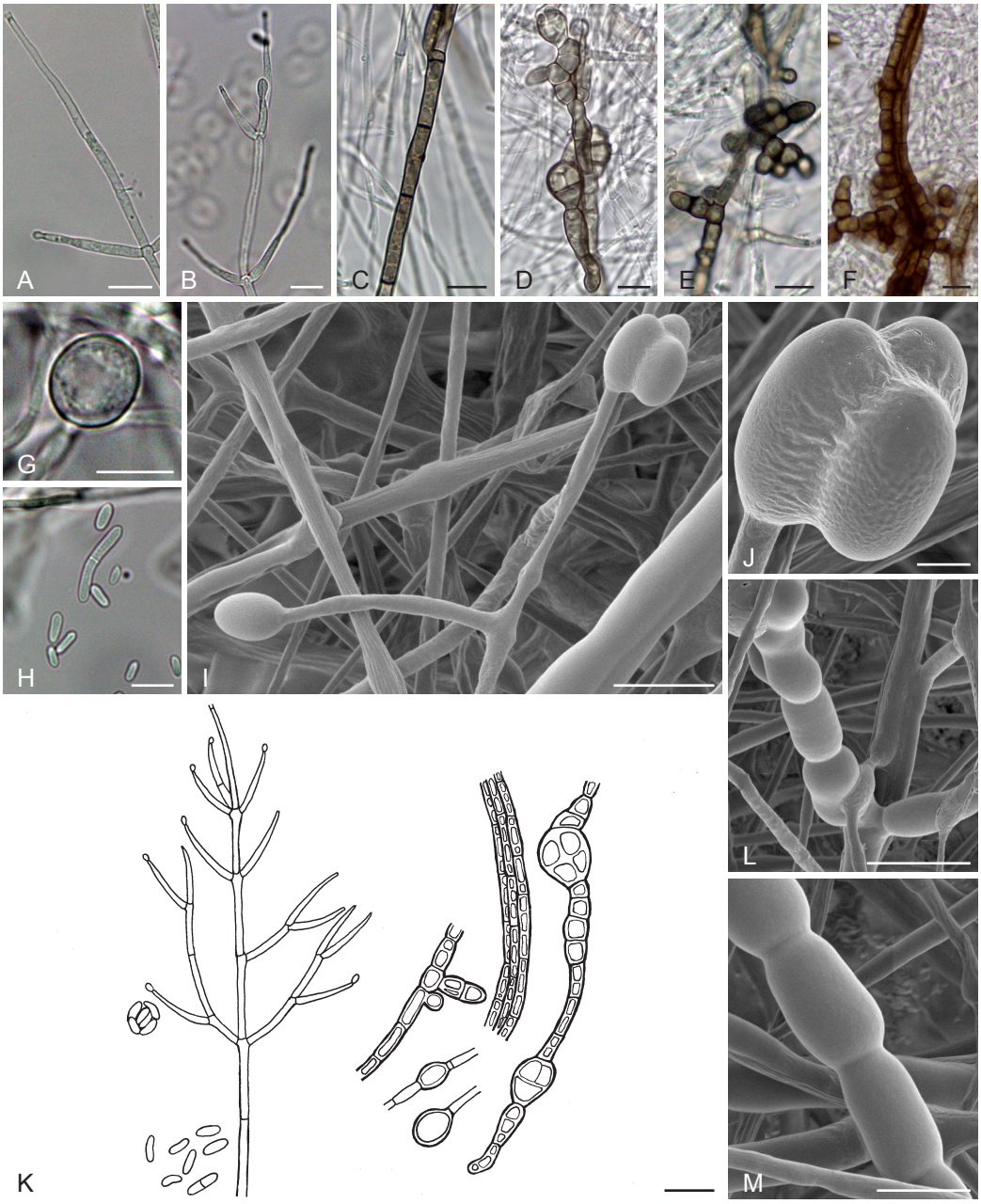


FIG. 12. *Verticillium zaregamsianum* V204 (= CBS 137624): **A, B.** Conidiophores (LM). **C.** Resting mycelium (LM). **D–F.** Microsclerotia (LM). **G.** Chlamydospore (LM). **H.** Conidia (LM). **I.** Conidiophore with conidia (cryoSEM). **J.** Clump of conidia (cryoSEM). **K.** Conidiophore, conidia, chlamydospores, resting mycelium and microsclerotia. **L, M.** Microsclerotia (cryoSEM). *Scale bars:* **A–H, K, L** = 10 µm; **I, M** = 5 µm; **J** = 1 µm.

(86/0.97) within a group of *S. alkalinus* isolates, while *S. magadii* (MAG2^T) is placed more basally to the *S. alkalinus* group, closer to *Acremonium alcalophilum* CBS 114.92^T ex-type isolate. *Sodiomyces* therefore has been sized up to 21 isolates grouped in 3 species. The morphology of new species is shown in **FIG. 27, 28**, and we also made a comparative morphological chart with three species of *Sodiomyces* showing differentiating criteria between the species (**FIG. 29**).

Verticillium sensu stricto (Act, GAPDH, TS, ITS) Robust identification of four *Verticillium* strains (V201, V202, V203, V204) recovered from soda soil at different depths at Orongoyskoe Lake required the analysis of extra loci, other than ITS and LSU alone. For this, we followed the **INDERBITZIN ET AL. (2011B)** and **HYDE ET AL. (2014)** studies utilizing Act, GAPDH, TS, along with ITS sequences. The phylogenetic analysis of these loci for *Verticillium* s. str. clade (*Plectosphaerellaceae*) is shown in **FIG. 11**. The final concatenated matrix contained 1840 characters with 31 % of them being phylogenetically informative. Members of the sister genus *Gibellulopsis* was used to root the tree. Our isolates group within the *Verticillium zaregamsianum* clade of reference isolates falling into *Flavexundans*-clade with good support values (80/0.99), and therefore identified as such. *Verticillium* species of *Flavexundans*-clade are characterized by the presence of yellow pigment, which is also present in our isolates (see **FIG. 11**) adding extra evidence for proper identification of our strains. Morphological features of the *V. zaregamsianum* V204 isolate are depicted in **FIG. 12**. Interestingly, although four isolates of *V. zaregamsianum*, sampled at different depths, were identical across four studied loci, but the growth patterns though seem to differ. V201 and V202 isolates recovered from 30 to 70 cm depth, seem to be less alkalitolerant than V203 and V204 recovered at greater depth at about 60 to 95 cm.

Scopulariopsis (LSU, TEF1-α) Three *Scopulariopsis* isolates (*Microascales*) with moderate alkalitolerance were identified as *S. brevicaulis* and *S. fusca* based on both morphological data and two-locus phylogenetic analysis (see **FIG. 10, 13** and **14**). Double-loci, LSU and TEF1-α, concatenated phylogenetic matrix contained 1429 characters, which include 13 % of phylogenetically informative characters (mostly in TEF1-α gene). The reference species designation was taken from **SANDOVAL-DENIS ET AL. (2013)**. Two isolates, G413 and G415, fall within the well-supported *S. brevicaulis*-clade (85/1.0), whereas isolate G414 falls within the sister group – *S. candida* complex. The identification of the G414 isolate to the species level was largely based on the morphological features.

Lasiosphaeriaceae/Chaetomiaceae (LSU, ITS) Two obligate alkaliphilic strains recovered from Kunkur Steppe (Russia) and Ararat Valley (Armenia) – M316 and M317 fall into the *Chaetomiaceae* grouping with *Thielavia australiensis* ATCC 28236^T ex-type strain with good support values (83/1.0), as seen in **FIG. 15**. Unfortunately, M316 and M317 isolates have not formed morphological structures based onwhich we could describe these strains in detail and they are very closely related phylogenetically. We found two synonymous mutations in RPB2 gene and one insertion mutation in ITS region between M316 and M317 isolates. Interestingly, the eye-catching feature of M316 is that it contains a group 1 intron in the SSU gene spanning for 416 nucleotides as opposed to M317 isolate. In spite of phylogenetic identity of LSU and ITS to *Thielavia australiensis* ATCC 28236^T ex-type, we therefore shall identify them as members of *Thielavia*, even though

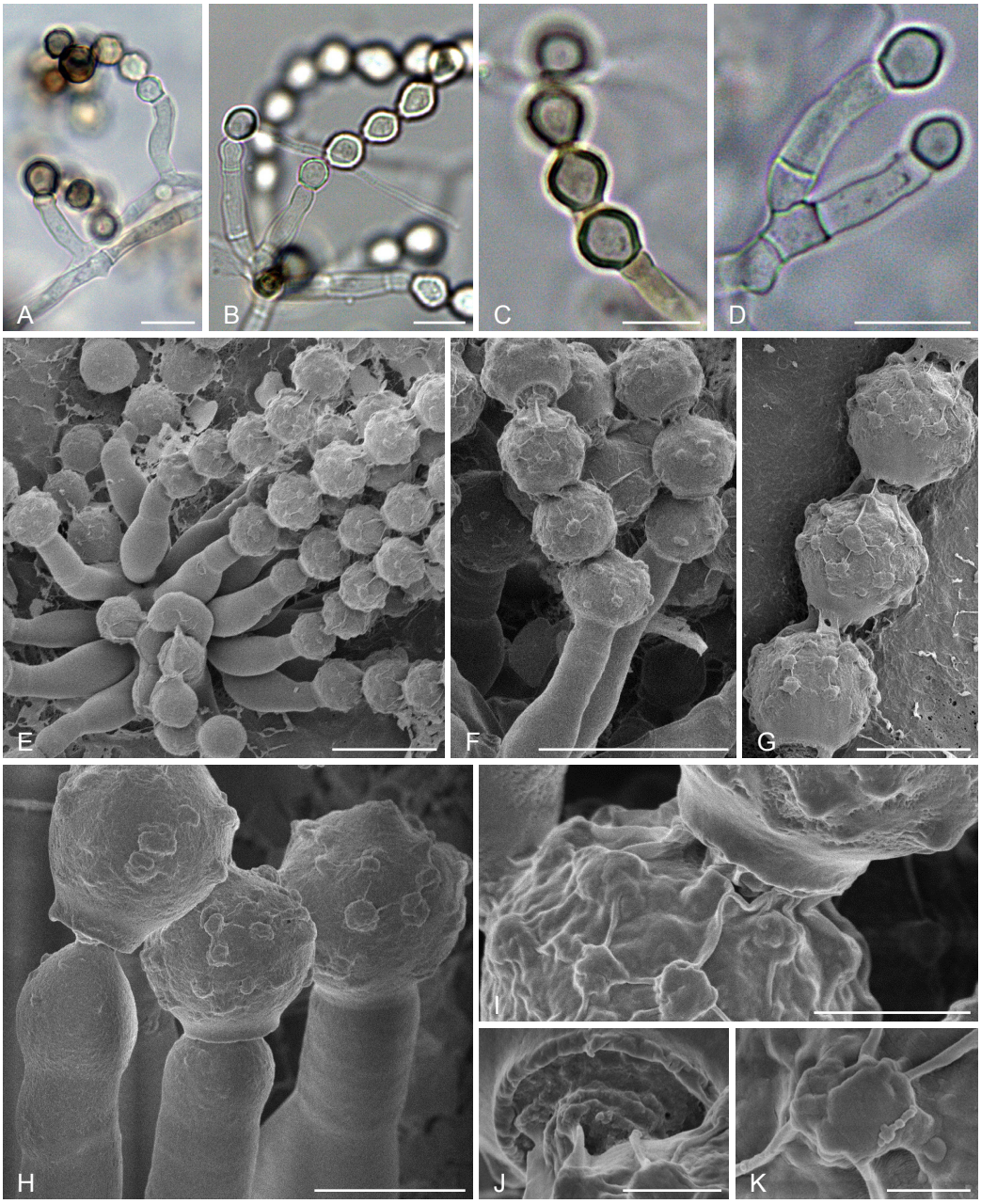


FIG. 13. *Scopulariopsis fusca* G414 (= CBS 138116): **A–D.** Conidiophores and conidia (LM). **E, F.** Conidiophores and conidia (cryoSEM). **G.** Chain of conidia (cryoSEM). **H.** Annellidic conidiogenesis (cryoSEM). **I.** Conidia junction (cryoSEM). **J.** Conidia junction as seen from beneath (cryoSEM). **K.** Conidia surface element (cryoSEM). *Scale bars:* **A–F** = 10 µm; **G** = 5 µm; **H** = 4 µm; **I** = 2 µm; **J** = 1 µm; **K** = 0.5 µm.

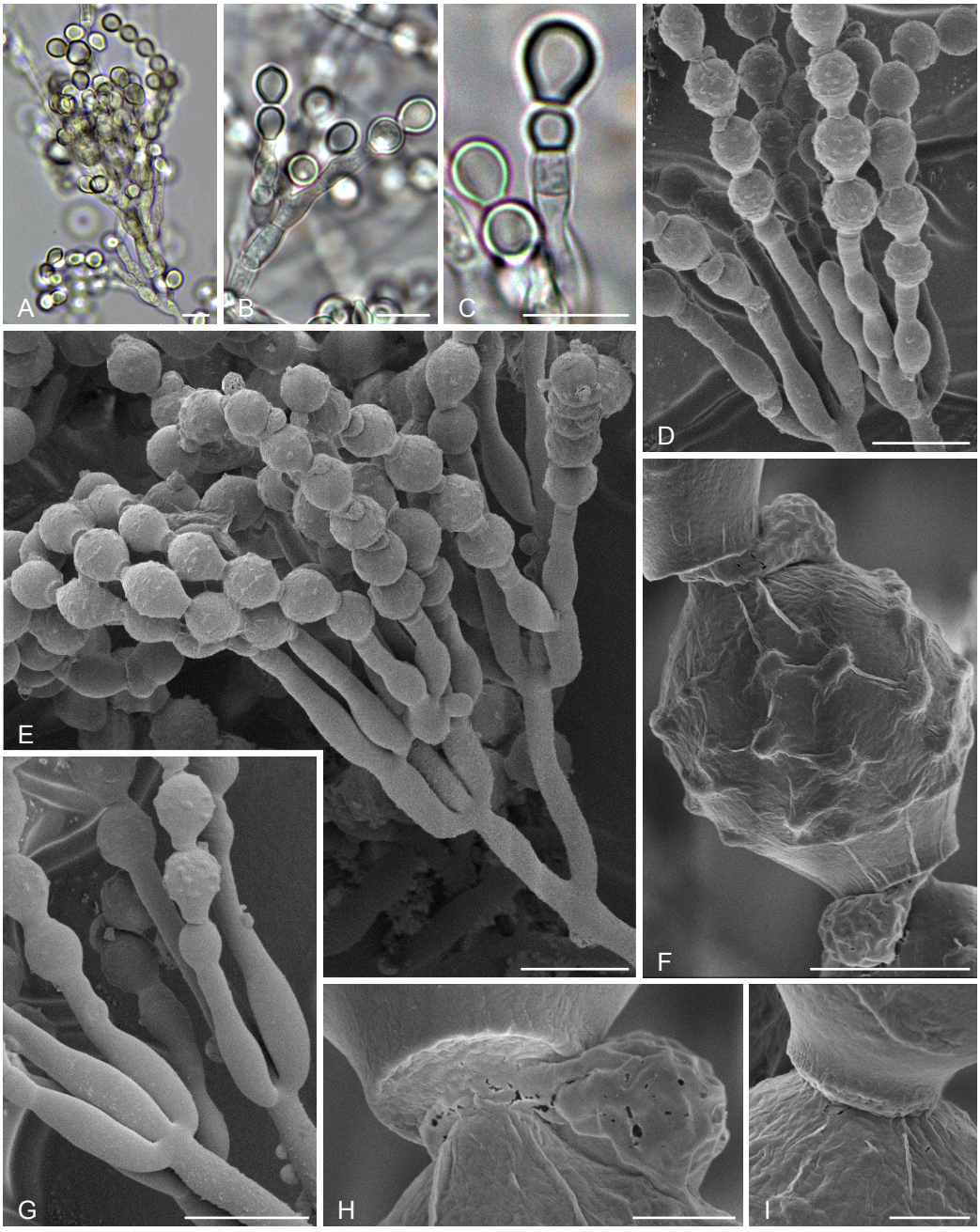


FIG. 14. *Scopulariopsis brevicaulis* G415 (= CBS 137632): **A–C.** Conidiophores and conidia (LM). **D, E, G.** Conidiophores and conidia (cryoSEM). **F.** Conidium within a conidia chain (cryoSEM). **H.** Conidia junction as seen from beneath (cryoSEM). **I.** Conidia junction as seen atop (cryoSEM). *Scale bars:* **A–E, G** = 10 µm; **F** = 3 µm; **H, I** = 1 µm.

Thielavia species display a large degree of polyphyly across *Chaetomiaceae*. One ambiguous isolate M314 group within sister *Lasiosphaeriaceae* and have been sterile during our investigation. This isolate falls into the polyphyletic area of genera *Zopfiella*, *Cercophora*, *Cladorrhinum*, which makes proper identification based on sequencing data impossible. We keep the *Lasiosphaeriaceae* sp. designation for the M314 strain (**FIG. 16**).

Pleosporaceae (SSU, LSU, RPB2, ITS) Fourteen isolates of various alkalitolerance are members of *Pleosporaceae* (see **FIG. 17**). Eight grouped within *Alternaria* and spread across three sections (*Alternata*, *Phragmosporae*, and *Soda*). Based largely on morphology, isolate G408 was identified as *A. alternata*, which is grouped within *Alternata* section. The other strains (M301 and M312) fall into *Phragmosporae* section. One of them, M312, produced conidia and was identified as *A. molesta*, the other one has been sterile and we keep the *Alternaria* sp. designation for the strain M301. Five isolates of *Alternaria* (M304^T, M307^T, M309, M310, and M313^T) are clearly diverging from the rest sister sections of *Alternaria* and we accommodate those isolates into a new section *Soda*, which is treated below. Six isolates are placed basal to the *Alternaria*, having grouped with *Pleospora*-allied genera. Unfortunately, as these six isolates have been sterile, we were not able to study them thoroughly. Interestingly, some of these isolates might represent new genera as, for example M306, M305, M311, and M302, distinctly diverged from the known reference species (**FIG. 17, 18** and **19**).

Alternaria sect. Soda (GAPDH) In order to follow the evolutionary relationship between the isolates in a new section *Soda* and allied sections we constructed a separate phylogenetic tree based on the partial sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene containing introns which contribute well to the clade differentiation within the section rank (**FIG. 20**). The matrix contained 23% of phylogenetically informative characters and was rooted to *Pleospora tarda* CBS 714.68^T type. Our analysis shows that our five new isolates clearly diverge from the sister sections of *Alternaria* – sect. *Infectoriae*, *Embellisia* and *Chalastospora*, confirming similar observation based on amore conserved dataset as seen before in **FIG. 17**. GAPDH data provides evidence that five isolates split into two or three species. Here, we designate three *A. kulundii* sp. nov. (M309, M310, and M313^T), *A. petuchovskii* sp. nov. (M304^T), and *A. shukurtuzii* sp. nov. (M307^T). Although the statistical support for *A. kulundii* clade is extremely low (57/0.54), we shall designate it as a separate species from the M304^T isolate, in spite the fact that the clade of *A. kulundii* together with M304^T received maximal supports. Our decision to split this four-isolate cluster strongly relied on the growth patterns. The growth experiments provide the evidence for the species differentiation, as in **FIG. 19, 21** it is seen that isolates of *A. kulundii* M309, M310, M313^T behave somewhat similar on various media, however, *A. petuchovskii* M304^T and *A. shukurtuzii* M307^T perform differently with a highly reduced growth rate on neutral media and overall colony morphology without Na^+ supplementation. The addition of extra Na^+ strongly reinforced the growth at neutral pH for those species. *A. shukurtuzii* M307^T is placed outside *A. kulundii* and *A. petuchovskii* isolates. Unfortunately, we were not able to amplify GAPDH gene in M301 and M312 isolates, which fall into *Phragmosporae* section, thus M312 isolate as one producing conidia was identified by morphology as *A. molesta* (**FIG. 26, E–J**), and M301 being sterile was left as *Alternaria*

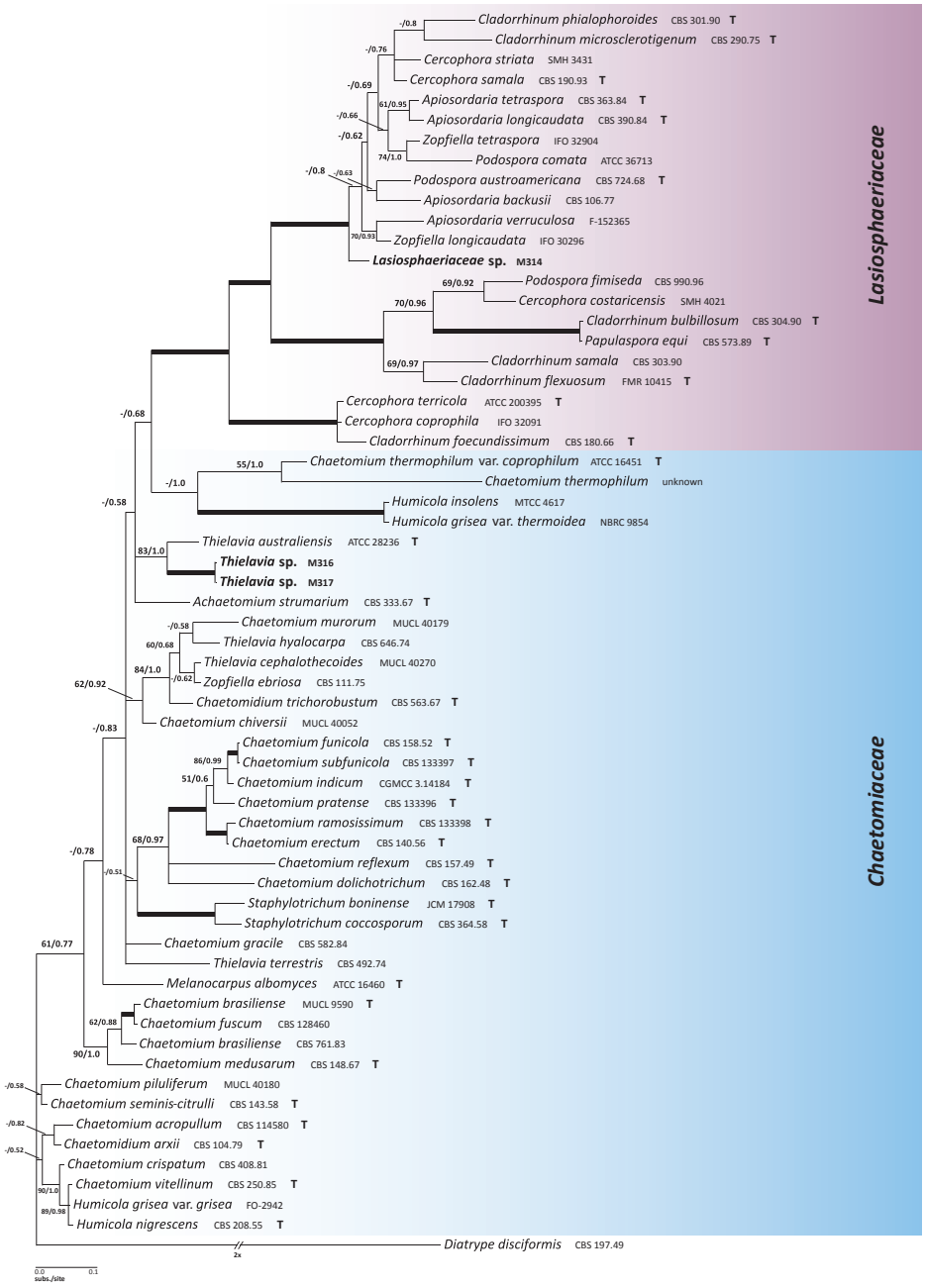


FIG. 15. Position of the two obligate alkaliphilic isolates of *Thielavia* sp. within *Chaetomiaceae*. Ambiguous *Lasiosphaeriaceae* sp. M314 strain fall within sister family. Bayesian 50 % majority rule consensus tree as inferred from the phylogenetic analysis of two loci (LSU, ITS). Thickened branches indicate strong combined support (ML > 90, PP > 0.94).

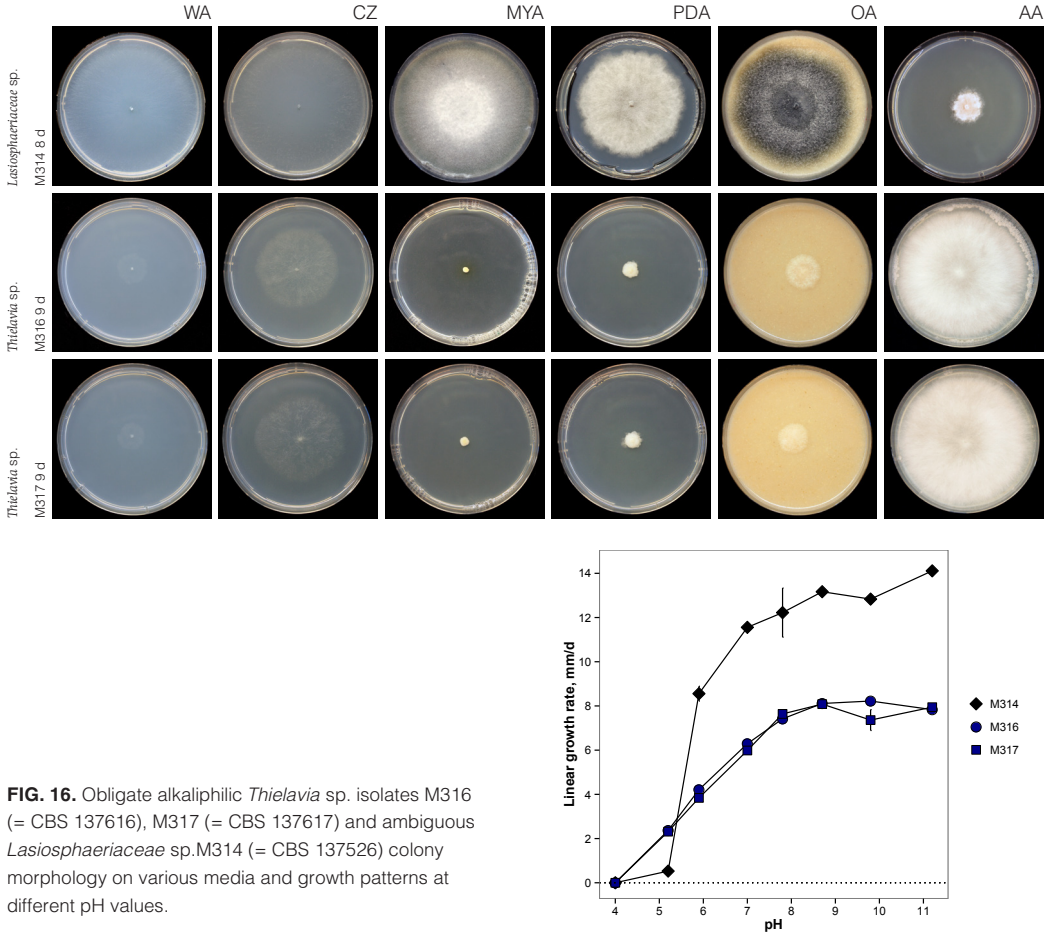


FIG. 16. Obligate alkaliphilic *Thielavia* sp. isolates M316 (= CBS 137616), M317 (= CBS 137617) and ambiguous *Lasiosphaeriaceae* sp.M314 (= CBS 137526) colony morphology on various media and growth patterns at different pH values.

sp. (**FIG. 22** and **23**).

By a combination of morphology and DNA information, we identified isolates G402, G406, G412, and G418 as *Cladosporium sphaerospermum*, *Purpureocillium lilacinum*, *Fusarium* sp., and *Penicillium* sp., respectively (**FIG. 22** and **23**).

Morphology

Simply-structured acremonium-like morphotypes dominate across our collection of alkalitolerant and alkaliphilic fungi. *Sodiomyces alkalinus*, *Acrostalagmus luteoalbus*, *Emericellopsis alkalina*, *Chordomyces antarcticum*, *Acremonium* sp. are the most represented taxa in our study, which produce such type of asexual morphology (**FIG. 8**, **30** and **31**). For the *Sodiomyces alkalinus*

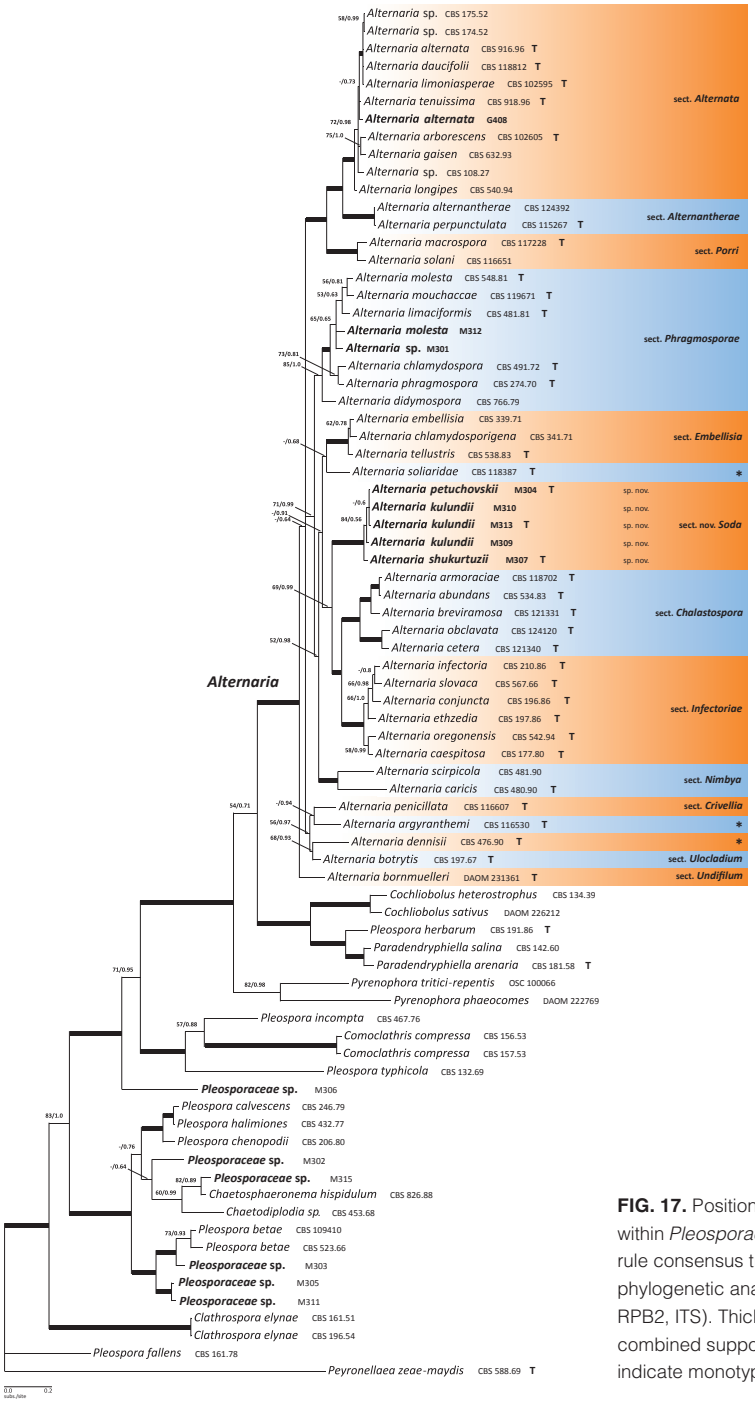


FIG. 17. Position of the alkalitolerant isolates within *Pleosporaceae*. Bayesian 50 % majority rule consensus tree as inferred from the phylogenetic analysis of four loci (SSU, LSU, RPB2, ITS). Thickened branches indicate strong combined support (ML > 90, PP > 0.94). Asterisks indicate monotypic sections.

and *Emericellopsis alkalina* morphology details, see **GRUM-GRZHIMAYLO ET AL. (2013A, B)**. Sister to *Emericellopsis* lineage, we observed moderate alkalitolerants of *Sarocladium* affinity and *Acremonium sclerotigenum*, which produce similar conidiation, as seen in **FIG. 24**. Above mentioned taxa represent a large part of the observed biodiversity from soda soils within our framework and are polyphyletic. Despite being polyphyletic, they produce similar conidiospores clumped up in compact heads, likely to be glued together by slime. Sister to *Emericellopsis*, we recovered a strong alkalitolerant isolate identified as *Acremonium roseolum* (strain A109) forming atypical catenate conidia (**FIG. 25**), which falls into the *inflatum*-clade, as outlined by **SUMMERBELL ET AL. (2011)**. At substantially lower frequencies we observed chains of dry airborne conidia in the species of *Alternaria*, *Cladosporium*, *Purpureocillium*, *Fusarium*, *Penicillium*, and Scopulariopsis (**FIG. 13, 14, 23, 26** and **32**). Unfortunately, a number of isolates have not developed reproductive stages during our investigation. Those include obligate alkaliphiles of *Chaetomiaceae* – *Thielavia* sp., single *Lasiosphaeriaceae* sp. isolate and *Pleospora*-related (*Pleosporaceae*) isolates of different affinity to pH. Ascomata formation has been observed only in *Sodiomyces* species, and two isolates of *Emericellopsis alkalina*. One of them, E101^{T}, was reported previously in **GRUM-GRZHIMAYLO ET AL. (2013B)**, however, later we observed the ascoma formation in another *E. alkalina* isolate – A117 (data not shown). Interestingly, we did not record ascospores inside the ascomata of *Sodiomyces tronii*. In *Alternaria* sect. *Soda* and *Verticillium* isolates we observed darkly pigmented thick-wall mycelium along with the formation of microsclerotia (**FIG. 12, 32** and **33**), which might be survival structures in harsh environments. We have not detected resting structures in facultative alkaliphiles like *Acrostalagmus*, *Chordomyces*. Obligate alkaliphilic *Sodiomyces tronii* and *S. magadii* have been able to produce single or short-chained chlamydospores (**FIG. 29**), as opposed to *S. alkalinus*, which did not form chlamydospores during our investigation. Curiously, obligate and facultative alkaliphilic isolates of our collection, *Thielavia* sp., *Sodiomyces* species, *Acrostalagmus luteoalbus*, *Chordomyces antarcticum*, *Emericellopsis alkalina* display brightly-coloured (white, salmon, pinkish, orange) colonies as seen on plates with the studied media, which is counterintuitive, as the stress-related and defensive morphology often encompasses darkly-coloured mycelium.

Taxonomy

SODIOMYCES A.A. Grum-Grzhim. et al., **PERSOONIA 31, 154, 2013.**

Type species — *Sodiomyces alkalinus* (Bilanenko & M. Ivanova) A.A. Grum-Grzhim. et al. **PERSOONIA 31, 157, 2013.**

= *Heleococcum alkalinum* Bilanenko & M. Ivanova, **MYCOTAXON 91, 501. 2005.**

A genus of the *Plectosphaerellaceae* (*Glomerellales*, *Hypocreomycetidae*).

Colonies on alkaline agar (AA, pH 10.0–10.2) rather fastgrowing, on MEA (pH 6.0–6.5) growing more slowly. Young colonies white; later, darkening zones appearing as a result of formation of abundant ascomata, velvety to woolly. Reverse colourless. Odour pleasant. Exudate absent. Mycelium superficial or partly immersed, consisting of hyaline, smooth-walled septate hyphae, often fasciculate. Decumbent vegetative hyphae thin-walled, hyaline.

Asexual morph acremonium- and gliocladium-like. *Conidiation* nematogenous and plectonematogenous. *Conidiophores* are characterized by considerable variation in the branching patterns including solitary phialides early formed laterally from simple and fasciculate vegetative hyphae, basitonously verticillate, penicillate (monoverticillate and biverticillate) arrangement. *Phialides* variable, gradually tapering towards the apex, rather thin walled. *Conidia* aseptate, hyaline, subglobose, oval, ellipsoidal, cylindrical, with rounded apices, smooth as observed by SEM, aggregated in slimy masses. *Chlamydospores* absent or present, 1–2-multi-celled, intercalary, terminal, in chains, darkening with age, forming bands and microsclerotia.

Ascomata cleistothecial, dark-brown, globose, superficial. *Peridium* multi-layered, pseudoparenchymatous. *Paraphyses* absent. *Asci* thin-walled, without apical apparatus, saccate, unitunicate, scattered irregularly in the ascocarp, embedded in a gelatinous matrix. *Ascospores* ellipsoidal or ovoid, medially 1-septate, not constricted at the septum, thick-walled, pale brown, smooth. *Ascospores* released by dissolution of the ascus wall before maturity, accumulating within the ascocarp, released in a slimy mass, liberated by pressure within the ascocarp.

Key to *Sodiomyces* species

1. Ascospores present on alkaline agar, asexual morph acremonium-like or absent, conidia subglobose, L/B<2, chlamydospores not in chains if present.....**(2)**

Ascospores absent on alkaline agar, asexual morph gliocladium-like, conidia cylindrical, L/B>2, chlamydospores in chains.....(*S. tronii*)

2. Ascospores length more than 10 µm.....(*S. alkalinus*)

Ascospores length less than 10 µm.....(*S. magadii*)

SODIOMYCES MAGADII Bondarenko et al., *sp. nov.* — MycoBank **MB811263**; **FIG. 27**.

Etymology — Name refers to the Magadi Lake in Kenya (Africa), where the fungus was isolated.

Ascomata dark brown, superficial on the substratum, globose, 90–220 (on average 160) µm diam, non-ostiolate, cleistothecial, not changing colour in 3 % KOH and lactic acid. *Peridium* multi-layered, pseudoparenchymatous, exoperidium on average 9 µm thick, composed of 3–5 layers of flattened cells. *Paraphyses* absent. *Asci* not observed. *Ascospores* ovoid, 6–8 x 4–7 µm, medially 1-septate at maturity, not constricted at the septum, thick-walled, pale brown, smooth.

Asexual morph unknown.

Culture characteristics — Colonies on alkaline agar (AA, pH 10.0–10.2) reaching 40–50 mm diam in 10 d at 25 °C. On MEA (pH 6.5) growth is extremely restricted. Young colonies white; later, black punctuated zones appearing as a result of formation of abundant ascomata, fluffy to woolly. Reverse colourless. Exudate absent. Odour pleasant. Decumbent vegetative hyphae thin-walled, hyaline, 0.5–2.0 µm wide. Mycelium consisting of hyaline, smooth-walled, septate hyphae, 1–3 µm wide, often fasciculate. *Chlamydospores* 1- and 2-celled, intercalary and terminal, 8–12 µm diam.

Specimen examined — **KENYA**, soda soil (pH 11) at the edge of Magadi Lake, Jan. 2013,

S. Bondarenko (CBS H-21958 – holotype); culture ex-type MAG2 = CBS 137619 = VKM F-4583.

SODIOMYCES TRONII Bondarenko et al., *sp. nov.* — MycoBank **MB811264**; **FIG. 28.**

Etymology — Name refers to the 'trona' salt (carbonate mineral), which is abundant in Magadi Lake in Kenya (Africa), where the fungus was isolated.

Ascomata dark brown, superficial on the substratum, globose, 100–180 (on average 150) µm diam, non-ostiolate, cleistothecial, not changing colour in 3 % KOH and lactic acid. *Peridium* multi-layered, pseudoparenchymatous, exoperidium up to 40 µm thick, composed of 5–8 layers of angular cells. Strings of thick-walled brown cells can take part in exoperidium formation. *Asci* thin-walled, without apical apparatus, unitunicate, scattered irregularly in the ascocarp, embedded in a gelatinous matrix. *Ascospores* not observed.

Asexual morph gliocladium-like. *Conidiation* abundant mostly plectonematogenous, partially nematogenous and is characterized by considerable variation in the branching patterns of primary and secondary conidiophores. Primary *conidiophores* in young cultures are predominantly penicillate, symmetrical, with monoverticillate arrangement, 30–60 µm long, phialides somewhat divergent or adpressed, solitary phialides 25–40 µm long are early formed laterally from simple and fasciculate vegetative hyphae. At maturity, secondary *conidiophores* with narrowly penicillate, biverticillate, branches including the phialides adpressed predominate, secondary conidiophores up to 70–80 µmlong, with thick-walled supporting cell and thin-walled branches. *Phialides* variable, 20–40 µm long, gradually tapering towards the apex from 2 to 4 µm at the base to 1–2 µm at the tip, rather thin-walled. *Conidia* aseptate, cylindrical, with rounded apices, to oval, allantoid or tapering at times, aggregated in spherical slimy masses, later confluent in common slimy mass, 5–11 x 2–4 µm, smooth as observed by SEM, hyaline.

Culture characteristics — Colonies on alkaline agar (AA, pH 10.0–10.2) rather fast-growing, reaching 40–80 mm diam in 10 d at 25 °C. On MEA (pH 6.0) growth is limited, colonies reaching 10–15 mm diam in 10 d at 25 °C. Young colonies white; after 2–3 month on alkaline agar or after month on Czapek agar (CZ, pH 6.2) darkening punctuated zones appearing as a result of formation of ascomata. Colonies fluffy to woolly. Reverse colourless. Exudate absent. Decumbent vegetative hyphae thin-walled, hyaline, 0.5–2.0 µm wide. Mycelium consisting of hyaline, smooth-walled, septate hyphae, 1–3 µm wide, often fasciculate. *Chlamydospores* present in old cultures, predominantly in chains, darkening with age, forming bands and microsclerotia.

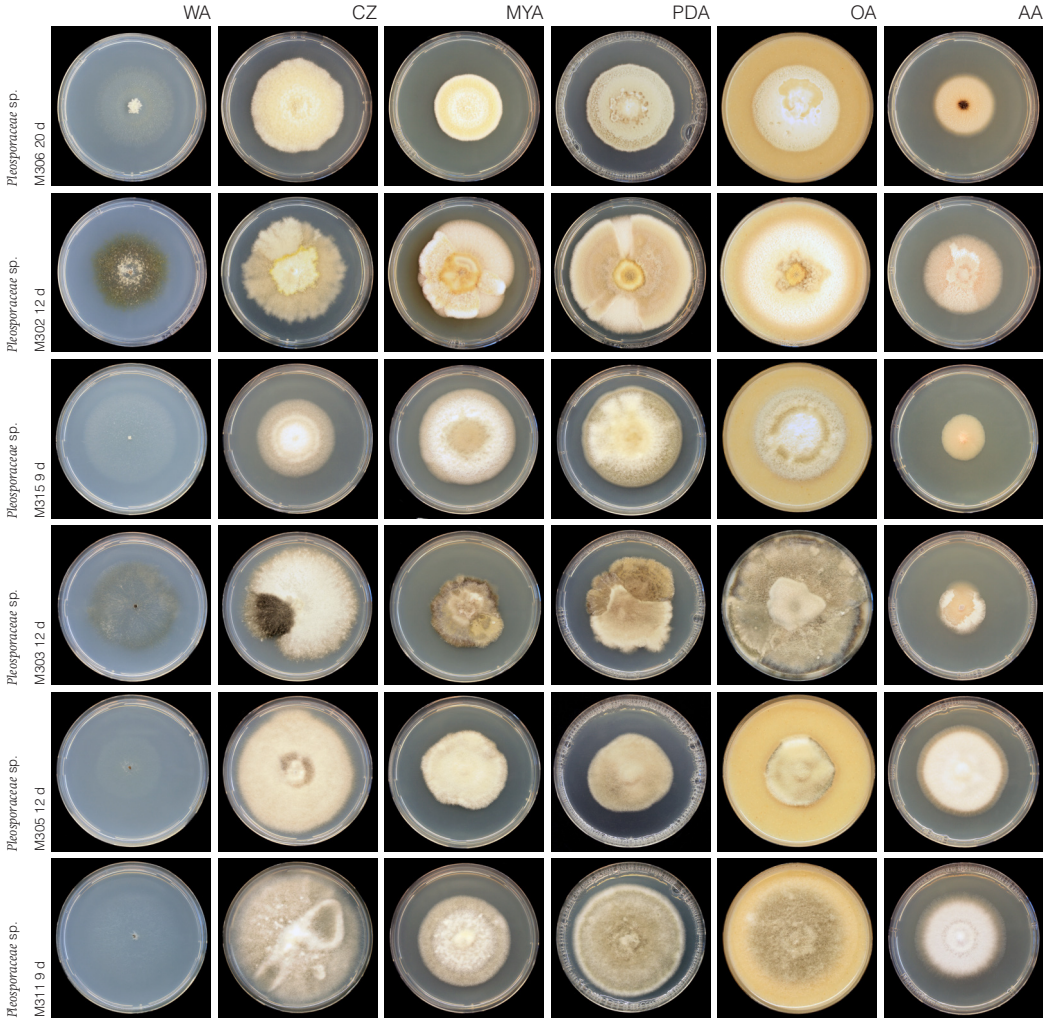
Specimen examined — **KENYA**, soda soil (pH 11) at the edge of Magadi Lake, Jan. 2013, *S. Bondarenko* (CBS H-21957 – holotype); culture ex-type MAG1 = CBS 137618 = VKM F-4582. **KENYA**, soda soil (pH 11) at the edge of Magadi Lake, Jan. 2013, *S. Bondarenko*, MAG3 = CBS 137620.

Notes — The comparative morphological chart for the *Sodiomyces* species is shown in **FIG. 29**.

CHORDOMYCES Bilanenko et al., *gen. nov.* — MycoBank **MB811265**

Etymology — from the English chord and Latin mycetes, referring to the synnemata covering the surface of the fungal colony.

Type species — *Chordomyces antarcticum* Bilanenko et al.



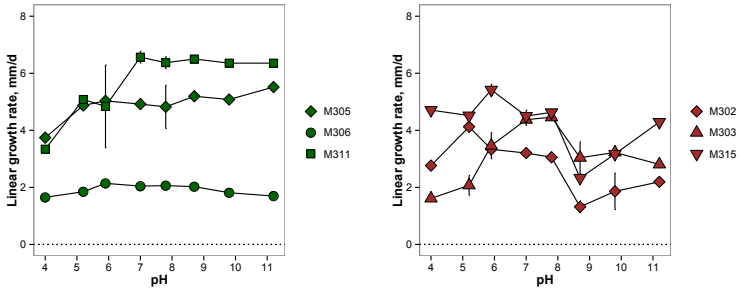


FIG. 18. Alkalitolerant isolates of sterile *Pleospora* related species (*Pleosporaceae*). Colony morphology on various media and growth patterns at different pH values.

A genus of the *Plectosphaerellaceae* (*Glomerellales*, *Hypocreomycetidae*).

Colonies white, tufted, characterized by bundles of hyaline vegetative hyphae, or synnemata, gradually tapering towards the apex, sometimes repeatedly branched, tufts appearing bristling or fimbriate because of numerous radiating phialides. Contrasting with synnemata in *Tilachlidium*, the mycelial tufts are not separated from vegetative mycelium by sterile base. *Conidiation* abundant, mainly synnematogenous, partly plectonematogenous, the tufts bearing simple orthophialides and branched conidiophores. *Stroma*, *setae* and *hyphopodia* absent. *Conidiophores* branched (in different manner, often verticillate) or consisting of simple phialides. *Conidiogenous cells* phialidic (schizophialide more rarely), tapering to the apex, hyaline. *Conidia* catenate or adhering in a slimy mass, ellipsoid to cylindrical, rounded at ends, hyaline, smooth-walled. *Sexual morph* unknown.

CHORDOMYCES ANTARCTICUM Bilanenko et al., *sp. nov.* — MycoBank **MB811266**; **FIG. 30, 31**.

Etymology — Epithet taken from the *Acremonium antarcticum* (Speg.) D. Hawksw.

Conidiation at first with solitary conidiophores, plectonematogenous, later in synnemata, reaching 10 mm in length, hyaline. *Synnemata* without differential sterile base, sometimes branched, appearing fimbriate due to radiating phialides. Thick parts of synnemata with a sterile tip, thiner ones (branches of synnemata) sporulating all over their length. *Conidiophores* often verticillate on short broad basal cells or consisting of simple phialides on thickened basal hyphae. *Phialides* (20)28–30(45) µm long, tapering from 2.5 to 3.0 µm near the base to 0.8–1.0 µm on the tip; tip with a short (length up to 2 µm) *collarette*, often proliferating sympodially, with branches often longer than the primary phialide tip (schizophialides), usually without a septum at the proliferation. *Conidia* held in slimy spherical heads (as seen with LM and cryoSEM), ellipsoidal to cylindrical, rounded at ends, with protuberant hilum at detachment (as seen with cryoSEM), hyaline, smooth-walled, 3.8–6.5 x 1.8–2.3 µm, L/W 2.2–2.8, predominantly 1-celled, 2-celled conidia with the middle septa are rare in occurrence. *Chlamydospores* absent.

Culture characteristics — Colonies rather fast-growing on alkaline media, reaching 20–24 mm on AA (pH 10), 16–20 mm on OA in 10 d, slow-growing and restricted on MYA and PDA, white, tomentose to synnematous (especially on AA). Mycelium mainly superficial on AA, partly superficial, partly immersed on OA, webby and mainly immersed on CZ. Odour not present.

Specimen examined — **RUSSIA**, Altai, Kulunda Steppe, soda soil (pH 9.8) at the edge of Karakul Lake, Nov. 2002, *M. Georgieva* (CBS H-21956 – holotype); culture ex-type M27 = CBS 120045 = VKM FW-3041. **RUSSIA**, Altai, Kulunda Steppe, soda soil (pH 9.4) at the edge of Petuchovskoe Lake, Aug. 2002, *D. Y. Sorokin*, A134 = CBS 137606. **RUSSIA**, Altai, Kulunda Steppe, soda soil (pH 10.1) at the edge of Berdabay Lake, Aug. 2005, *D. Y. Sorokin*, A135 = CBS 137607. **RUSSIA**, Trans-Baikal, soda soil (pH 10.1) at the edge of Nuhe-Nur Lake, Aug. 2003, *M. P. Lebedeva*, A137 = CBS 137608. **MONGOLIA**, North Gobi, Bayan-Zag area, soda soil (pH 9.3), Sep. 2003, *I. A. Yamnova*, A140 = CBS 137609. **KAZAKHSTAN**, from *Suaeda salsa* on the coast of the Aral Lake, Dec. 2003. *F. V. Sapozhnikov*, A141 = CBS 137610. **KAZAKHSTAN**, from *Suaeda salsa* on the coast of the Aral Lake, Dec. 2003, *F. V. Sapozhnikov*, A142 = CBS 137611. **MONGOLIA**, North Gobi, Bayan-Zag area, soda soil (pH 8.9), Aug.

2003, *I. A. Yamnova*, M10 = CBS 120042 = VKM FW-3039. **RUSSIA**, Altai, Kulunda Steppe, soda soil (pH 9.8) at the edge of Uzkoe Lake, Aug. 2002, *D. Y. Sorokin*, M30 = CBS 120046 = VKM FW-3042. **RUSSIA**, Altai, Kulunda Steppe, soda soil (pH 10) at the edge of Solyonoe Lake, Aug. 2002, *D. Y. Sorokin*, M31 = CBS 120047 = VKM FW-3906. **RUSSIA**, Altai, Kulunda Steppe, soda soil (pH 9.9) at the edge of Petuhovskoe Lake, Aug. 2003, *D. Y. Sorokin*, M41 = CBS 120048 = VKM FW-3034. **RUSSIA**, Altai, Kulunda Steppe, soda soil (pH 10.1) at the edge of Bezimyannoe Lake, Aug. 2002, *D. Y. Sorokin*, V213 = CBS 137630.

Notes — The sequences of 12 strains of *Chordomyces antarcticum* from soda soils differed from the CBS 987.87 isolate identified as *Acremonium antarcticum* (2 mutations in SSU across 1663 nucleotides, 3 mutations in LSU across 2023 nucleotides, 63 mutations in RPB2 across 1068 nucleotides, 8 mutations in TEF1-α across 903 nucleotides, 4 mutations in ITS across 479 nucleotides), which was isolated from lichen *Hypogymnia physodes* (the isolate seemed to be mycoparasitized by the hyphomycete *Hobsonia christiansenii*) in Luxembourg as was reported by **DIEDERICH ET AL. (1991)**. The species name comes from *Sporotrichum antarcticum* Spegazzini (the type of this taxon is a herbarium specimen LPS 21677 in the herbarium of La Plata, Argentina), growing on *Caloplaca* cf. *regalis* (Vain.) Zahlbr. from South Orkney Islands and described by David Hawksworth as a new combination – *Acremonium antarcticum* **HAWKSWORTH (1979)**. The CBS 987.87 isolate from Luxembourg has no nomenclatural status and there is no ex-type isolate available of the old species name – *Sporotrichum antarcticum* Speg. It is therefore not possible to say exactly whether or not our isolates and CBS 987.87 have a relation to the

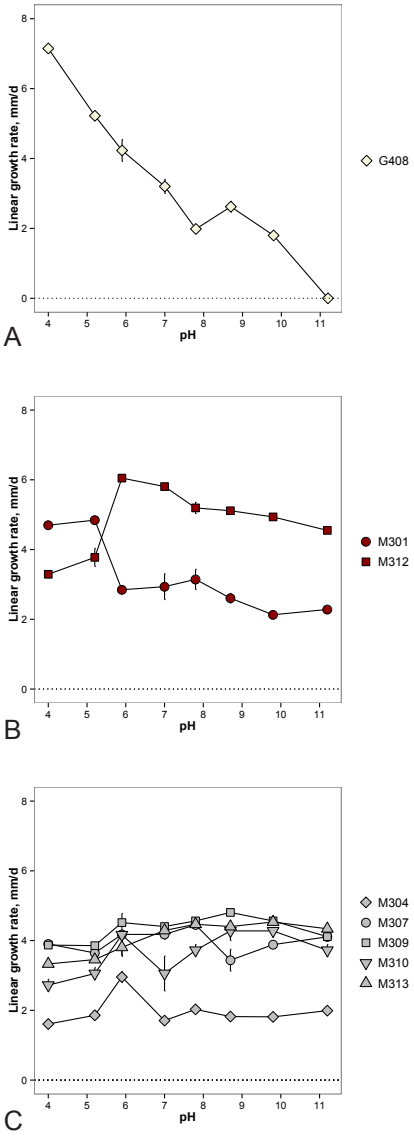


FIG. 19. Growth patterns at various pH values for Alternaria isolates. **A.** Weak alkalitolerant *Alternaria alternata* G408 (= CBS 137513). **B.** Strong alkalitolerant *Alternaria molesta* M312 (= CBS 137524) and moderate alkalitolerant *Alternaria* sp. M301 (= CBS 137514) (sect. *Phragmosporae*). **C.** Strong alkalitolerants and facultative alkaliphiles of *Alternaria* sect. *Soda* isolates.

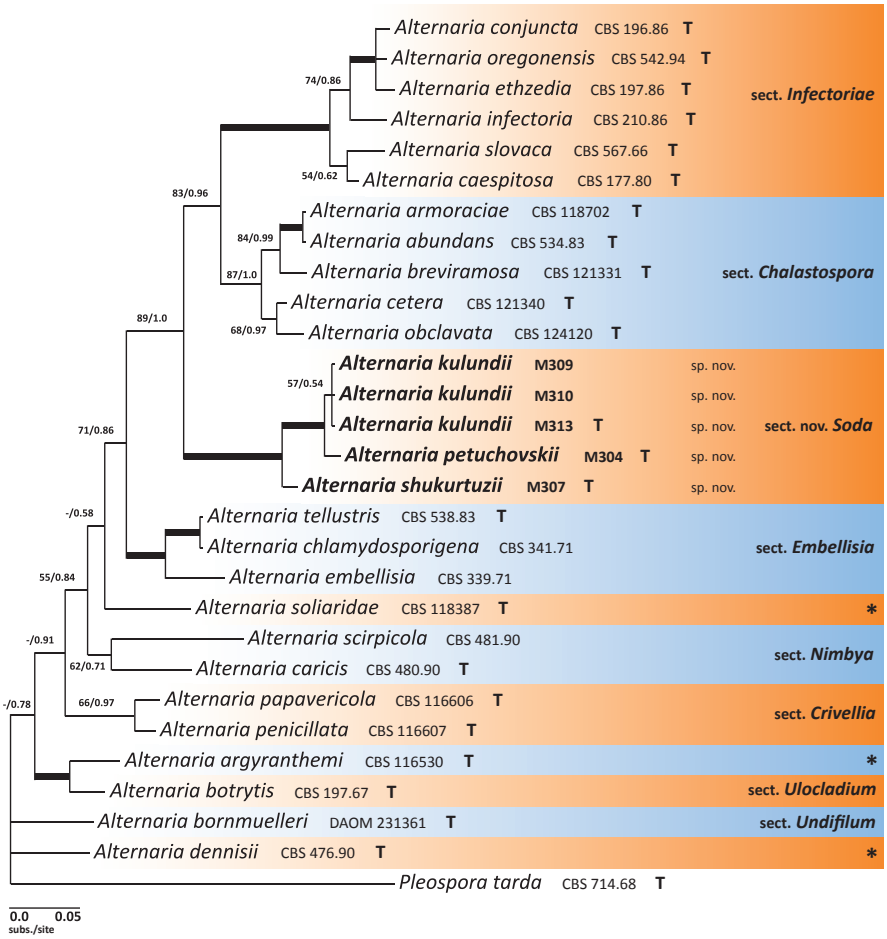


FIG. 20. Position of the new section *Soda* within sister *Alternaria* sections. Bayesian 50 % majority rule consensus tree as inferred from the phylogenetic analysis of GAPDH locus. Thickened branches indicate strong combined support (ML > 90, PP > 0.94). Asterisks indicate monotypic sections.

type of *A. antarcticum*, and the matter of linking the name *A. antarcticum* to any existing isolates is quite problematical. Moreover, the name *A. antarcticum* is initially associated with lichenicolous species, but the species from *Caloplaca* might not be strictly lichenicolous but just an opportunist. Both CBS 987.87 and the type of *A. antarcticum* colonized lichen were parasitized by a more aggressive fungus, and it is possible that the *Acremonium* in these cases was a mycoparasite on the invader, rather than on the lichen itself. Our 12 isolates from soda soils are not lichenicolous and might be associated with roots of halophyte plants. Despite the differences in sequencing data between our 12 new isolates and CBS 987.87 (especially in RPB2), we established a new

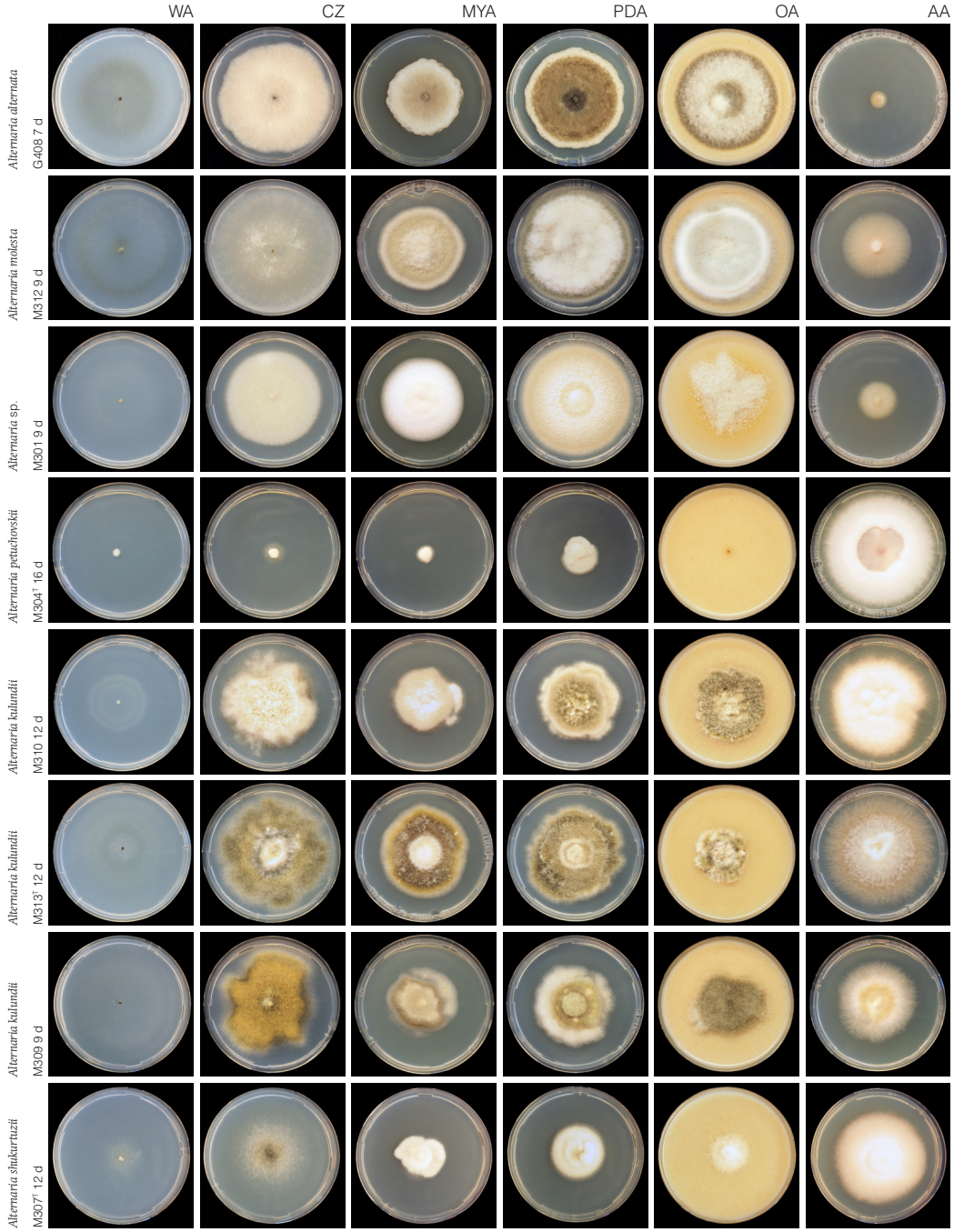


FIG. 21. Alkalitolerant *Alternaria* (*Pleosporaceae*) isolates colony morphology on various media.

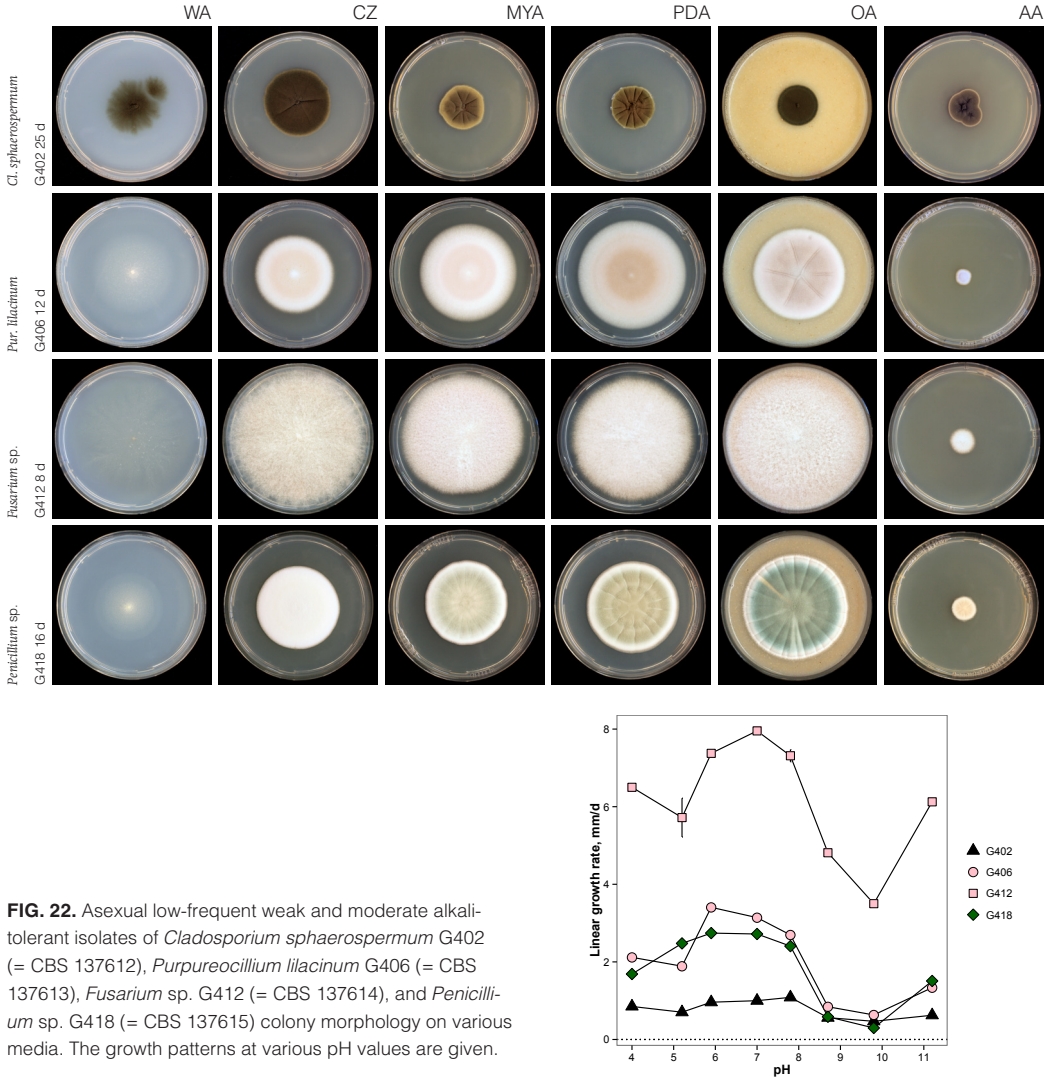


FIG. 22. Asexual low-frequent weak and moderate alkali-tolerant isolates of *Cladosporium sphaerospermum* G402 (= CBS 137612), *Purpureocillium lilacinum* G406 (= CBS 137613), *Fusarium* sp. G412 (= CBS 137614), and *Penicillium* sp. G418 (= CBS 137615) colony morphology on various media. The growth patterns at various pH values are given.

genus *Chrodomyces* with a single species *Chordomyces antarcticum*, accommodating the name *A. antarcticum* CBS 987.87 along with 12 our isolates. The clade harbouring 13 isolates received maximal statistical support in our *Plectosphaerellaceae* tree (LSU, ITS) (see **FIG. 5**).

ALTERNARIA Section ***SODA*** Bilanenko et al., *sect. nov.* — Mycobank **MB811267**
Type species — *Alternaria kulundii* Bilanenko et al.

Diagnosis — Section *Soda* contains simple or occasionally branched, short to moderately long, *conidiophores* with one conidiogenous locus. Apical or lateral short secondary *conidiophores* with a single conidiogenous locus may occur, conidiogenous tip can be enlarged. *Conidia* are solitary or in short to long, simple or branched chains, narrowly ellipsoid to long-ovoid or somewhat obclavate, moderate to very large in size, septate, with transverse and longitudinal septa, conspicuously constricted at most of the transverse septa. *Microsclerotia* or *chlamydospores* may occur, microsclerotia may germinate by conidial development. *Sexual morphs* have not been observed.

Notes — Section *Soda* contains 3 species (*A. kulundii*, *A. petuchovskii*, *A. shukurtuzii*), isolated from the soils at the different soda lakes in Kulunda Steppe in Western Siberia (Russia), pH of soils samples varied from 9.8 to 10.2. Species belonged to this section show strong alkalitolerant to facultative alkaliphilic type of the adaptation. Section *Soda* is well-defined from sister sections, *Infectoriae*, *Chalastospora*, and *Embellisia* based on partial sequences of SSU, LSU, RPB2, ITS, and GAPDH.

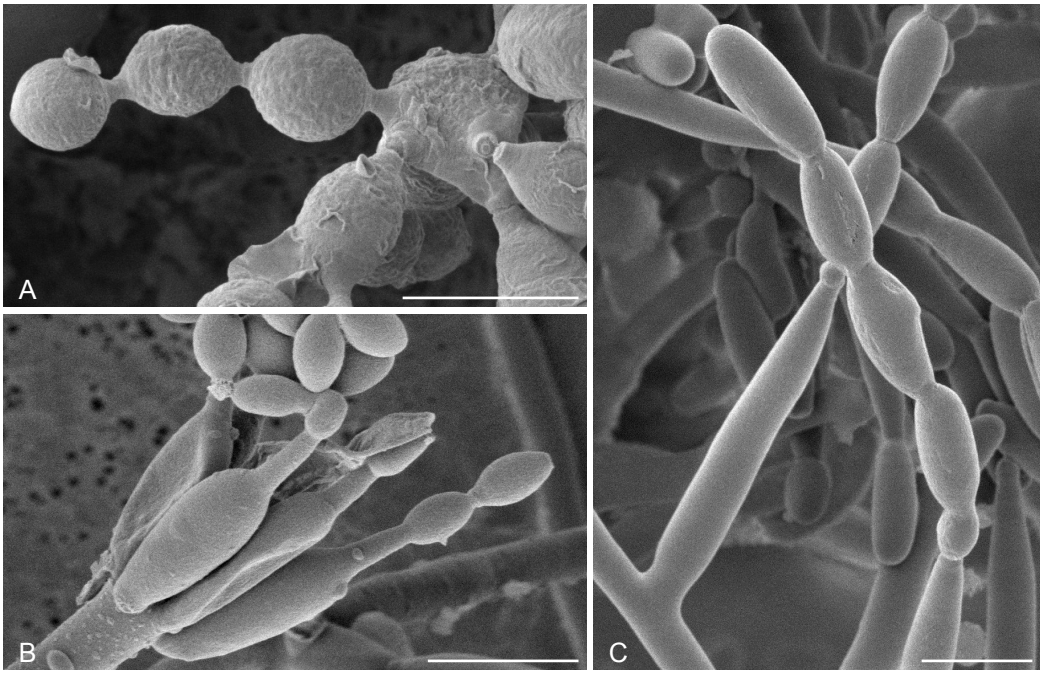


FIG. 23. Asexual low-frequent isolates from soda soils. **A.** Moderate alkalitolerant *Cladosporium sphaerospermum* G402 (= CBS 137612). **B.** Weak alkalitolerant *Purpureocillium lilacinum* G406 (= CBS 137613). **C.** Moderate alkalitolerant *Fusarium* sp. G412 (= CBS 137614). All images were taken with cryoSEM. *Scale bars:* **A–C** = 5 µm.

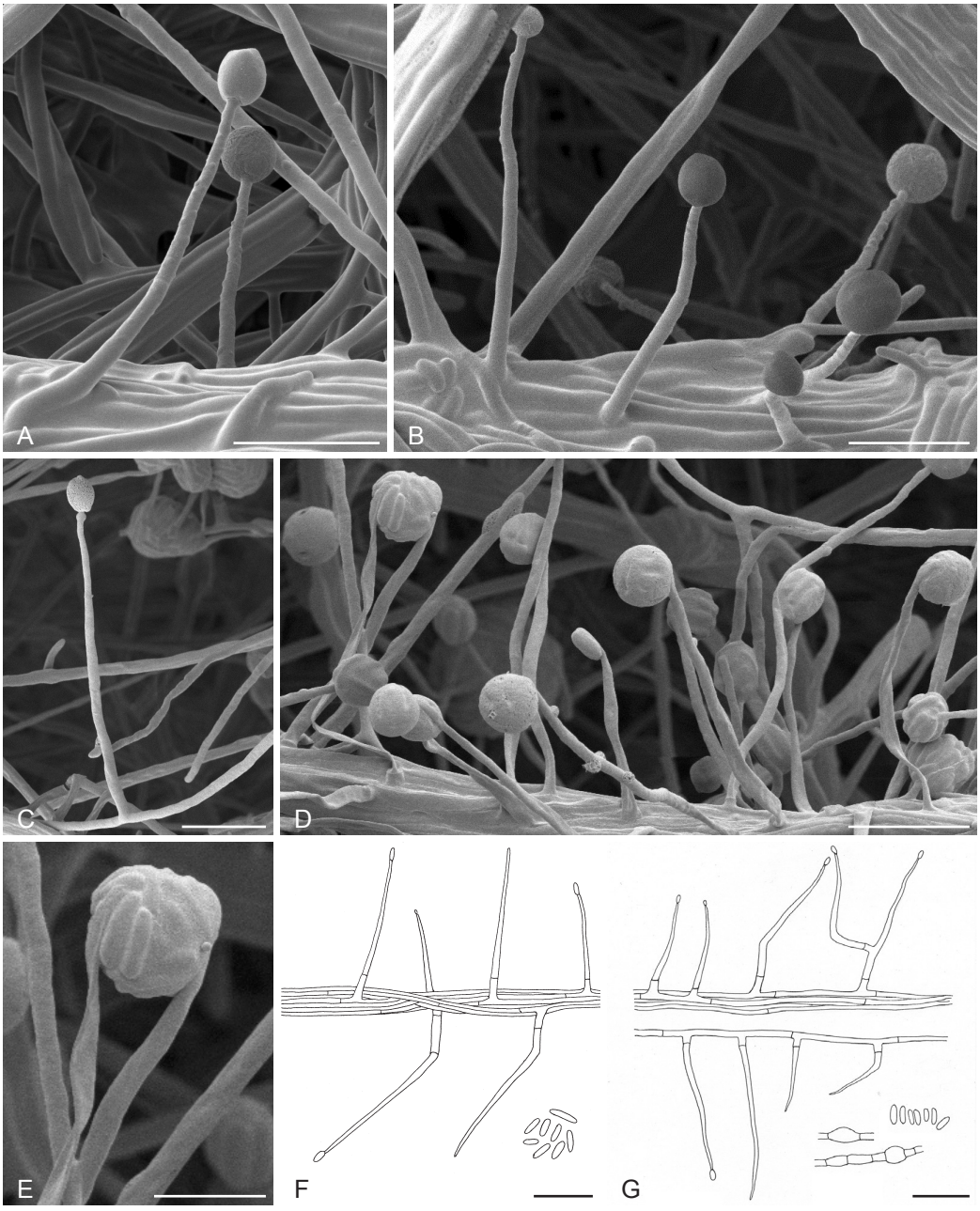


FIG. 24. Acremonium-like morphology of alkalitolerant isolates. **A, B, F.** *Acremonium sclerotigenum* A101 (= CBS 138752): hyphal chords, conidiophores, conidial heads (cryoSEM) and a drawing of the morphology observed. **C–E, G.** Sarocladium sp. A131 (= CBS 138121): hyphal chords, conidiophores, conidial heads (cryoSEM) and a drawing of the morphology observed. *Scale bars:* **A–D, F, G** = 10 µm; **E** = 5 µm.

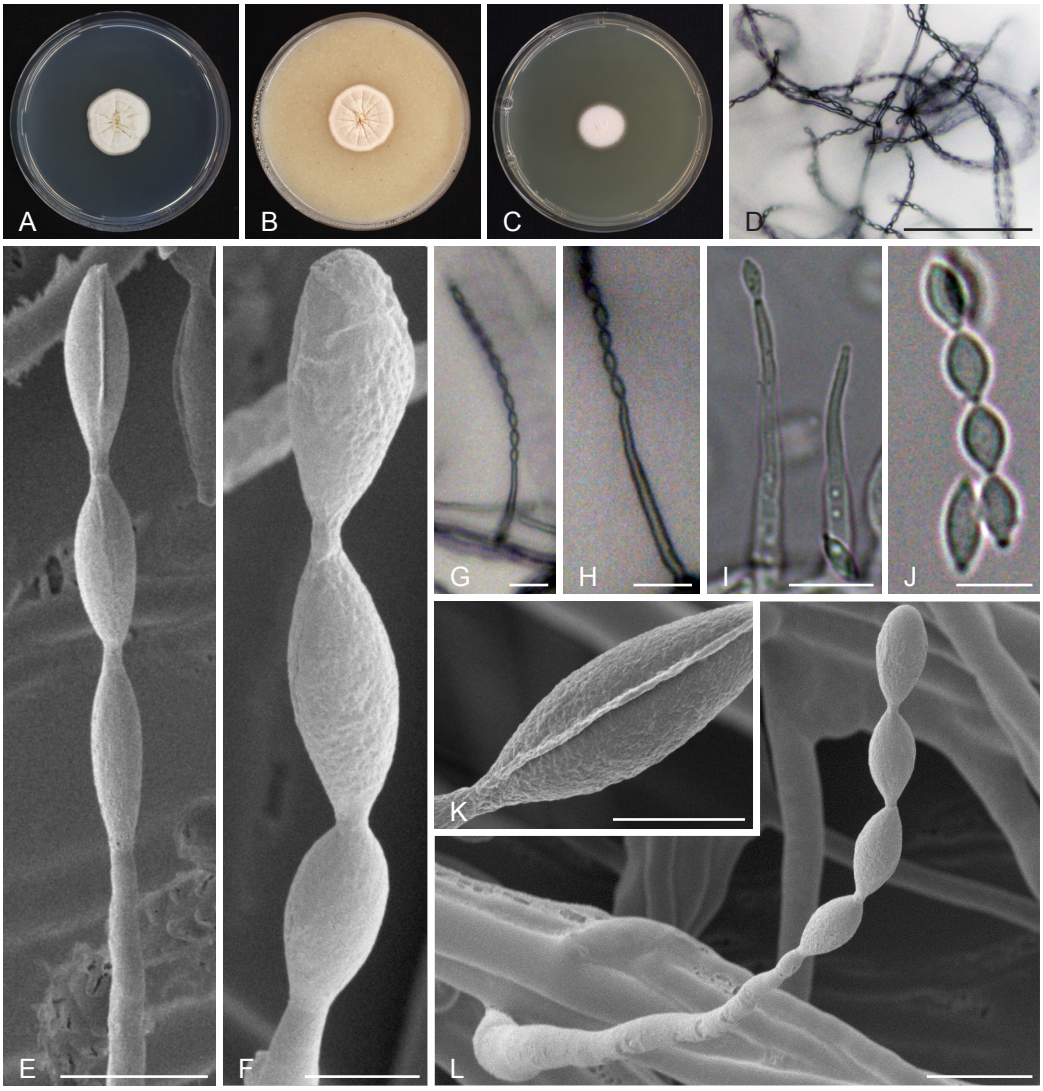


FIG. 25. Strong alkalitolerant *Acremonium roseolum* A109 (= CBS 138118). **A–C.** 11-d-old colony morphology on PDA, OA, and alkaline agar (AA), respectively (9 cm Petri dish). **D.** Catenate conidia in native culture (LM). **E, F.** Catenate conidia (cryo-SEM). **G–J.** Conidia and conidiophores (LM). **K.** Conidium surface (cryoSEM). **L.** Conidia being formed on a conidiophore emerging from a hyphal chord (cryoSEM). *Scale bars:* **D** = 50 µm; **E, L, J** = 5 µm; **F, K** = 2 µm; **G–I** = 10 µm.

ALTERNARIA KULUNDII Bilanenko et al., *sp. nov.* — Mycobank **MB811268**; **FIG. 32 (G–Q), 33**

Etymology — Name refers to the Kulunda steppe of Western Siberia in Russia, where the fungus was isolated from.

Colonies on CZ, MYA, PDA develop rather fast growing colonies (reaching 40–50 mm in diam after 7 d) with loose aerial brownish-pink-colored mycelium, light beige in colony center, reverse brownish. No *sporulation* on those media observed. Only *microsclerotia* and *chlamydospores* occured. Colonies on AA develop fast growing (colonies reaching 50–60 mm in diam after 7 d) pink-beige-colored mycelium, abundant light felt-fluffy mycelium in the center forming pinkish prostrate mycelium towards the margins of young colonies, reverse is similar in colour. No *conidiation*, *chlamydospores* occur. Colonies on SNA with a piece of autoclaved filter paper form poorly growing light beige mycelium. *Microsclerotia* are less developed in comparison with PDA. *Conidia* appeared within 7 d of incubation under CoolWhite fluorescent light with an 8 h photoperiod at room temperature. *Conidiation* on SNA unstable.

Conidiophores solitary simple, moderately long conidiophores with mostly one conidiogenous locus, conidiogenous tip is thick-walled and enlarged. Lateral short secondary conidiophores with a single conidiogenous locus may occur. *Conidia* solitary and in simple or branched chains, narrowly ellipsoid to long-ovoid or somewhat obclavate, dull pale tan to dark brown, conidial walls smooth to delicately roughened. Mature *conidia* large in size, mostly 80–110 x 15–25 µm, bulb-like in base, with 8–11, up to 14 transverse and several longitudinal septa, conspicuously constricted at the base transverse septa. Chains of oval *chlamydospores* form in young culture, *microsclerotia* are generated as a result of transverse and longitudinal septa formation, bulblike, dark brown.

Specimen examined — **RUSSIA**, Altai, the north of Kulunda Steppe, soda soil (pH 10.0), Aug. 2003, *M. Georgieva* (CBS H-21961 – holotype); culture ex-type M313 = CBS 137525. **RUSSIA**, Altai, Kulunda Steppe, at the edge of Tanatar Lake, soda soil (pH 10.2) Aug. 2002, *M. Georgieva*, M310 = CBS 137522. **RUSSIA**, Altai, Kulunda Steppe, at the edge of Uzkoe Lake, soda soil (pH 9.8) Aug. 2002, *M. Georgieva*, M309 = CBS 137521.

ALTERNARIA PETUCHOVSKII Bilanenko et al., *sp. nov.* — Mycobank **MB811269**; **FIG. 32 (A–F)**

Etymology — Name refers to the Petuchovskoe Lake in Kulunda steppe of Western Siberia (Russia), where the fungus was isolated.

Colonies on CZ, MYA, PDA develop slowly growing (colonies reaching up to 10–20 mm in diam after 7 d) light pink-beige colored mycelium. No *sporulation*, only *microsclerotia* or *chlamydospores* occur on those media. Colonies on AA develop fast growing (colonies reaching 60–70 mm in diam after 7 d) pink-beige-colored fluffy mycelium with prostrate mycelium in the center of colony and funiculose mycelium towards the margins; colony reverse in the similar colors. No *conidiation*, *chlamydospores* occur. Colonies on SNA with a piece of autoclaved filter paper form poorly growing light beige mycelium. *Microsclerotia* are less developed in comparison with PDA. *Conidia* appeared within 7 d incubation under Cool-White fluorescent light with an 8 h photoperiod at room temperature. *Conidiation* on SNA unstable.

Conidiophores solitary simple or occasionally branched, short or moderately long conidiophores with mostly one conidiogenous locus, conidiogenous tip is thick-walled and enlarged. Lateral short secondary conidiophores with a single conidiogenous locus may occur.

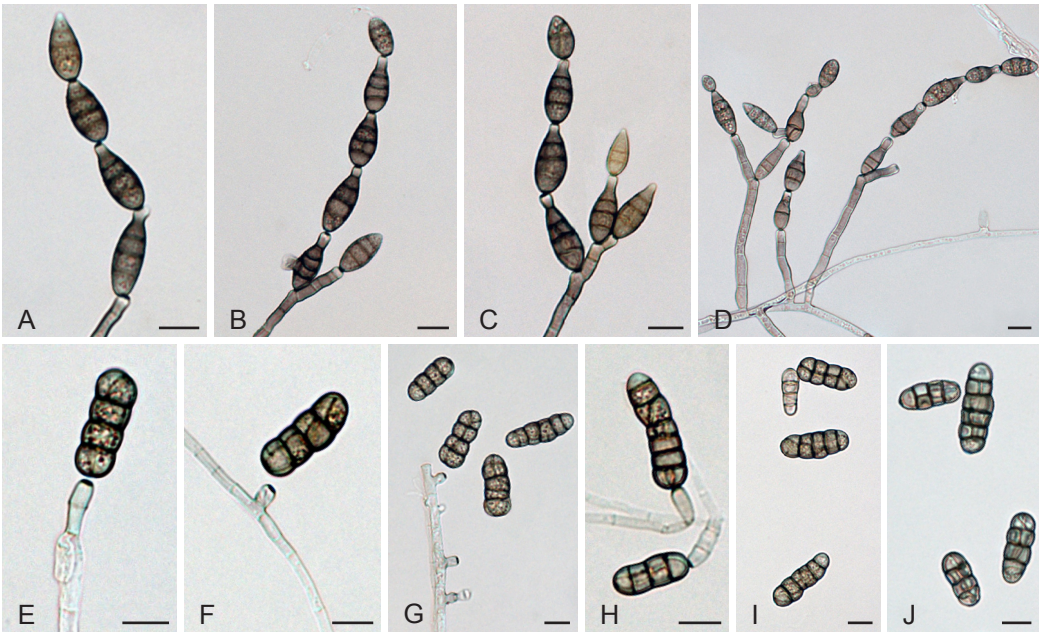


FIG. 26. Low-frequent *Alternaria* from soda soils. **A–D.** *Alternaria alternata* (sect. *Alternata*) G408 (= CBS 137513): conidia and conidiophores (LM). **E–J.** *Alternaria molesta* (sect. *Phragmosporae*) M312 (= CBS 137524): conidia and conidiophores (LM). *Scale bars:* **A–J** = 10 µm.

Conidia solitary and in simple or branched chains, narrowly ellipsoid to long-ovoid or somewhat obclavate, with bulb-like base, dull pale tan to dark brown, conidial walls smooth to delicately roughened. Mature *conidia* large, mostly 90–110 x 20–30 µm, up to 120 µm length, with 9–11, up to 18 or even more transverse and several longitudinal septa, conspicuously constricted at the base transverse septa. *Chlamydospores* in chains and bulb-like brown-colored *microsclerotia* present. *Microsclerotia* are generated as a result of transverse and longitudinal septa formation, bulb-like, dark brown. *Microsclerotia* may germinate by conidial development.

Specimen examined — **RUSSIA**, Altai, Kulunda Steppe, soda/chloride soil at the edge of the Petuchovskoe Lake (pH 9.9), Aug. 2003, *M. Georgieva* (CBS H-21959 – holotype); culture ex-type M304 = CBS 137517.

ALTERNARIA SHUKURTUZII Bilanenko et al., *sp. nov.* — Mycobank **MB811270**; **FIG. 32 (R–T)**

Etymology — Name refers to the Shukurtuz Lake in Kulunda steppe of Western Siberia (Russia), where the fungus was isolated.

Colonies on CZ, MYA, PDA develop moderately slowly growing (colonies reaching 20–30 mm in diam on MYA and PDA, 40–50 mm on CZ after 7 d) light pink-beige colored mycelium, brownish in center on CZ. No *sporulation*, only *chlamydospores* occur on those media. Colonies

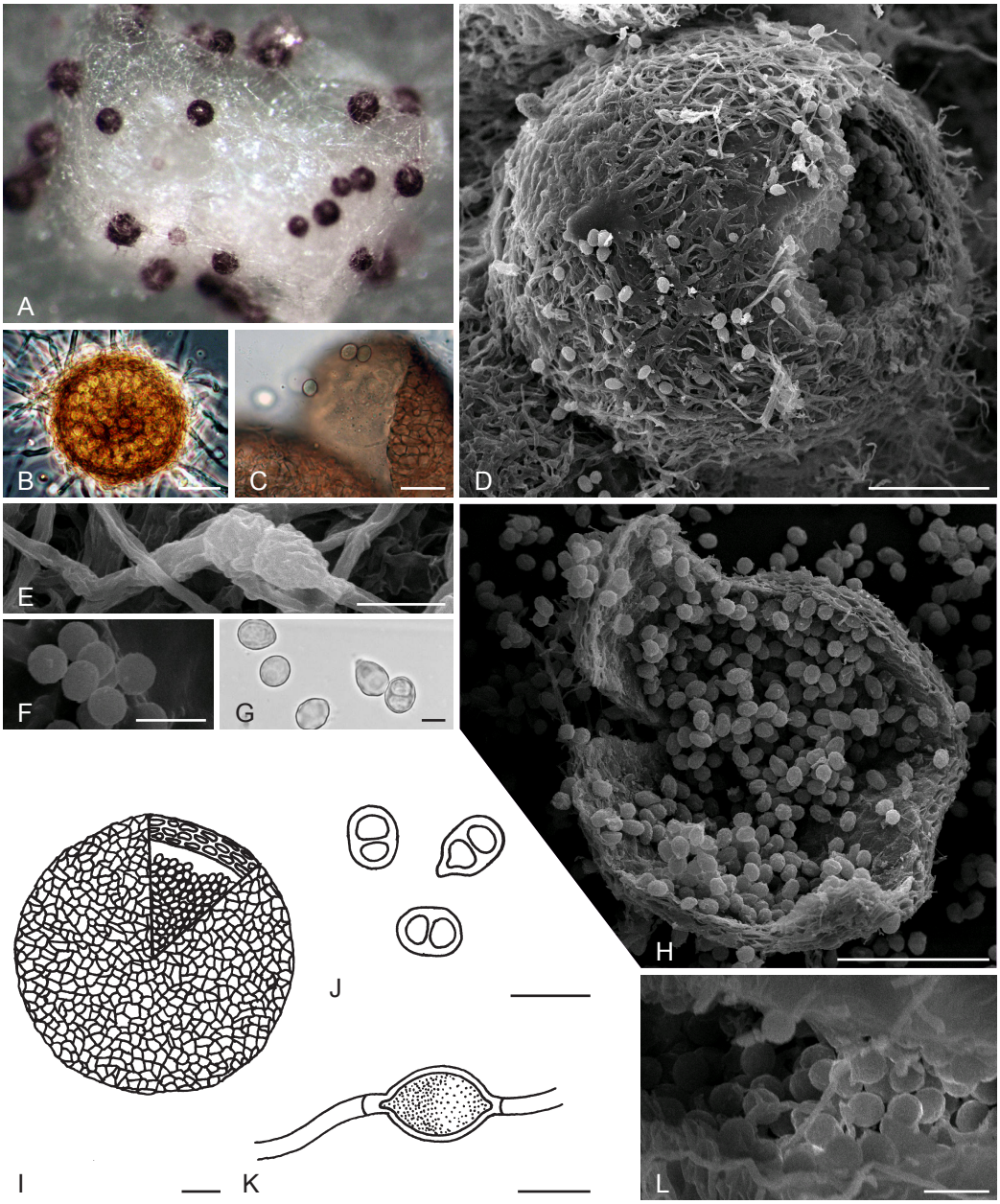


FIG. 27. *Sodiomyces magadii* MAG2 (= CBS 137619^{T}). **A.** Agar plug with fruiting bodies as seen through a binocular microscope. **B.** Intact fruiting body with ascospores inside (LM). **C.** Opened ascoma releasing ascospores. Note the peridium surface structure (LM). **D.** Fruiting body (SEM). **E.** Chlamydospores (SEM). **F.** Ascospores (SEM). **G.** Ascospores (note the septa) (LM). **H.** Broken fruiting body (SEM). **I, J, K.** Fruiting body, ascospores and chlamydospore, respectively. **L.** Cracked ascoma with ascospores (SEM). *Scale bars:* **B, C, I** = 20 µm; **D, H** = 50 µm; **E, G** = 5 µm; **F, J, K, L** = 10 µm.

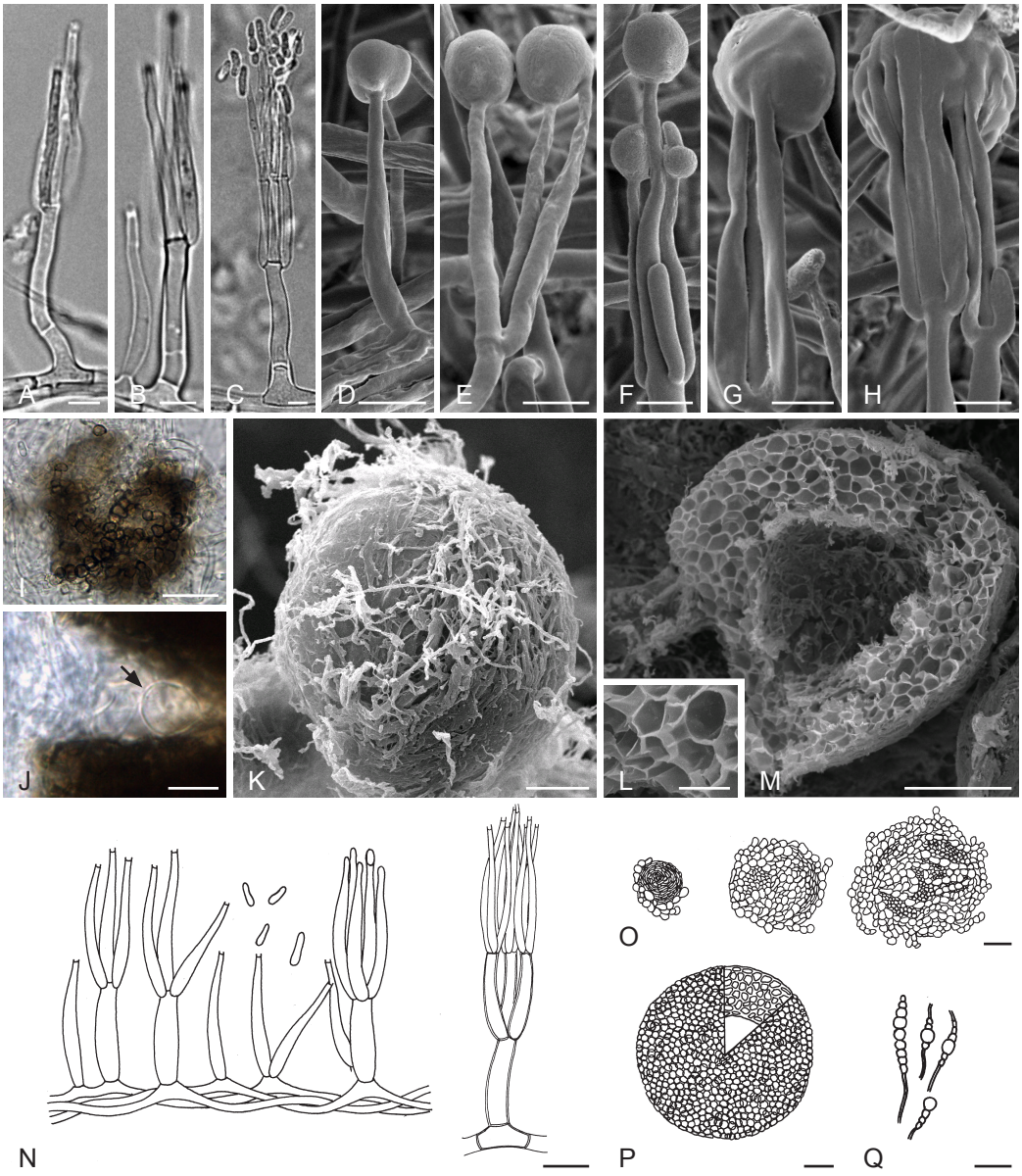


FIG. 28. *Sodiomyces tronii* MAG1 (= CBS 137618^{T}). **A, B.** Conidiophore development (LM). **C.** Mature conidiophore with conidia (LM). **D–H.** Early and late stages of conidiophore development (cryoSEM). **I.** Early stage of fruiting body development. Strings of thick-walled brown cells are involved in exoperidium formation (LM). **J.** Opened ascoma with an empty ascus (arrowed) (LM). **K.** Fruiting body (SEM). **L.** Peridium structure as seen on a cross-section (SEM). **M.** Broken fruiting body (SEM). **N.** A drawing of the asexual structures observed. **O.** Fruiting body development stages. Strings of thick-walled brown cells are involved in exoperidiumformation. **P.** Mature fruiting body (no ascospores have been observed). **Q.** Chains of thick-walled cells involved in exoperidium formation. *Scale bars:* **A–H** = 5 µm; **I, K, O–Q** = 20 µm; **J, L, N** = 10 µm; **M** = 50 µm.

on AA develop fast growing (colonies reaching 50–60mmin diam after 7 d) pink-beige-coloured fluffy mycelium, reverse in the similar colours. No *sporulation*, only *chlamydospores* occur. Colonies on SNA with a piece of autoclaved filter paper form poorly growing mycelium. *Chlamydospores* occur. *Conidia* appeared within 7 d incubation under CoolWhite fluorescent light with an 8 h photoperiod at room temperature. *Conidiation* on SNA unstable. No *microsclerotia* observed.

Conidiophores solitary simple or occasionally branched, with mostly one conidiogenous locus, conidiogenous tip is thick-walled and enlarged. Lateral short secondary conidiophores with a single conidiogenous locus may occur. *Conidia* solitary and in simple or branched chains, narrowly ellipsoid to long-ovoid, dull pale tan to dark brown, conidial walls smooth to delicately roughened. Mature *conidia* mostly 60–80 x 7–10 µm, with 7–9 transverse and 0–1 longitudinal septa, not conspicuously constricted at the septa. *Chlamydospores* occur.

Specimen examined — **RUSSIA**, Altai, Kulunda Steppe, the edge of the Shukurtuz Lake, soda/chloride/sulfate soil (pH 9.9), Aug. 2002, *M. Georgieva* (CBS H-21960 – holotype); culture ex-type M307 = CBS 137520.

Discussion

The present study systematically characterizes the alkaliphilic and alkalitolerant filamentous fungi isolated from alkaline (soda) soils. Until now, our understanding of the biodiversity of the alkaliphiles and alkalitolerants has been based on occasional reports that lack both sequencing data and detailed morphological descriptions. The demand for sequencing data is especially high, since most alkaliphiles and alkalitolerants have only simple asexual morphology (polyphyletic acremonium-like development) impairing proper identification of the species. The acremonium-, verticillium-, and gliocladium-type asexual morphological development has also been demonstrated for most of our alkaliphilic and strongly alkalitolerant fungi. To strengthen our descriptions and analyses, we coupled our phylogenetic analyses to the morphology data and pH-dependent growth rate data for essentially every isolate we obtained. The growth experiments at various pH values helped us to assess the potential ecological contribution of the isolated fungi in soda soils and to discriminate between accidental transient species and effective alkaliphiles. Though the growth patterns were often too complex to explicitly interpret, we were able to assign the patterns into one of five 'growth types': obligate alkaliphiles and facultative alkaliphiles, strong, moderate and weak alkalitolerants. In several cases this estimation was complicated by the fact that the pH-tolerance is highly affected by the presence of extra Na^+ in the growth medium. Based on such adopted classification, we were able to create a combined framework of phylogenetic, morphological and growth data that could serve as a foundation for future studies on physiology and evolution of alkaliphilic filamentous fungi.

Overall, we showed that soda soils are in fact inhabited with a substantial number of filamentous fungal species, which are able not only to withstand high ambient pH, but for some of them high pH is essential for optimal growth. One of the prominent conclusions

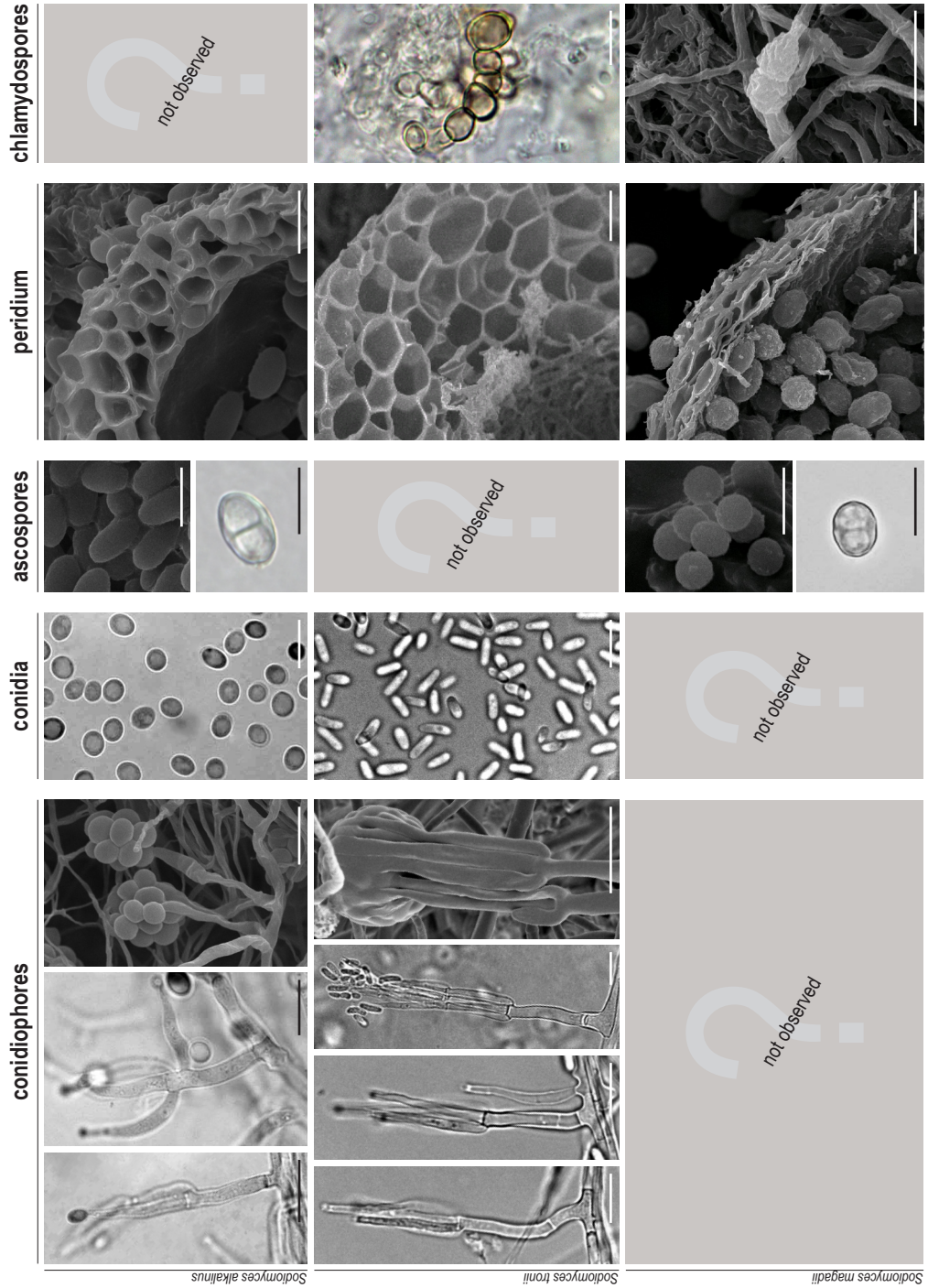


FIG. 29. Comparative morphological chart for the species of *Sodiomyces*. *Scale bars:* 10 µm.

from our data is that alkaliphilic and alkalitolerant fungi are polyphyletic and spread across several subphyla in *Ascomycota*. This result suggests independent evolution and several origins of the alkaliphilic trait in filamentous fungi. Still, most of the alkaliphilic species are within the *Hypocreomycetidae*. This is the taxonomic location where obligate and facultative alkaliphiles are overrepresented. Notably, *Emericellopsis* (*Bionectriaceae*) and *Plectosphaerellaceae* lineages show particular enrichment; nearly half of our isolates belong to either of those two groups. The facultative alkaliphile *Emericellopsis alkalina* lineage appears to have evolved from marine isolates of *Emericellopsis* (**GRUM-GRZHIMAYLO ET AL. 2013B**). Even though the highest frequency of alkalitolerant fungi was observed within *Hypocreomycetidae*, we found two obligate alkaliphiles, which belong to the *Thielavia* of the *Chaetomiaceae* (*Sordariomycetidae*) and over ten strong alkalitolerant isolates are nested within the *Pleosporaceae* (*Dothideomycetes*).

Interestingly, in the present study, we obtained two *Alternaria* sect. *Phragmosporae* isolates (M301 and M312); to our knowledge, this is the only section of *Alternaria* containing marine-borne isolates, providing extra evidence for the link between marine habitats and soda soils – terrestrial counterparts with similar abiotic conditions. Allied to the *Alternaria*, six of our isolates are taxonomically spread around *Pleospora*. Several close reference strains, e.g., *Pleospora halimiones* CBS 432.77, *Chaetodiplodia* sp. CBS 453.68 and others, have been isolated from the associated halophytic grasses. The enriched biodiversity of the alkalitolerant fungal strains provides evidence for the ecological overlap between the halophyte-associated isolates and soda lakes in the *Pleospora*-related species.

The growth experiments using common media coupled with the pH preference test in race tubes allowed us to study the structure of the soda soils fungal community. This approach showed differences in the pH and Na^+ preferences of our isolates, which enabled us to reveal true alkaliphiles and distinguish them from the moderate and weak alkalitolerant species. In many cases the preference to grow at high ambient pH relative to neutral pH, coincided with the presence of extra Na^+ in the medium, which points towards the fact that high pH and high salts stresses are linked, and probably have co-evolved. Also alkaliphilic bacteria require Na^+ cations to tolerate high pH, as the Na^+-pumps seem to be one of the major players in pH homeostasis of the cell (**KRULWICH ET AL. 2011**). Extra evidence for such a tight association has been shown in *Fusarium oxysporum* (**CARACUEL ET AL. 2003**). For example, as seen in **FIG. 6**, strong alkalitolerant *Chordomyces* isolates prefer alkaline agar (AA) medium (pH 10, which contains 5 g/l NaCl) rather than common neutral media like PDA, MYA without extra NaCl. However, if the NaCl concentration is kept constant, but the pHs are varied, as we set up in race tubes – we see no clear preference to ambient pH, as seen in graphs in **FIG. 6**. That observation implies that for *Chordomyces* (*Plectosphaerellaceae*), Na^+ is necessary for optimal growth regardless of the tested ambient pH. Similar observations hold for our *Verticillium zaregamsianum*, several *Alternaria* sect. *Soda* strains, and to some extent, for *Acrostalagmus luteoalbus* strains. Conversely, we have recovered truly alkaliphilic fungi that prefer high ambient pH regardless of the presence of extra Na^+ in the growth medium. For instance, species of *Sodiomyces* (*Plectosphaerellaceae*) and sterile *Thielavia* sp. (*Chaetomiaceae*) showed clear alkaliphilic phenotype both in the media

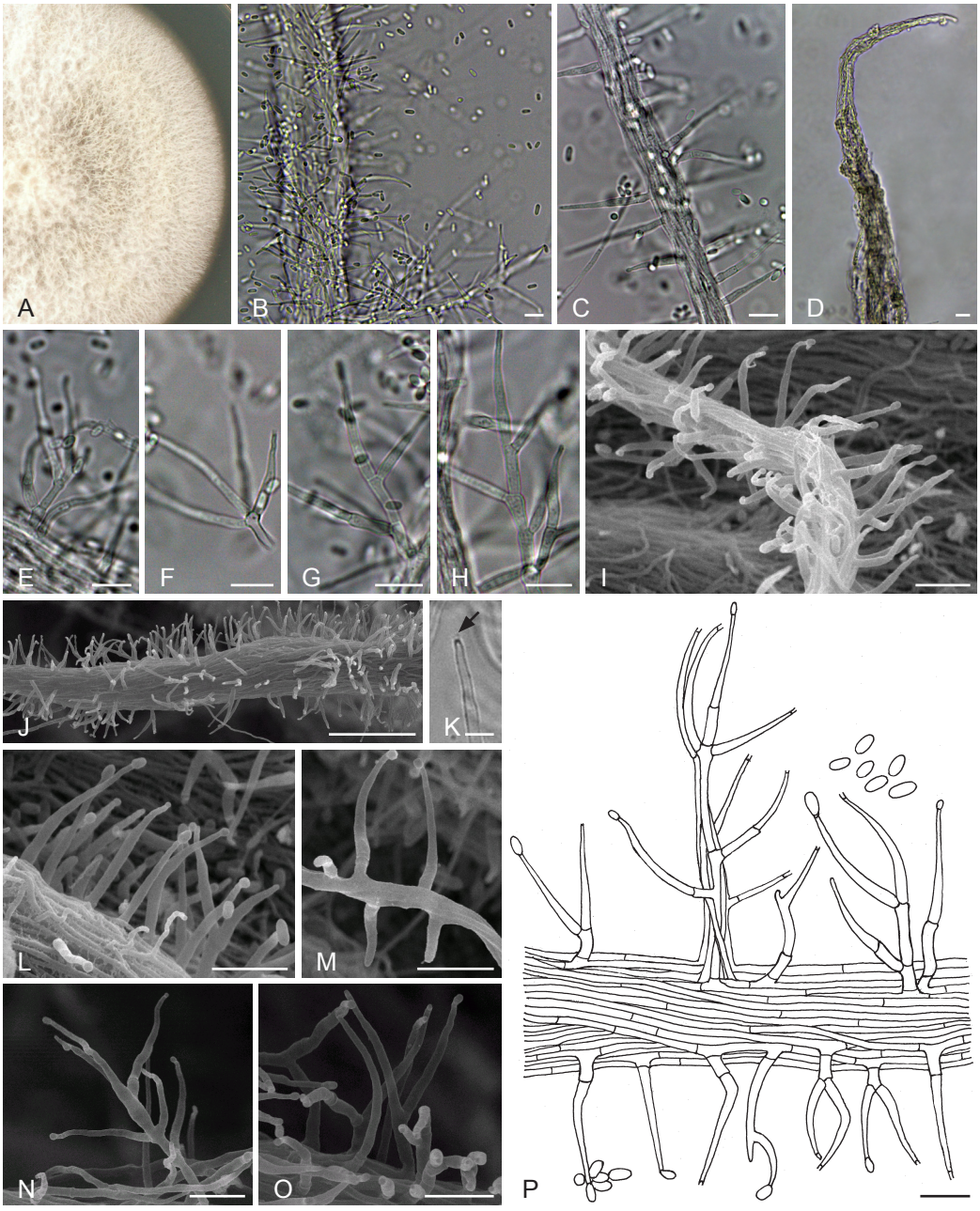


FIG. 30. *Chordomyces antarcticum* M27 (= CBS 120045^{T}). **A.** 20-d-old colony on AA agar (9 cm Petri dish). **B.** Hyphal chord with branching (LM). **C.** Hyphal chord with unbranched conidiopohres (LM). **D.** Sterilic hyphal chord tip (LM). **E–H.** Various types of branching conidiophores (LM). **I, J.** Hyphal chords with conidiation (SEM). **K.** Conidiophore collarette (arrowed) (LM). **L–O.** Various types of conidiophores (SEM). **P.** Drawing of the morphology observed. *Scale bars:* **B–I, K–P** = 10 µm; **J** = 50 µm.

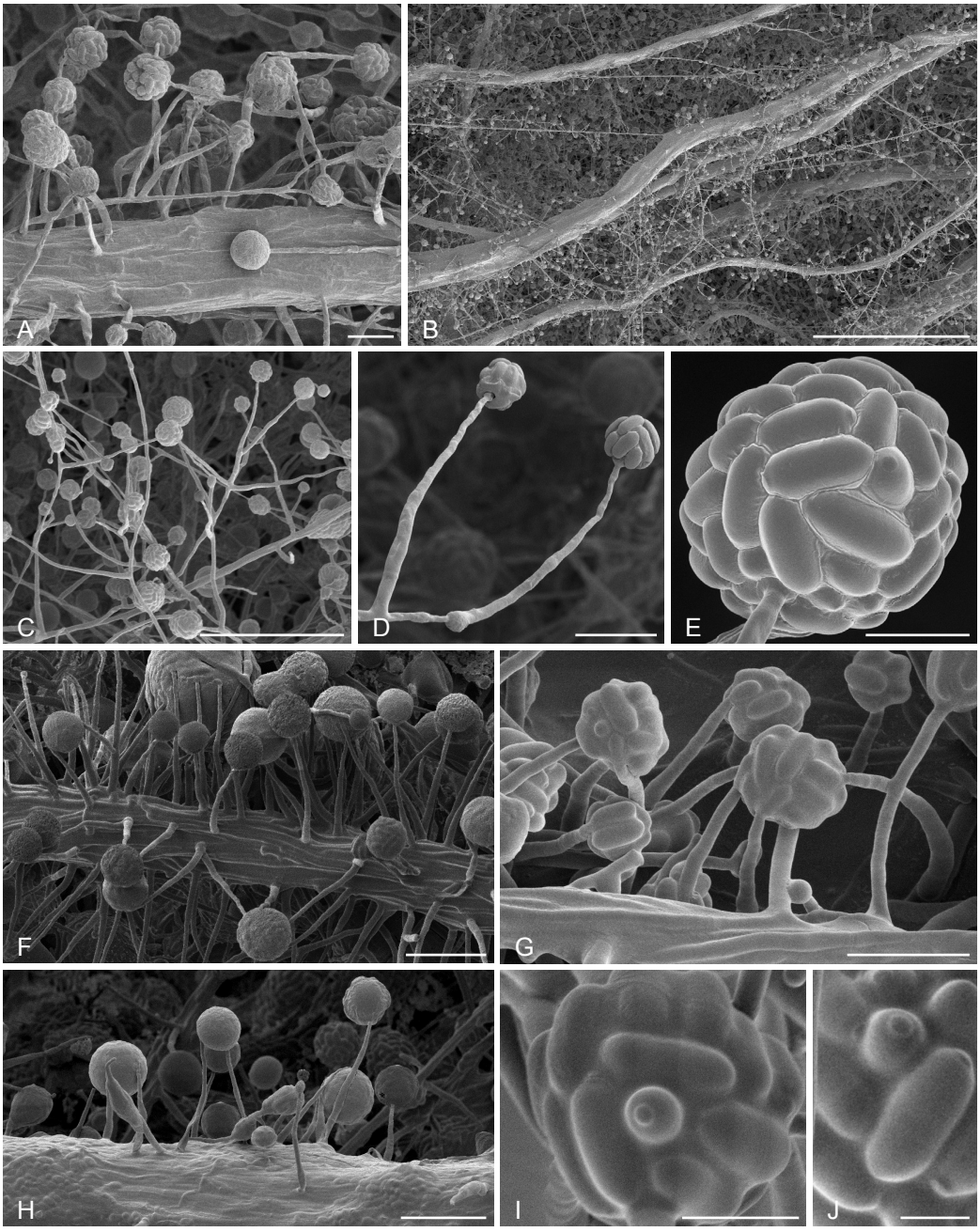


FIG. 31. *Chordomyces antarcticum* morphology as seen with cryoSEM. **A–E.** Strain A141 (= CBS 137610). **F.** Strain M10 (= CBS 120042). **G, I, J.** Strain V213 (= CBS 137630). **H.** Strain CBS 987.87. *Scale bars:* **A, D, G** = 10 µm; **B** = 300 µm; **C** = 50 µm; **E, I** = 4 µm; **F, H** = 20 µm; **J** = 2 µm.

complemented with NaCl and without it (**FIG. 9, 16**). As *Chordomyces* and *Sodiomyces* belong to taxonomically distinct clades with strong support values, it could be that there is different sensitivity to Na^+ ions in these species, which results in utilizing different strategies of dealing with high ambient pH. Strikingly, our newly defined section *Soda* in *Alternaria* prominently stands out from the rest, implying specific evolution of this clade within *Alternaria* of strong alkalitolerance for the members of section *Soda*.

Our growth experiments also revealed moderate and weak alkalitolerants such as *Scopulariopsis brevicaulis*, *Alternaria alternata* (sect. *Alternata*), *Purpureocillium lilacinum*, *Penicillium* sp., *Cladosporium sphaerospermum*, and *Fusarium* sp. with the growth optimum at neutral or below neutral pH values. These species partly also appear in the existing reports on the alkalitolerant and halotolerant fungi (**KLADWANG ET AL. 2003, GUNDE-CIMERMAN ET AL. 2009**). Darkly pigmented *Cladosporium sphaerospermum* is a ubiquitous fungus, often isolated from hypersaline environments throughout the world (**ZALAR ET AL. 2007, BENSCH ET AL. 2012**); however, in our study this species was not observed frequently and did not exhibit alkaliphilic abilities. The inability of the above named weak and moderate alkalitolerants to grow optimally at high pH values as well as their low abundancy, leads us to consider them as transition species in the alkaline soils, as they are also known to inhabit neutral soils worldwide.

The morphology studies of our alkalitolerant and alkaliphilic fungi provided evidence for the relation between the morphotype of the strain and its ability to tolerate the pH. The strong alkalitolerants and effective alkaliphiles, e.g., *Emericellopsis alkalina*, *Sodiomyces* species, *Acrostalagmus luteoalbus*, *Verticillium zaregamsianum* display acremonium-, verticillium-, and gliocladium-type asexual morphology (see examples in **FIG. 8, 12** and **28**) with conidia being clumped in heads, as better seen with cryogenic scanning electron microscopy. The same type of asexual morphology is observed in moderate alkalitolerants, sister to the *Emericellopsis* lineage, like *Sarocladium* sp. and *Acremonium sclerotigenum*. Ascomata of the isolates *Sodiomyces* species and *Emericellopsis alkalina* are of cleistothecial type, see **FIG. 27, 28** and **29**, and in **GRUM-GRZHIMAYLO ET AL. (2013A, B)**. Ascospores in those isolates are being formed in asci, walls of which lyse shortly, releasing ascospores in the ascoma matrix. Therefore, by the time the ascoma is fully developed, the ascospores are sunk in the common matrix with no signs of asci left, which in turn would release through the crack in the ascoma. Such type of ascomata, ascospore development and release might be adaptive to harsh alkaline and salty environments. Conidia clumping by slime and common ascospore matrix might create a favorable local environment prolonging the viability of both asexual and sexual spores. Slime can act as a buffering barrier lowering local pH around the spores, and may provide extra protection against osmotic shock. In a similar way, the halophilic basidiomycete *Wallemia ichthyophaga*, forms sarcina-like cell clumps (**ZALAR ET AL. 2005**). However, we saw no such gluing slime of conidia in strong alkalitolerants of *Alternaria* sect. *Soda* species. They rather often formed abundant dark thick-walled resting mycelium along with the dark microsclerotia, which are known to be stress-related morphological structures (**FIG. 32, 33**).

The ecology of the obligate alkaliphilic species like members of *Sodiomyces* is disputable.

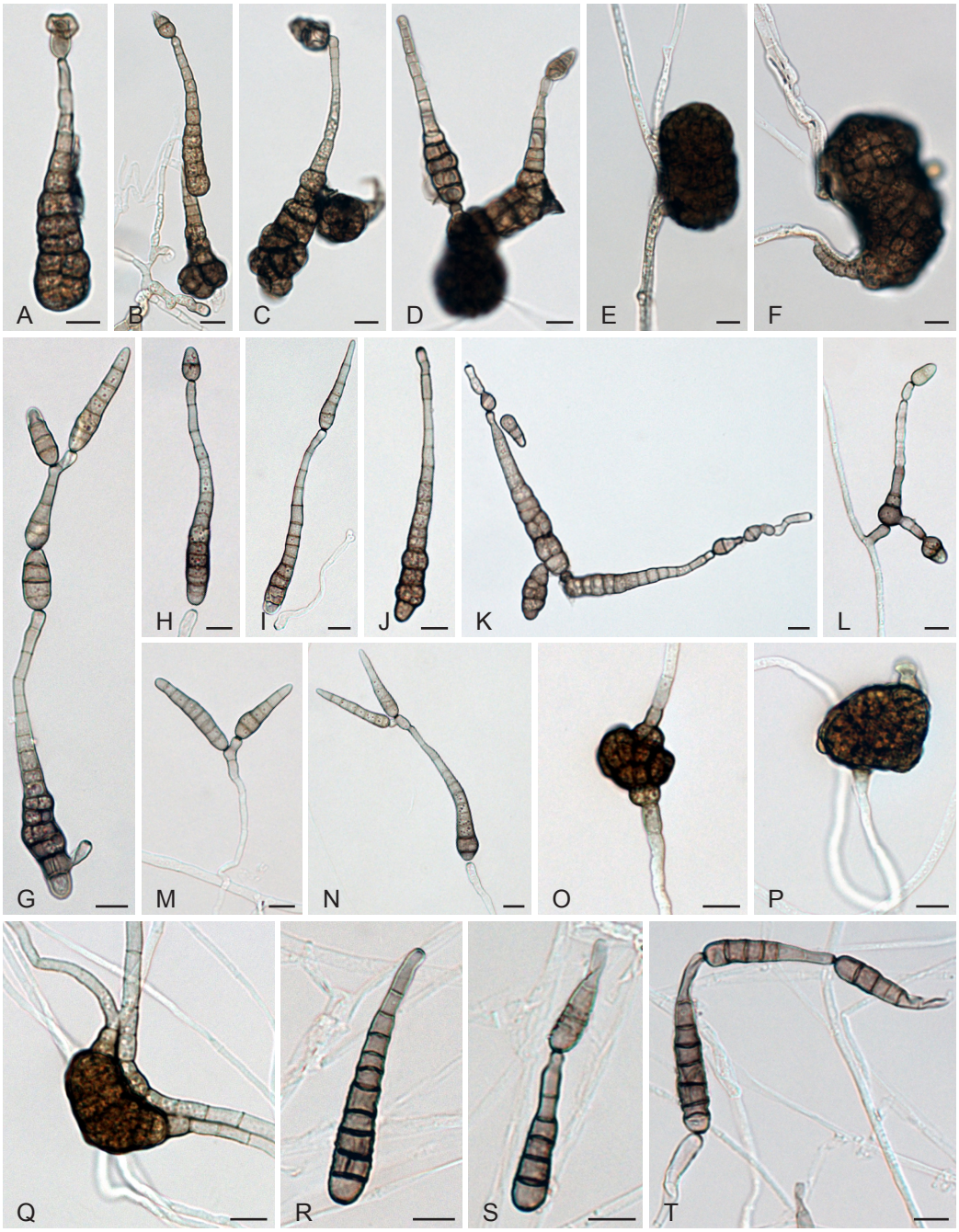


FIG. 32. *Alternaria* sect. *Soda*: conidia, conidiophores, and microsclerotia (LM). **A–F.** *A. petuchovskii* M304 (= CBS 137517^{T}). **G–Q.** *A. kulundii* M310 (= CBS 137522). **R–T.** *A. shukurtuzii* M307 (= CBS 137520^{T}). *Scale bars:* 10 µm.

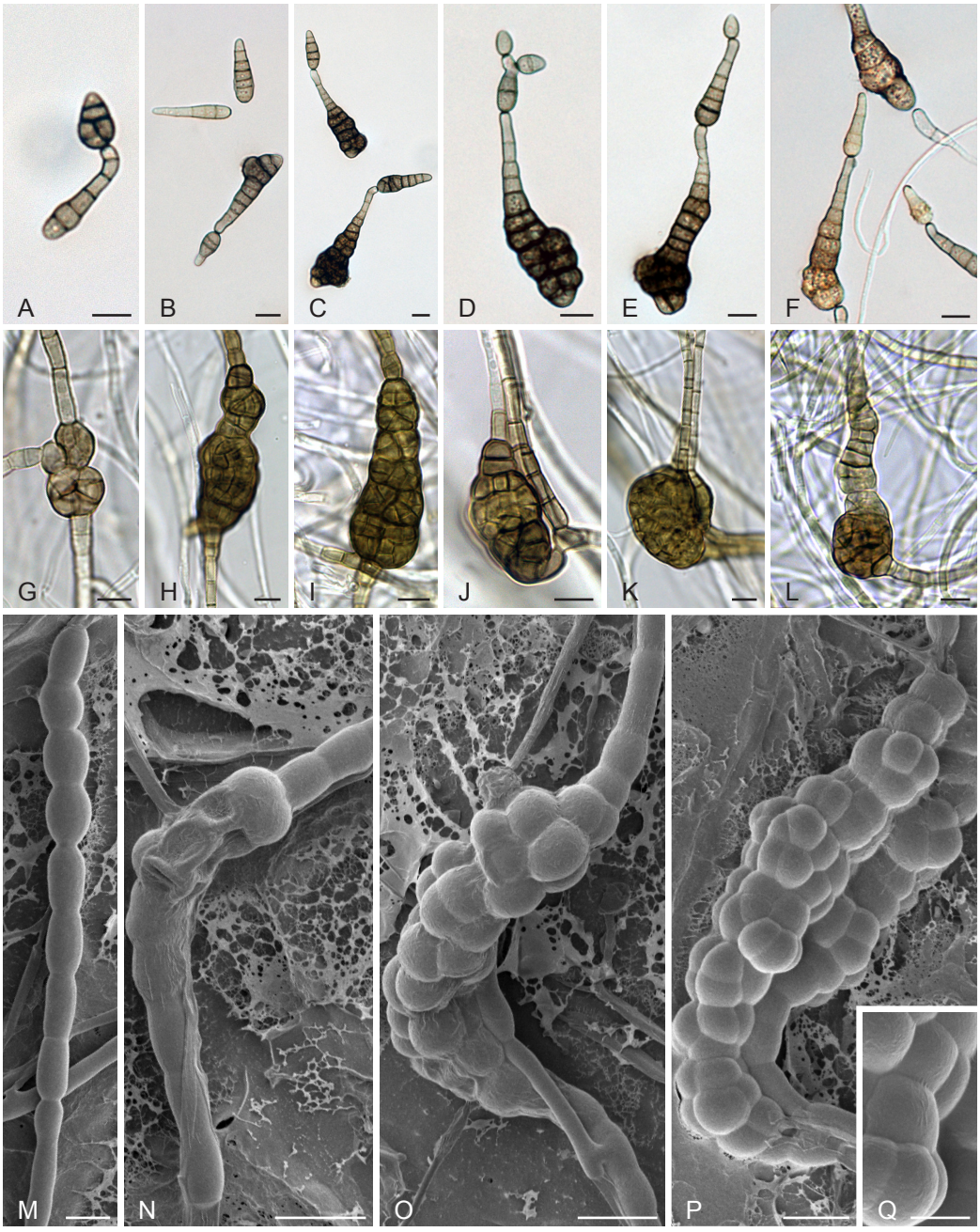


FIG. 33. *Alternaria kulundii* (sect. *Soda*) M313 (= CBS 137525^{T}). **A–F.** Conidia and conidiophores (LM). **G–L.** Microsclerotia (LM). **M–P.** Development stages of a microsclerotium (cryoSEM). **Q.** Close-up view on the cell surface of the microsclerotium (cryoSEM). *Scale bars:* **A–L, N– P** = 10 µm; **M, Q** = 5 µm.

Our previous growth experiments of *Sodiomyces alkalinus* ex-type strain CBS 110278^{T} showed the preference towards complex plant material carbon sources, like cotton seeds, alfalfameal and soybean hulls. That is not surprising, as those substrates are complemented with proteins and vitamins, broadening the diet for the fungus. Most probable substrates for fungi in soda soils include plant biomass from halophytic grasses, chitin carapaces and protein-rich eggs of brine shrimps, and possibly bird feathers. *Sodiomyces* might be tightly associated with bacteria as we often encountered them along the fungal hypha, which makes it challenging to separate from each other. Another large group of our strains, facultative alkaliphilic *Emericellopsis alkalina* is associated with marine-borne isolates, probably utilizing similar strategies as coping with marine environments, enabling them to thrive in soda soils as well (**GRUM-GRZHIMAYLO ET AL. 2013B**). *Acrostalagmus luteoalbus* and *Verticillium zaregamsianum* isolated from soda soils display facultative alkaliphilic and a strong alkalitolerant phenotype. The first one is known to be a widely distributed fungus inhabiting the rhizosphere (**DOMSCH ET AL. 2007**), while the latter has only been found in Japan associated with lettuce and tenweek stocks (**INDERBITZIN ET AL. 2011B**). The facultative alkaliphilic *Chordomyces antarcticum* fungus is enigmatic. All 12 isolates group unambiguously to the known CBS 987.87 isolate. This isolate was recovered from the *Hypogymnia physodes* lichen thallus, which was already infected by another fungus. It is therefore unclear whether the CBS 987.87 isolate was mycoparasitizing or was involved in lichen degradation. *Acremonium furcatum* CBS 122.42^{T} ex-type and *Acremonium stromaticum* CBS 863.73^{T} ex-type are sister species to *Chordomyces antarcticum* species group. By our unpublished data, *Acremonium furcatum* has been isolated frequently from the lichen thalli indeed; however, the type strains of *A. furcatum* and *A. stromaticum* were recovered from dune sands and rhizome, respectively. What makes fungi from soda soils and lichen thalli to be possibly associated is a mystery to us.

The present study has provided insight on the morphological and phylogenetic properties of alkalitolerant and alkaliphilic filamentous fungi recovered from soda soils. We attempted to make a systematic overview of the fungal biodiversity in soda soils; however, we expect different fungi to appear depending on the recovery medium. Therefore, this study is a starting point for subsequent recoveries of alkalitolerant and alkaliphilic fungi in the future. Notably, we have shown comprehensive polyphyletic diversity of filamentous fungi at soda lakes, which we hope will initiate more research and lead to subsequent discoveries of this peculiar ecological group of fungi. It is interesting to speculate if future isolations would confirm our observations about the several dominant lineages of strong alkalitolerants and effective alkaliphiles (i. e. families *Plectosphaerellaceae* and *Pleosporaceae*; genera *Emericellopsis* and *Thielavia*). Another intriguing aspect is the physiology of these fungi. Alkaliphilic fungi are likely to possess industrially valuable metabolites and enzymes, which are active at high pH. Whether alkaliphiles in different taxonomic lineages display convergent evolution in the alkaliphilic traits, or utilize different mechanisms enabling them to survive and grow best at high pH, remains to be discovered.

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SUPPLEMENTARY TABLE 1. Strains used in the study with the data on the habitats they were isolated from.

Species	Growth type*	Isolation medium	Strain code	CBS accession	VKM accession	Isolation area	Isolation place	mmol/ kg	ug ekv/100 g soil	pH	Total salts (g/kg)	Depth (cm)	Mineralization type	Isolation date	Reference
Acremonium roseolum		WA	A109	CBS 138118		Trans-Baikal, Russia	Kuchiger area	-	-	9	-	0 to 5	sulphate	Aug-03	1
Acremonium sclerotigenum		AA	A101	CBS 138752		Trans-Baikal, Russia	near Alla River	-	-	8	-	0 to 2	sulfate	Aug-03	1
Acremonium sclerotigenum		AA	A130			Trans-Baikal, Russia	near Alla River	-	-	8	-	0 to 2	sulfate	Aug-03	1
Acremonium sp.		AA	A104	CBS 138753		Kulunda Steppe, Altai, Russia	-	taken from Atriplex verrucifera MB.					soda	Aug-02	1
Acremonium sp.		WA/CZ	A105			Trans-Baikal, Russia	Orongoyskoe Lake	-	0.7	7.8	26	0 to 2	soda-sulfate	Aug-03	1
Acremonium sp.		AA	A106	CBS 138117		Trans-Baikal, Russia	Sulfatnoe Lake	-	0.94	8.5	3.7	25 to 38	sulfate-soda	Aug-03	1
Acremonium sp.		AA	A107			Trans-Baikal, Russia	Chedder Lake	-	-	9.1	-	0 to 5	soda	Aug-01	1
Acremonium sp.		AA	A108			Aral Lake, Kazakhstan	Aktumsyk Cape	taken from Suaeda salsa					chloride-sulfate	Sep-03	1
Acremonium sp.		MYA	A110			Trans-Baikal, Russia	Sulfatnoe Lake	-	31.22	10.3	139.4	0 to 2	sulfate-soda	Aug-03	1
Acremonium sp.		AA	A111	CBS 138119		Aral Lake, Kazakhstan	Aktumsyk Cape	-	-	8	-	0 to 5	chloride-sulfate	Sep-03	1
Acremonium sp.		AA	E102			Kulunda Steppe, Altai, Russia	noname lake	11	-	9.1	47	0 to 5	chloride	Aug-03	1
Acrostalagmus luteoalbus		AA	V206	CBS 137626		Kulunda Steppe, Altai, Russia	Solyonoe Lake	74	-	10	187	0 to 5	chloride	Aug-02	this study
Acrostalagmus luteoalbus		AA	V207	CBS 137627		Kulunda Steppe, Altai, Russia	Zheltir' Lake	230	-	9.6	137	0 to 5	soda-chloride	Aug-02	this study
Acrostalagmus luteoalbus		AA	V208	CBS 137628		Kulunda Steppe, Altai, Russia	Bezimyannoe Lake	70	-	10	38	0 to 5	chloride-sulfate	Aug-02	this study
Acrostalagmus luteoalbus		AA	V209	CBS 137629		Kulunda Steppe, Altai, Russia	Glauberovoe Lake	73	-	9.5	154	0 to 5	chloride-sulfate	Aug-02	this study
Acrostalagmus luteoalbus		AA	V205	CBS 137625		Kulunda Steppe, Altai, Russia	Bezimyannoe Lake	54	-	9.6	90	0 to 5	chloride	Aug-03	this study
Alternaria alternata		MYA	G408	CBS 137513		Kulunda Steppe, Altai, Russia	Petuchovskoe Lake	77	-	10.1	55	0 to 5	soda-chloride	Aug-03	this study
Alternaria kulundii		AA	M309	CBS 137521		Kulunda Steppe, Altai, Russia	Uzkoe Lake	1520	-	9.8	137	0 to 5	soda-chloride	Aug-02	this study
Alternaria kulundii		AA	M310	CBS 137522		Kulunda Steppe, Altai, Russia	Tanatar Lake	1155	-	10.2	73	0 to 5	soda	Aug-02	this study
Alternaria kulundii T		AA	M313	CBS 137525		Kulunda Steppe, Altai, Russia	north	134	-	10	22	0 to 5	soda	Aug-05	this study
Alternaria molesta		AA	M312	CBS 137524		Kulunda Steppe, Altai, Russia	Tanatar Lake	taken from Suaeda corniculata C.A.Mey					soda	Aug-02	this study
Alternaria petuchovskii T		AA	M304	CBS 137517		Kulunda Steppe, Altai, Russia	Petuchovskoe Lake	140	-	9.9	45	0 to 5	soda-chloride	Aug-03	this study
Alternaria shukurtuzii T		AA	M307	CBS 137520		Kulunda Steppe, Altai, Russia	Shukurtuz Lake	480	-	9.9	53	0 to 5	chloride-sulfate	Aug-02	this study
Alternaria sp.		AA	M301	CBS 137514		Kulunda Steppe, Altai, Russia	Solyonoe Lake	74	-	10	187	0 to 5	chloride	Aug-02	this study
Chordomyces antarcticum		AA	A134	CBS 137606		Kulunda Steppe, Altai, Russia	Petuchovskoe Lake	32	-	9.4	230	0 to 5	chloride	Aug-02	this study
Chordomyces antarcticum		AA	A135	CBS 137607		Kulunda Steppe, Altai, Russia	Berdabay Lake	242	-	10.1	60	0 to 5	soda	Aug-05	this study
Chordomyces antarcticum		AA	A137	CBS 137608		Trans-Baikal, Russia	Nuhe-Nur Lake	-	7.28	10.1	10	0 to 2	soda	Aug-03	this study
Chordomyces antarcticum		AA	A140	CBS 137609		North Gobi, Mongolia	Bayan-Zag area	-	1.08	9.3	43	1 to 2	soda-sulfate	Sep-03	this study
Chordomyces antarcticum		AA	A141	CBS 137610		Aral Lake, Kazakhstan	coast	taken from Suaeda salsa					chloride-sulfate	Dec-03	this study
Chordomyces antarcticum		AA	A142	CBS 137611		Aral Lake, Kazakhstan	coast	taken from Suaeda salsa					chloride-sulfate	Dec-03	this study
Chordomyces antarcticum		AA	M10	CBS 120042	FW-3039	North Gobi, Mongolia	Bayan-Zag area	-	0.5	8.9	21	0 to 1	sulfate-soda	Aug-03	this study
Chordomyces antarcticum T		AA	M27	CBS 120045	FW-3041	Kulunda Steppe, Altai, Russia	Karakul' Lake	265	-	9.8	144	0 to 5	soda	Aug-02	this study
Chordomyces antarcticum		AA	M30	CBS 120046	FW-3042	Kulunda Steppe, Altai, Russia	Uzkoe Lake	1520	-	9.8	137	0 to 5	soda-chloride	Aug-02	this study
Chordomyces antarcticum		AA	M31	CBS 120047	FW-3906	Kulunda Steppe, Altai, Russia	Solyonoe Lake	74	-	10	187	0 to 5	chloride	Aug-02	this study
Chordomyces antarcticum		AA	M41	CBS 120048	FW-3043	Kulunda Steppe, Altai, Russia	Petuchovskoe Lake	140	-	9.9	45	0 to 5	soda-chloride	Aug-03	this study
Chordomyces antarcticum		AA	V213	CBS 137630		Kulunda Steppe, Altai, Russia	Bezimyannoe Lake	70	-	10.1	38	0 to 5	chloride-sulfate	Aug-02	this study
Cladosporium sphaerospermum		AA	G402	CBS 137612		Trans-Baikal, Russia	near Alla River	-	-	8	-	0 to 5	sulfate	Aug-01	this study
Emericellopsis alkalina		AA	A103			Kulunda Steppe, Altai, Russia	Mirabilit Lake	295	-	9.6	100	0 to 5	soda-chloride-sulfate	Aug-02	1
Emericellopsis alkalina		AA	A112			North-East Mongolia	Burd Lake	23	-	10.1	33	0 to 5	soda	1999	1
Emericellopsis alkalina		AA	A113		FW-1476	Choibalsan area, North-East Mongolia	-	474	-	11	57	0 to 5	soda	1999	1
Emericellopsis alkalina		AA	A114		FW-1473	Kulunda Steppe, Altai, Russia	Solyonoe Lake	74	-	10	187	0 to 5	chloride	Aug-02	1
Emericellopsis alkalina		AA	A115		FW-1474	Kulunda Steppe, Altai, Russia	noname lake (near Sazadi Lake)	220	-	9.6	225	0 to 5	chloride-sulfate	Aug-02	1
Emericellopsis alkalina		AA	A116			Kulunda Steppe, Altai, Russia	Mirabilit Lake	295	-	9.6	100	0 to 5	soda-chloride-sulfate	Aug-02	1
Emericellopsis alkalina		AA	A117	CBS 138120	FW-1471	Kulunda Steppe, Altai, Russia	Shukurtuz Lake	480	-	9.9	53	0 to 5	chloride-sulfate	Aug-02	1
Emericellopsis alkalina		AA	A118			Kulunda Steppe, Altai, Russia	Zheltir' Lake	230	-	9.6	137	0 to 5	soda-chloride	Aug-02	1
Emericellopsis alkalina		AA	A119			Kulunda Steppe, Altai, Russia	Bezimyannoe Lake	70	-	10.1	38	0 to 5	chloride-sulfate	Aug-02	1
Emericellopsis alkalina		AA	A120			Kulunda Steppe, Altai, Russia	Bezimyannoe Lake	1410	-	9.9	310	0 to 5	soda	Aug-02	1
Emericellopsis alkalina		AA	A121			Kulunda Steppe, Altai, Russia	Tanatar Lake	1155	-	10.2	73	0 to 5	soda	Aug-02	1
Emericellopsis alkalina		AA	A122			Kulunda Steppe, Altai, Russia	near Solonovka village	54	-	9.5	65	0 to 5	chloride	Aug-02	1
Emericellopsis alkalina		AA	A123			Kulunda Steppe, Altai, Russia	-	taken from Salicornia europaea L.					soda	Aug-03	1
Emericellopsis alkalina		AA	A124			Kulunda Steppe, Altai, Russia	south, Berdabay	-	242	10.1	60	0 to 5	soda	Aug-05	1
Emericellopsis alkalina		AA	A125			Trans-Baikal, Russia	Nuhe-Nur Lake	-	6.36	10.1	7.1	0 to 2	soda	Aug-03	1
Emericellopsis alkalina		AA	A126			Trans-Baikal, Russia	Nuhe-Nur Lake	-	2.12	10.1	1.9	3 to 12	soda	Aug-03	1
Emericellopsis alkalina		AA	A127			Trans-Baikal, Russia	Nuhe-Nur Lake	-	2.12	10.1	1.9	3 to 12	soda	Aug-03	1
Emericellopsis alkalina		AA	A128			Trans-Baikal, Russia	Sulfatnoe Lake	-	31.22	10.3	139.4	0 to 2	sulfate-soda	Aug-03	1
Emericellopsis alkalina T		AA	E101	CBS 127350	F-4108	Kulunda Steppe, Altai, Russia	Tanatar Lake	1155	-	10.1	73	0 to 5	soda	Aug-02	1
Emericellopsis alkalina		AA	M14	CBS 120043	F-3905	Kulunda Steppe, Altai, Russia	Bezimyannoe Lake	1410	-	9.9	310	0 to 5	soda	Aug-02	1
Emericellopsis alkalina		AA	M20	CBS 120044	FW-3040	Kulunda Steppe, Altai, Russia	Zheltir' Lake	230	-	9.6	137	0 to 5	soda-chloride	Aug-02	1
Emericellopsis alkalina		AA	M71	CBS 120049	F-3907	Trans-Baikal, Russia	Sulfatnoe Lake	-	31.22	10.3	139	0 to 2	sulfate-soda	Aug-03	1
Fusarium sp.		AA	G412	CBS 137614		Aral Lake, Kazakhstan	Aktumsyk Cape	-	-	8	-	0 to 5	chloride-sulfate	Sep-03	this study
Lasiosphaeriaceae sp.	?	AA	M314	CBS 137526		Choibalsan area, North-East Mongolia	Burd Lake	23	-	10.1	33	0 to 5	soda	1999	this study
Penicillium sp.		CZ	G418	CBS 137615		Trans-Baikal, Russia	Sulfatnoe Lake	-	0.59	8.1	11.1	6 to 24	sulfate-soda	Aug-03	this study
Pleosporaceae sp.		AA	M302	CBS 137515		Kulunda Steppe, Altai, Russia	Petuchovskoe Lake	77	-	10.1	55	0 to 5	soda-chloride	Aug-03	this study
Pleosporaceae sp.		AA	M303	CBS 137516		Kulunda Steppe, Altai, Russia	Petuchovskoe Lake	140	-	9.9	45	0 to 5	soda-chloride	Aug-03	this study
Pleosporaceae sp.		AA	M305	CBS 137518		Kulunda Steppe, Altai, Russia	Belen'koe Lake	610	-	9.6	91	0 to 5	soda-chloride-sulfate	Aug-02	this study
Pleosporaceae sp.		AA	M306	CBS 137519		Kulunda Steppe, Altai, Russia	noname lake	220	-	9.6	225	0 to 5	chloride-sulfate	Aug-02	this study
Pleosporaceae sp.		AA	M311	CBS 137523		Kulunda Steppe, Altai, Russia	Tanatar Lake	taken from Suaeda acuminata C.A.Mey					soda	Aug-02	this study
Pleosporaceae sp.		AA	M315	CBS 137527		North Gobi, Mongolia	Ulan-Nur Lake	-	0.8	7.8	13	0 to 1	sulfate-soda	Aug-03	this study
Purpureocillium lilacinum		CZ	G406	CBS 137613		Trans-Baikal, Russia	Nuhe-Nur Lake	-	7.28	10.1	9.7	0 to 2	soda	Aug-03	this study
Sarocladium sp.		AA	A131	CBS 138121		Aral Lake, Kazakhstan	Aktumsyk Cape	-	-	8.3	-	0 to 5	chloride-sulfate	Sep-03	1
Scopulariopsis brevicaulis		AA	G413	CBS 137631		Kulunda Steppe, Altai, Russia	Mirabilit Lake	870	-	9.7	165	0 to 5	soda-chloride-sulfate	Aug-02	this study

Scopulariopsis brevicaulis		AA	G415	CBS 137632		Trans-Baikal, Russia	Kuchiger	-	-	8	-	0 to 5	sulfate	Aug-03	this study
Scopulariopsis fusca		AA	G414	CBS 138116		Trans-Baikal, Russia	Kuchiger	-	-	9	-	0 to 5	sulfate	Aug-03	this study
Sodiomyces alkalinus		AA	F7	CBS 132729		Kunkur Steppe, Chitinskaya area, Russia	low-salt soda lake	-	-	-	-	0 to 5	soda	1999	2
Sodiomyces alkalinus		AA	F8	CBS 133680		Kunkur Steppe, Chitinskaya area, Russia	low-salt soda lake	-	-	-	-	0 to 5	soda	1999	2
Sodiomyces alkalinus		AA	F9	CBS 133681		Kunkur Steppe, Chitinskaya area, Russia	low-salt soda lake	-	-	-	-	0 to 5	soda	1999	2
Sodiomyces alkalinus		AA	F10	CBS 132730		Natron Lake, Tanzania	soda soil on the edge of the lake	-	-	10 (water)		0 to 5	soda	1999	2
Sodiomyces alkalinus T		AA	F11	CBS 110278	F-3762	Choibalsan area, North-East Mongolia	Shar-Burdiyn Lake	419	-	10.7	49	0 to 5	soda	1999	2
Sodiomyces alkalinus		AA	F12	CBS 132731		North-East Mongolia	Barun-Undziyn Lake	69	-	10.5	82	0 to 5	soda	1999	2
Sodiomyces alkalinus		AA	F13	CBS 132732		Kulunda Steppe, Altai, Russia	Solyonoe Lake	74	-	10	187	0 to 5	chloride	Aug-02	2
Sodiomyces alkalinus		AA	F14	CBS 133682		Kulunda Steppe, Altai, Russia	Karakul' Lake	265	-	9.8	144	0 to 5	soda	Aug-02	2
Sodiomyces alkalinus		AA	F15	CBS 133683		Kulunda Steppe, Altai, Russia	Mirabilit Lake	870	-	9.7	165	0 to 5	soda-chloride-sulfate	Aug-02	2
Sodiomyces alkalinus		AA	F16	CBS 133684		Kulunda Steppe, Altai, Russia	Petuchovskoe Lake	1160	-	10.2	163	0 to 5	soda	Aug-02	2
Sodiomyces alkalinus		AA	F17	CBS 133685		Kulunda Steppe, Altai, Russia	Bezimyannoe Lake	1410	-	9.9	310	0 to 5	soda	Aug-02	2
Sodiomyces alkalinus		AA	F18	CBS 132733		Kulunda Steppe, Altai, Russia	Tanatar Lake	1155	-	10.2	73	0 to 5	soda	Aug-02	2
Sodiomyces alkalinus		AA	F19	CBS 133686		Kulunda Steppe, Altai, Russia	Mirabilit Lake	295	-	9.6	100	0 to 5	soda-chloride-sulfate	Aug-02	2
Sodiomyces alkalinus		AA	F20	CBS 133687		North Gobi, Mongolia	Bayan-Zag area	-	1.08	9.3	43	1 to 2	sulfate-soda	Aug-03	2
Sodiomyces alkalinus		AA	F21	CBS 133688		North Gobi, Mongolia	Bayan-Zag area	-	1.7	9.2	6	10 to 18	sulfate-soda	Aug-03	2
Sodiomyces alkalinus		AA	F22	CBS 133689		Kulunda Steppe, Altai, Russia	north	134	-	10	22	0 to 5	soda	Aug-05	2
Sodiomyces alkalinus		AA	F23	CBS 133690		Kulunda Steppe, Altai, Russia	Karagay Lake	345	-	9.9	43	0 to 5	soda	Aug-05	2
Sodiomyces alkalinus		AA	F24	CBS 133691		Kulunda Steppe, Altai, Russia	Gor'koye Lake	366	-	10.4	30	0 to 5	soda	Aug-05	2
Sodiomyces magadii T		AA	MAG2	CBS 137619	F-4583	Magadi Lake, Kenya	soda soil on the edge of the lake	-	-	11	-	-	-	Jan-13	this study
Sodiomyces tronii T		AA	MAG1	CBS 137618	F-4582	Magadi Lake, Kenya	soda soil on the edge of the lake	-	-	11	-	-	-	Jan-13	this study
Sodiomyces tronii		AA	MAG3	CBS 137620		Magadi Lake, Kenya	soda soil on the edge of the lake	-	-	11	-	-	-	Jan-13	this study
Thielavia sp.		AA	M316	CBS 137616		Kunkur Steppe, Chitinskaya area, Russia	Holvo-Torum Lake	38	-	9.2	76	0 to 5	soda	1999	this study
Thielavia sp.		AA	M317	CBS 137617		Ararat Valley, Armenia	Yeraskhahun	62	-	10.2	42	0 to 5	soda	1999	this study
Verticillium zaregamsianum		AA	V201	CBS 137621		Trans-Baikal, Russia	Orongoyskoe Lake	-	0.3	7.6	4.6	31 to 48	soda-sulfate	Aug-03	this study
Verticillium zaregamsianum		AA	V202	CBS 137622		Trans-Baikal, Russia	Orongoyskoe Lake	-	0.43	7.9	1.7	48 to 70	soda-sulfate	Aug-03	this study
Verticillium zaregamsianum		AA	V203	CBS 137623		Trans-Baikal, Russia	Orongoyskoe Lake	-	0.57	8.1	1.6	63 to 71	soda-sulfate	Aug-03	this study
Verticillium zaregamsianum		AA	V204	CBS 137624		Trans-Baikal, Russia	Orongoyskoe Lake	-	0.65	8.2	1.4	90 to 95	soda-sulfate	Aug-03	this study

1 — GRUM-GRZHIMAYLO 2013B

2 — GRUM-GRZHIMAYLO 2013A

* — obligate alkaliphile
— facultative alkaliphile
— strong alkalitolerant
— moderate alkalitolerant
— weak alkalitolerant

SUPPLEMENTARY TABLE 2. *Ascomycota* tree GenBank accessions. New isolates from soda soils are in bold.

taxon	voucher	SSU
Alternaria alternata	CBS 916.96	KC584507
Alternaria petuchovskii	**M304 (= CBS 137517)**	**KJ443079**
Ambrosiella xylebori	CBS 110.61	DQ471031
Armillaria mellea	PBM2470	AY787217
Aspergillus fumigatus	JCM1738	AB008401
Aureobasidium pullulans	CBS 584.75	DQ471004
Bionectria ochroleuca	GJS90-227	AY489684
Bombardia bombarda	AFTOL-ID 967	DQ471021
Botryotinia fuckeliana	OSC 100012	AY544695
Bulgaria inquinans	CBS 118.31	DQ471008
Caliciopsis orientalis	CBS 138.64	DQ471039
Camarops microspora	CBS 649.92	DQ471036
Capronia pilosella	WUC28	DQ823106
Chaetomium globosum	CBSc 15-5973	AY545725
Cladonia caroliniana	AFTOL-ID 3	AY584664
Cladosporium cladosporioides	CBS 170.54	DQ678004
Cladosporium sphaerospermum	**G402 (= CBS 137612)**	**KJ443070**
Coccomyces strobi	CBS 202.91	DQ471027
Cordyceps cardinalis	OSC 93609	AY184973
Cryphonectria parasitica	ATCC 38755	DQ862048
Dermatocarpon miniatum	AFTOL-ID 91	AY584668
Diaporthe eres	CBS 109767	DQ471015
Diatrype disciformis	CBS 197.49	DQ471012
Dothidea insculpta	CBS 189.58	DQ247810
Emericellopsis alkalina	CBS 127350	KC987209

Emericellopsis maritima	CBS 491.71	FJ176807
Eutypa lata	CBS 208.87	DQ836896
Exophiala pisciphila	WUC137	DQ823108
***Fusarium* sp.**	**G412 (= CBS 137614)**	**KJ443073**
Gelatinospora tetrasperma	CBS 178.33	DQ471032
Gibberella fujikuroi	CBS 221.76	AB725604
Glomerella cingulata	FAU 553	AF543762
Gnomonia gnomon	CBS 199.53	DQ471019
Heleococcum japonense	CBS 397.67	JX158485
Hypocrea lutea	ATCC 208838	AF543768
***Lasiosphaeriaceae* sp.**	**M314 (= CBS 137526)**	**KJ443088**
Lecanora hybocarpa	AFTOL-ID 639	DQ782883
Lindra thalassiae	JK 5090A	DQ470994
Lobaria scrobiculata	AFTOL-ID 128	AY584679
Magnaporthe grisea	Ina168	AB026819
Melanconis alni	AR 3500	DQ862052
Melanopsamma pomiformis	ATCC 18873	AY489677
Microascus trigonosporus	CBS 218.31	DQ471006
Mollisia cinerea	OSC 100029	DQ470990
Neurospora crassa	Broad	X04971
Paradendryphiella arenaria	CBS 181.58	KC793336
Peltigera degenii	AFTOL-ID 134	AY584681
***Penicillium* sp.**	**G418 (= CBS 137615)**	**KJ443076**
Petriella setifera	CBS 437.75	DQ471020
Pezicula carpinea	CBS 282.39	DQ471016
Peziza vesiculosa	OSC 126	DQ470995
Physcia aipolia	AFTOL-ID 84	DQ782876
Pleospora herbarum	CBS 191.86	DQ247812
***Pleosporaceae* sp.**	**M305 (= CBS 137518)**	**KJ443080**
Purpureocillium lilacinum	CBS 284.36	AY526475
Purpureocillium lilacinum	**G406 (= CBS 137613)**	**KJ443071**
Saccharomyces castellii	CBS 4309	Z75577
Sclerotinia sclerotiorum	CBS 499.50	DQ471013
Scopulariopsis brevicaulis	**G413 (= CBS 137631)**	**KJ443074**
Sodiomyces alkalinus	CBS 110278	JX158471
***Thielavia* sp.**	**M316 (= CBS 137616)**	**KJ443090**
Umbilicaria mammulata	96042	AY648114
Usnea antarctica	AFTOL-ID 813	DQ883702
Verticillium dahliae	ATCC 16535	AY489705
Westerdykella cylindrica	CBS 454.72	AY016355
Xylaria acuta	ATCC 56487	AY544719

SUPPLEMENTARY TABLE 3. *Scopulariopsis* tree GenBank accessions. New isolates from soda soils are in bold.

taxon	voucher	LSU	TEF1-α
Graphium tectonae	CBS 127.84	EF151332	_
Microascuc manginii	MUCL 41467	HG380433	HG380356
Microascuc manginii	UTHSC 09-2576	HG380459	HG380382
Microascuc manginii	UTHSC 09-3241	HG380460	HG380383
Microascuc manginii	UTHSC 10-3405	HG380461	HG380384
Microascuc manginii^{T}	MUCL 40743	HG380458	HG380381
Microascus cinereus^{T}	CBS 365.65	HG380423	HG380346
Microascus cirrosus^{T}	CBS 217.31	JQ434680	HG380352
Microascus niger	MUCL 40746	HG380434	HG380357
Microascus pyramidus^{T}	CBS 212.65	HG380435	HG380358
Microascus trigonosporus^{T}	CBS 218.31	JQ434679	HG380359
Petriella setifera	CBS 437.75	DQ470969	DQ836911
Scopulariopsis acremonium^{T}	MUCL 9028	HG380439	HG380362
Scopulariopsis asperula	CBS 853.68	JQ434669	JQ434621

Scopulariopsis brevicaulis	**G413 (= CBS 137631)**	**KJ443117**	**KJ443205**
Scopulariopsis brevicaulis	**G415 (= CBS 137632)**	**KJ443119**	**KJ443207**
Scopulariopsis brevicaulis T	MUCL 40726	HG380440	HG380363
Scopulariopsis brevicaulis	UTHSC 06-277	HG380441	HG380364
Scopulariopsis brevicaulis	UTHSC 06-619	HG380442	HG380365
Scopulariopsis brevicaulis	UTHSC 09-1092	HG380445	HG380368
Scopulariopsis brevicaulis	UTHSC 11-1240	HG380446	HG380369
Scopulariopsis brevicaulis	UTHSC 11-427	HG380448	HG380371
Scopulariopsis brumptii T	MUCL 9003	HG380450	HG380373
Scopulariopsis carbonaria T	MUCL 9027	HG380462	HG380385
Scopulariopsis chartarum T	MUCL 9001	HG380463	HG380386
Scopulariopsis flava	LCP 10.5710	JQ434646	JQ434631
Scopulariopsis flava	MUCL 9031	HG380464	HG380387
Scopulariopsis fusca	**G414 (= CBS 138116)**	**KJ443118**	**KJ443206**
Scopulariopsis fusca	MUCL 9032	HG380465	HG380388
Scopulariopsis gracilis T	CBS 369.70	HG380467	HG380390
Scopulariopsis murina T	IHEM 18567	HG380481	HG380404
Scopulariopsis sp.	UTHSC 05-3453	HG380498	HG380421
Scopulariopsis sp.	UTHSC 09-866	HG380499	HG380422

SUPPLEMENTARY TABLE 4. *Plectosphaerellaceae* tree GenBank accessions. New isolates from soda soils are in bold.

taxon	voucher	LSU	ITS
Acremonium alcalophilum T	CBS 114.92	JX158443	JX158421
Acremonium brunnescens T	CBS 559.73	HQ231966	_
Acremonium collariferum	JW016P.1	FJ765366	FJ765367
Acremonium cucurbitacearum	CBS 683.88	HQ231968	DQ825968
Acremonium furcatum T	CBS 122.42	EF543831	_
Acremonium nepalense T	CBS 971.72	HQ231970	DQ825971
Acremonium restrictum T	CBS 178.40	HQ232119	_
Acremonium stromaticum T	CBS 863.73	HQ232143	DQ825969
Acrostalagmus annulatus	DAOM 212126	GU180646	GU180632
Acrostalagmus luteoalbus	CBS 194.87	EF543826	_
Acrostalagmus luteoalbus	**V205 (= CBS 137625)**	**KJ443141**	**KJ443271**
Acrostalagmus luteoalbus	**V206 (= CBS 137626)**	**KJ443142**	**KJ443272**
Acrostalagmus luteoalbus	**V207 (= CBS 137627)**	**KJ443143**	**KJ443273**
Acrostalagmus luteoalbus	**V208 (= CBS 137628)**	**KJ443144**	**KJ443274**
Acrostalagmus luteoalbus	**V209 (= CBS 137629)**	**KJ443145**	**KJ443275**
Cephalosporium serrae var. *fuscum*	CBS 120818	_	EF543844
Cephalosporium serrae var. *fuscum* T	CBS 560.65	_	EF543845
Chordomyces antarcticum	**A134 (= CBS 137606)**	**KJ443102**	**KJ443234**
Chordomyces antarcticum	**A135 (= CBS 137607)**	**KJ443103**	**KJ443235**
Chordomyces antarcticum	**A137 (= CBS 137608)**	**KJ443104**	**KJ443236**
Chordomyces antarcticum	**A140 (= CBS 137609)**	**KJ443105**	**KJ443237**
Chordomyces antarcticum	**A141 (= CBS 137610)**	**KJ443106**	**KJ443238**
Chordomyces antarcticum	**A142 (= CBS 137611)**	**KJ443107**	**KJ443239**
Chordomyces antarcticum	CBS 987.87	JX158444	JX158422
Chordomyces antarcticum	**M10 (= CBS 120042)**	**KJ443108**	**KJ443240**
Chordomyces antarcticum T	**M27 (= CBS 120045)**	**KJ443109**	**KJ443241**
Chordomyces antarcticum	**M30 (= CBS 120046)**	**KJ443110**	**KJ443242**
Chordomyces antarcticum	**M31 (= CBS 120047)**	**KJ443111**	**KJ443243**
Chordomyces antarcticum	**M41 (= CBS 120049)**	**KJ443112**	**KJ443244**
Chordomyces antarcticum	**V213 (= CBS 137630)**	**KJ443146**	**KJ443276**
Colletotrichum boninense	CMT74	JQ754138	JQ754046
Gibellulopsis chrysanthemi T	MAFF 242621	KC287230	KC287235
Gibellulopsis nigrescens	CBS 577.50	EF543841	EF543856
Gibellulopsis nigrescens	DAOM 226890	GU180648	GU180631
Gibellulopsis piscis T	CBS 892.70	EF543835	DQ825985
Gliocladium cibotii T	CBS 109240	EF543842	DQ825980

Glomerella cingulata	FAU 553	AF543786	_
Lectera colletotrichoides	IMI 265740	_	JQ647449
Lectera longa ᵀ	IMI 181698	_	JQ647448
Musicillium theobromae	CBS 385.32	EF543836	_
Musicillium theobromae	CBS 458.51	EF543837	EF543858
Musicillium theobromae ᵀ	CBS 968.72	EF543838	EF543859
Plectosphaerella alismatis ᵀ	CBS 113362	JF780521	JF780523
Plectosphaerella citrullae	Plect 189	HQ239050	HQ238964
Plectosphaerella citrullae ᵀ	CBS 131741	HQ239048	HQ238962
Plectosphaerella cucumerina	DAOM 226828	GU180647	GU180630
Plectosphaerella cucumerina	FAU 508	U17399	_
Plectosphaerella cucumerina	P16	AF222497	_
Plectosphaerella delsorboi ᵀ	CBS 116708	EF543843	DQ825986
Plectosphaerella melonis	Plect 148	HQ239007	HQ238968
Plectosphaerella pauciseptata ᵀ	CBS 131745	HQ239012	HQ238971
Plectosphaerella plurivora ᵀ	CBS 131742	HQ239015	HQ238975
Plectosphaerella ramiseptata	CBS 131743	HQ239049	HQ238963
Sodiomyces alkalinus ᵀ	CBS 110278	JX158427	JX158405
Sodiomyces magadii ᵀ	**MAG2 (= CBS 137619)**	**KJ443148**	**KJ443278**
Sodiomyces tronii ᵀ	**MAG1 (= CBS 137618)**	**KJ443147**	**KJ443277**
Sodiomyces tronii	**MAG3 (= CBS 137620)**	**KJ443149**	**KJ443279**
Stachylidium bicolor	DAOM 226658	GU180651	_
Verticillium albo-atrum	CBS 130.51	HQ231976	DQ825977
Verticillium dahliae	76 Greece	AF104926	AF104926
Verticillium dahliae	ATCC 16535	DQ470945	_
Verticillium zaregamsianum	**V201 (= CBS 137621)**	**KJ443137**	**KJ443267**
Verticillium zaregamsianum	**V202 (= CBS 137622)**	**KJ443138**	**KJ443268**
Verticillium zaregamsianum	**V203 (= CBS 137623)**	**KJ443139**	**KJ443269**
Verticillium zaregamsianum	**V204 (= CBS 137624)**	**KJ443140**	**KJ443270**

SUPPLEMENTARY TABLE 5. *Verticillium* sensu stricto tree GenBank accessions. New isolates from soda soils are in bold.

taxon	voucher	Act	GAPDH	TS	ITS
Gibellulopsis nigrescens	PD595	JN188102	JN188166	JN188038	JN187976
Gibellulopsis nigrescens	PD711	JN188127	JN188191	JN188063	JN187999
Verticillium albo-atrum	PD693	JN188122	JN188186	JN188058	JN187994
Verticillium albo-atrum ᵀ	PD747	JN188144	JN188208	JN188080	JN188016
Verticillium albo-atrum	PD748	JN188145	JN188209	JN188081	JN188017
Verticillium alfalfae	PD353	HQ206933	HQ414731	HQ414921	HQ206742
Verticillium alfalfae ᵀ	PD489	JN188097	JN188161	JN188033	JN187971
Verticillium alfalfae	PD620	HQ206965	HQ414763	HQ414953	HQ206851
Verticillium dahliae ᵀ	PD322	HQ206921	HQ414719	HQ414909	HQ206718
Verticillium dahliae	PD502	HQ206942	HQ414740	HQ414930	HQ206813
Verticillium isaacii	PD343	JN188090	JN188154	JN188026	JN187964
Verticillium isaacii	PD619	JN188110	JN188174	JN188046	JN187984
Verticillium isaacii ᵀ	PD660	HQ206985	HQ414783	HQ414973	HQ206873
Verticillium klebahnii	PD347	JN188091	JN188155	JN188027	JN187965
Verticillium klebahnii ᵀ	PD401	JN188093	JN188157	JN188029	JN187967
Verticillium klebahnii	PD658	JN188113	JN188177	JN188049	JN187987
Verticillium longisporum ᵀ	PD687 allele A1	HQ206993	HQ414791	HQ414981	_
Verticillium longisporum ᵀ	PD687 allele D2	HQ206994	HQ414792	HQ414982	_
Verticillium nonalfalfae ᵀ	PD592	JN188099	JN188163	JN188035	JN187973
Verticillium nonalfalfae	PD744	JN188141	JN188205	JN188077	JN188013
Verticillium nonalfalfae	PD810	JN188150	JN188214	JN188086	JN188022
Verticillium nubilum	PD621	HQ206966	HQ414764	HQ414954	HQ206852
Verticillium nubilum	PD702	JN188123	JN188187	JN188059	JN187995
Verticillium nubilum ⊥	PD742	JN188139	JN188203	JN188075	JN188011
Verticillium tricorpus	PD594	JN188101	JN188165	JN188037	JN187975
Verticillium tricorpus ᵀ	PD690	JN188121	JN188185	JN188057	JN187993

Verticillium tricorpus	PD703	JN188124	JN188188	JN188060	JN187996
Verticillium zaregamsianum	PD586	JN188098	JN188162	JN188034	JN187972
Verticillium zaregamsianum	PD735	JN188132	JN188196	JN188068	JN188004
Verticillium zaregamsianum T	PD736	JN188133	JN188197	JN188069	JN188005
Verticillium zaregamsianum	PD738	JN188135	JN188199	JN188071	JN188007
Verticillium zaregamsianum	PD739	JN188136	JN188200	JN188072	JN188008
Verticillium zaregamsianum	PD740	JN188137	JN188201	JN188073	JN188009
Verticillium zaregamsianum	**V201 (= CBS 137621)**	**KJ443280**	**KJ443284**	**KJ443288**	**KJ443267**
Verticillium zaregamsianum	**V202 (= CBS 137622)**	**KJ443281**	**KJ443285**	**KJ443289**	**KJ443268**
Verticillium zaregamsianum	**V203 (= CBS 137623)**	**KJ443282**	**KJ443286**	**KJ443290**	**KJ443269**
Verticillium zaregamsianum	**V204 (= CBS 137624)**	**KJ443283**	**KJ443287**	**KJ443291**	**KJ443270**

SUPPLEMENTARY TABLE 6. *Sodiomyces* tree GenBank accessions. New isolates from soda soils are in bold.

taxon	voucher	ITS
Acremonium alcalophilum T	CBS 114.92	JX158421
Sodiomyces alkalinus T	CBS 110278	JX158405
Sodiomyces alkalinus	CBS 132729	JX158401
Sodiomyces alkalinus	CBS 132730	JX158404
Sodiomyces alkalinus	CBS 132731	JX158406
Sodiomyces alkalinus	CBS 132732	JX158407
Sodiomyces alkalinus	CBS 132733	JX158412
Sodiomyces alkalinus	CBS 133680	JX158402
Sodiomyces alkalinus	CBS 133681	JX158403
Sodiomyces alkalinus	CBS 133682	JX158408
Sodiomyces alkalinus	CBS 133683	JX158409
Sodiomyces alkalinus	CBS 133684	JX158410
Sodiomyces alkalinus	CBS 133685	JX158411
Sodiomyces alkalinus	CBS 133686	JX158413
Sodiomyces alkalinus	CBS 133687	JX158414
Sodiomyces alkalinus	CBS 133688	JX158415
Sodiomyces alkalinus	CBS 133689	JX158416
Sodiomyces alkalinus	CBS 133690	JX158417
Sodiomyces alkalinus	CBS 133691	JX158418
Sodiomyces magadii T	**MAG2 (= CBS 137619)**	**KJ443278**
Sodiomyces tronii T	**MAG1 (= CBS 137618)**	**KJ443277**
Sodiomyces tronii	**MAG3 (= CBS 137620)**	**KJ443279**

SUPPLEMENTARY TABLE 7. *Pleosporaceae* tree GenBank accessions. New isolates from soda soils are in bold.

taxon	voucher	SSU	LSU	RPB2	ITS
Alternaria abundans T	CBS 534.83	KC584581	KC584323	KC584448	AB120848
Alternaria alternantherae	CBS 124392	KC584506	KC584251	KC584374	KC584179
Alternaria alternata T	CBS 916.96	KC584507	DQ678082	KC584375	FJ196306
Alternaria alternata	**G408 (= CBS 137513)**	**KJ443072**	**KJ443115**	**KJ443163**	**KJ443247**
Alternaria arborescens T	CBS 102605	KC584509	KC584253	KC584377	_
Alternaria argyranthemi T	CBS 116530	KC584510	KC584254	KC584378	KC584181
Alternaria armoraciae T	CBS 118702	KC584511	KC584255	KC584379	KC584182
Alternaria bornmuelleri T	DAOM 231361	KC584624	KC584366	KC584491	FJ357317
Alternaria botrytis T	CBS 197.67	KC584609	KC584351	KC584476	KC584243
Alternaria breviramosa T	CBS 121331	KC584574	KC584318	KC584442	FJ839608
Alternaria caespitosa T	CBS 177.80	KC584625	KC584367	KC584492	KC584250
Alternaria caricis T	CBS 480.90	KC584600	KC584342	KC584467	_
Alternaria cetera T	CBS 121340	KC584573	KC584317	KC584441	FJ839607

Alternaria chlamydospora T	CBS 491.72	KC584520	KC584264	KC584388	KC584189
Alternaria chlamydosporigena	CBS 341.71	KC584584	KC584326	KC584451	KC584231
Alternaria conjuncta T	CBS 196.86	KC584522	KC584266	KC584390	_
Alternaria daucifolii T	CBS 118812	KC584525	KC584269	KC584393	KC584193
Alternaria dennisii T	CBS 476.90	KC584587	KC584329	KC584454	_
Alternaria didymospora	CBS 766.79	KC584588	KC584330	KC584455	FJ357312
Alternaria embellisia	CBS 339.71	KC584582	KC584324	KC584449	KC584230
Alternaria ethzedia T	CBS 197.86	KC584530	KC584274	KC584398	_
Alternaria gaisen	CBS 632.93	KC584531	KC584275	KC584399	KC584197
Alternaria infectoria T	CBS 210.86	KC584536	KC584280	KC584404	FM958526
Alternaria kulundii	**M309 (= CBS 137521)**	**KJ443083**	**KJ443128**	_	**KJ443258**
Alternaria kulundii	**M310 (= CBS 137522)**	**KJ443084**	**KJ443129**	**KJ443173**	**KJ443259**
Alternaria kulundii T	**M313 (= CBS 137525)**	**KJ443087**	**KJ443132**	**KJ443176**	**KJ443262**
Alternaria limaciformis T	CBS 481.81	KC584539	KC584283	KC584407	KC584203
Alternaria limoniasperae T	CBS 102595	KC584540	KC584284	KC584408	_
Alternaria longipes	CBS 540.94	KC584541	KC584285	KC584409	_
Alternaria macrospora T	CBS 117228	KC584542	KC584286	KC584410	KC584204
Alternaria molesta T	CBS 548.81	KC584544	KC584288	KC584412	KC584205
Alternaria molesta	**M312 (= CBS 137524)**	**KJ443086**	**KJ443131**	**KJ443175**	**KJ443261**
Alternaria mouchaccae T	CBS 119671	KC584545	KC584289	KC584413	KC584206
Alternaria obclavata T	CBS 124120	KC584575	FJ839651	KC584443	KC584225
Alternaria oregonensis T	CBS 542.94	KC584548	KC584292	KC584416	_
Alternaria penicillata T	CBS 116607	KC584580	KC584322	KC584447	KC584229
Alternaria perpunctulata T	CBS 115267	KC584550	KC584294	KC584418	KC584210
Alternaria petuchovskii T	**M304 (= CBS 137517)**	**KJ443079**	**KJ443124**	**KJ443170**	**KJ443254**
Alternaria phragmospora T	CBS 274.70	KC584595	KC584337	KC584462	_
Alternaria scirpicola	CBS 481.90	KC584602	KC584344	KC584469	KC584237
Alternaria shukurtuzii T	**M307 (= CBS 137520)**	**KJ443082**	**KJ443127**	**KJ443172**	**KJ443257**
Alternaria slovaca T	CBS 567.66	KC584576	KC584319	KC584444	KC584226
Alternaria solani	CBS 116651	KC584562	KC584306	KC584430	KC584217
Alternaria soliaridae T	CBS 118387	KC584563	KC584307	KC584431	KC584218
Alternaria sp.	CBS 108.27	KC584601	KC584343	KC584468	KC584236
Alternaria sp.	CBS 174.52	DQ678016	DQ678068	DQ677964	KC584228
Alternaria sp.	CBS 175.52	KC584577	KC584320	KC584445	KC584227
***Alternaria* sp.**	**M301 (= CBS 137514)**	_	**KJ443121**	_	_
Alternaria tellustris T	CBS 538.83	KC584598	KC584340	KC584465	_
Alternaria tenuissima	CBS 918.96	KC584567	KC584311	KC584435	_
Chaetodiplodia sp.	CBS 453.68	DQ678001	DQ678054	DQ677948	_
Chaetosphaeronema hispidulum	CBS 826.88	EU754046	EU754145	_	_
Clathrospora elynae	CBS 161.51	KC584628	KC584370	KC584495	_
Clathrospora elynae	CBS 196.54	KC584629	GU323214	KC584496	_
Cochliobolus heterostrophus	CBS 134.39	AY544727	AY544645	DQ247790	DQ491489
Cochliobolus sativus	DAOM 226212	DQ677995	DQ678045	DQ677939	_
Comoclathris compressa	CBS 156.53	KC584630	KC584372	KC584497	_
Comoclathris compressa	CBS 157.53	KC584631	KC584373	KC584498	_
Paradendryphiella arenaria	CBS 181.58	DQ471022	DQ470971	DQ470924	DQ411539
Paradendryphiella salina	CBS 142.60	KC793337	KC793339	KC793340	DQ411540
Peyronellaea zeae-maydis	CBS 588.69	EU754093	EU754192	GU371782	FJ427086
Pleospora betae	CBS 109410	EU754079	EU754178	GU371774	_
Pleospora betae	CBS 523.66	EU754080	EU754179	_	FJ426981
Pleospora calvescens	CBS 246.79	EU754032	EU754131	KC584500	_
Pleospora chenopodii	CBS 206.80	JF740095	JF740266	KC584501	_
Pleospora fallens	CBS 161.78	GU238215	GU238074	KC584502	_
Pleospora halimiones	CBS 432.77	JF740096	JF740267	KC584503	GU230752
Pleospora herbarum	CBS 191.86	DQ247812	GU238160	DQ247794	DQ491516
Pleospora incompta	CBS 467.76	GU238220	GU238087	KC584504	_
Pleospora typhicola	CBS 132.69	JF740105	JF740325	KC584505	_
***Pleosporaceae* sp.**	**M302 (= CBS 137515)**	**KJ443077**	**KJ443122**	**KJ443168**	**KJ443252**
***Pleosporaceae* sp.**	**M303 (= CBS 137516)**	**KJ443078**	**KJ443123**	**KJ443169**	**KJ443253**
***Pleosporaceae* sp.**	**M305 (= CBS 137518)**	**KJ443080**	**KJ443125**	_	**KJ443255**
***Pleosporaceae* sp.**	**M306 (= CBS 137519)**	**KJ443081**	**KJ443126**	**KJ443171**	**KJ443256**
***Pleosporaceae* sp.**	**M311 (= CBS 137523)**	**KJ443085**	**KJ443130**	**KJ443174**	**KJ443260**
***Pleosporaceae* sp.**	**M315 (= CBS 137527)**	**KJ443089**	**KJ443134**	_	**KJ443264**
Pyrenophora phaeocomes	DAOM 222769	DQ499595	DQ499596	DQ497614	DQ491507

Pyrenophora tritici-repentis	OSC 100066	AY544716	AY544672	_	_

SUPPLEMENTARY TABLE 8. *Lasiosphaeriaceae & Chaetomiaceae* tree GenBank accessions. New isolates from soda soils are in bold.

taxon	voucher	LSU	ITS
Achaetomium strumarium T	CBS 333.67	AY681170	AY681204
Apiosordaria backusii	CBS 106.77	AY780051	GQ922520
Apiosordaria longicaudata T	CBS 390.84	FR692340	_
Apiosordaria tetraspora T	CBS 363.84	FR692341	_
Apiosordaria verruculosa	F-152365	AY346258	_
Cercophora coprophila	IFO 32091	AY999112	AY999136
Cercophora costaricensis	SMH4021	AY780059	_
Cercophora samala T	CBS 109.93	AY999111	AY999134
Cercophora striata	SMH3431	AY780065	_
Cercophora terricola T	ATCC 200395	AY780067	_
Chaetomidium arxii T	CBS 104.79	FJ666359	JX280770
Chaetomidium trichorobustum T	CBS 563.67	FJ666352	_
Chaetomium acropullum T	CBS 114580	JX280662	JX280763
Chaetomium brasiliense	CBS 761.83	JX280674	JX280780
Chaetomium brasiliense T	MUCL 9590	AF286396	_
Chaetomium chiversii	MUCL 40052	AF286398	_
Chaetomium crispatum	CBS 408.81	JX280675	JX280781
Chaetomium dolichotrichum T	CBS 162.48	HM449063	HM449049
Chaetomium erectum T	CBS 140.56	HM449058	HM449044
Chaetomium funicola T	CBS 158.52	GU563354	GU563369
Chaetomium fuscum	CBS 128460	JX280680	JX280788
Chaetomium gracile	CBS 582.84	JX280710	JX280823
Chaetomium indicum T	CGMCC 3.14184	GU563360	GU563367
Chaetomium medusarum T	CBS 148.67	JX280716	JX280829
Chaetomium murorum	MUCL 40179	AF286405	_
Chaetomium piluliferum	MUCL 40180	AF286406	_
Chaetomium pratense T	CBS 133396	GU563357	GU563372
Chaetomium ramosissimum T	CBS 133398	GU563361	GU563371
Chaetomium seminis-citrulli T	CBS 143.58	JX280729	JX280841
Chaetomium subfunicola T	CBS 133397	JX867125	JX867125
Chaetomium thermophilum	_	DQ010014	_
Chaetomium thermophilum var. *coprophilum* T	ATCC 16451	JQ067895	JF412013
Chaetomium vitellinum T	CBS 250.85	JX280740	JX280859
Chatomium reflexum T	CBS 157.49	HM449055	HM449051
Cladorrhinum bulbillosum T	CBS 304.90	FR692339	FM955448
Cladorrhinum flexuosum T	FMR 10415	FN662477	FN662474
Cladorrhinum foecundissimum T	CBS 180.66	FR692343	FM955445
Cladorrhinum microsclerotigenum T	CBS 290.75	FR692342	FN662475
Cladorrhinum phialophoroides T	CBS 301.90	FR692344	FM955444
Cladorrhinum samala	CBS 303.90	FR692338	FM955447
Diatrype disciformis	CBS 197.49	DQ470964	_
Humicola grisea var. *grisea*	FO-2942	AB625577	AB625590
Humicola grisea var. *thermoidea*	NBRC 9854	AB625578	AB625591
Humicola insolens	MTCC 4617	EU257375	EF550968
Humicola nigrescens T	CBS 208.55	AB625579	AB625592
***Lasiosphaeriaceae* sp.**	**M314 (= CBS 137526)**	**KJ443133**	**KJ443263**
Melanocarpus albomyces T	ATCC 16460	JQ067902	JF412014
Papulaspora equi T	CBS 573.89	JX280755	JX280870
Podospora austro-americana T	CBS 724.68	AY999101	AY999124
Podospora comata	ATCC 36713	AY780072	_
Podospora fimiseda	CBS 990.96	AY346296	AY515361
Staphylotrichum boninense T	JCM 17908	AB625568	AB625580

Staphylotrichum coccosporum[T]	CBS 364.58	AB625572	AB625584
Thielavia australiensis[T]	ATCC 28236	JQ067899	JF412017
Thielavia cephalothecoides	MUCL 40270	AF286413	_
Thielavia hyalocarpa	CBS 646.74	JX280759	AJ271583
***Thielavia* sp.**	**M316 (= CBS 137616)**	**KJ443135**	**KJ443265**
***Thielavia* sp.**	**M317 (= CBS 137617)**	**KJ443136**	**KJ443266**
Thielavia terrestris	CBS 492.74	JX280761	JX280874
Zopfiella ebriosa	CBS 111.75	AY346305	_
Zopfiella longicaudata	IFO 30296	AY999109	AY999131
Zopfiella tetraspora	IFO 32904	AY999108	AY999130

SUPPLEMENTARY TABLE 9. *Alternaria* section *Soda* tree GenBank accessions. New isolates from soda soils are in bold.

taxon	voucher	GAPDH
Alternaria abundans[T]	CBS 534.83	KC584154
Alternaria argyranthemi[T]	CBS 116530	KC584098
Alternaria armoraciae[T]	CBS 118702	KC584099
Alternaria bornmuelleri[T]	DAOM 231361	FJ357305
Alternaria botrytis[T]	CBS 197.67	KC584168
Alternaria breviramosa[T]	CBS 121331	KC584148
Alternaria caespitosa[T]	CBS 177.80	KC584178
Alternaria caricis[T]	CBS 480.90	AY278826
Alternaria cetera[T]	CBS 121340	AY562398
Alternaria chlamydosporigena	CBS 341.71	KC584156
Alternaria conjuncta[T]	CBS 196.86	AY562401
Alternaria dennisii[T]	CBS 476.90	JN383469
Alternaria embellisia	CBS 339.71	KC584155
Alternaria ethzedia[T]	CBS 197.86	AY278795
Alternaria infectoria[T]	CBS 210.86	AY278793
Alternaria kulundii	**M309 (= CBS 137521)**	**KJ649619**
Alternaria kulundii	**M310 (= CBS 137522)**	**KJ649617**
***Alternaria kulundii*[T]**	**M313 (= CBS 137525)**	**KJ649618**
Alternaria obclavata[T]	CBS 124120	KC584149
Alternaria oregonensis[T]	CBS 542.94	FJ266491
Alternaria papavericola[T]	CBS 116606	FJ357298
Alternaria penicillata[T]	CBS 116607	KC584153
***Alternaria petuchovskii*[T]**	**M304 (= CBS 137517)**	**KJ649616**
Alternaria scirpicola	CBS 481.90	KC584163
***Alternaria shukurtuzii*[T]**	**M307 (= CBS 137520)**	**KJ649620**
Alternaria slovaca[T]	CBS 567.66	KC584150
Alternaria soliaridae[T]	CBS 118387	KC584140
Alternaria tellustris[T]	CBS 538.83	AY562419
Pleospora tarda[T]	CBS 714.68	AF443881

Chapter V

The alkaliphilic fungus *Sodiomyces alkalinus* produces enzymes active at high pH

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Abstract

Sodiomyces alkalinus is an alkaliphilic filamentous fungus isolated from saline alkaline soils across the world. As most fungi display a saprobic life-style utilizing dead plant biomass, we sought to assess the plant-degrading capabilities of *S. alkalinus*. We sequenced the genome of *S. alkalinus* and used *in silico* analysis to look into the distribution of carbohydrate-active enzymes and compare it to the industrial fungus *Aspergillus oryzae*. In addition, we employed enzyme bioassays coupled with previous growth data to directly estimate the degrading capacity of *S. alkalinus* on different carbon sources and pH values. We found that *S. alkalinus* is capable of producing endoglucanases and endoxylanases that are active at pH 10, as opposed to the enzymes from *A. oryzae*. The high number of cellulases encoded in the genome and growth experiments supports the idea that these enzymes can be induced at later stages of *S. alkalinus* cultivation. We also detected very strong alkaline proteases in *S. alkalinus*, which might account for its need to obtain nitrogen that is extremely limited in natural alkaline conditions of soda soils. *S. alkalinus* seems to be a promising producer of alkaline enzymes – a property that can be exploited in a number of commercial processes.

Key Words

Sodiomyces alkalinus, CAZymes, alkaline enzymes, cellulases, hemicellulases, proteases

Introduction

Among many types of so-called extreme environments, soda (alkaline) soils represent a habitat with an unusual repertoire of abiotic factors which long ago were considered unable to support life. It turned out that major stress factors associated with soda soils – high pH values, low water activity, and often elevated salt concentrations are in fact tolerable by many types of organisms. Soda soils usually flank shallow water basins, called soda lakes, which are considered as the most stable natural alkaline environments on earth. Soda lakes are found in arid zones and despite their wide distribution, are located in hard-to-reach continental interiors (**JONES ET AL. 1998**), which results in the relative scarcity of the information available on the diversity of organisms that populate these environments. Moreover, soda lakes are found throughout the world and vary in their physicochemical properties, which reflect local conditions and affect their microbial content. This variability in geological conditions hampers the attempts to build a unifying theory on the soda lakes and soda soils biology. Nonetheless, existing studies report that soda lakes are exclusively populated by prokaryotic and archaeal organisms with defined ecological roles that govern nutrient recycling (**JONES ET AL. 1998**). Soda soils have been proposed to be essentially closed ecosystems and therefore are capable of sustaining a complete nutrient cycle (**ZAVARZIN 1993**). The occurrence of various functional groups of microbial communities – heterotrophs, nitrifiers, methanogens, methanotrophs, phototrophs, sulfate reducers, sulfur oxidizers and others, suggests complex trophic interactions among microbes in soda lakes

(**ANTONY ET AL. 2013**).

Soda lakes are rich in CO_3^{2-} which maintains high pH. Virtually unlimited CO_2, high local temperatures and illuminations promote a high level of photosynthesis ranking these habitats among the most productive aquatic environments on earth (**MELACK & KILHAM 1974**). It is suggested that primary organic production in soda soils and lakes is build up by seasonal or permanent blooms of cyanobacteria and several flowering plant species, adapted to thrive in saline and alkaline conditions. Studies on several soda lakes report cyanobacterial members of *Arthrospira* (*Spirulina*), *Cyanospira*, *Synechococcus* and *Chroococcus* as dominant primary producers. At the environment with lower salinities, occasional blooms of *Navicula* and *Nitzschia* diatoms may represent a significant portion of the phototroph population (**GRANT ET AL. 1990**). Members of purple bacteria from *Ectothiorhodospira*, *Halorhodospira*, *Rhodobaca*, *Rubribacterium*, as anoxygenic phototrophs, have been noted as a possible contributor to the primary production (**GRANT 2004, BOLDAREVA ET AL. 2008, BOLDAREVA ET AL. 2009, ANTONY ET AL. 2013**). Importantly, cyanobacterial species are not only responsible for carbon build-up, but also for the production of oxygen and nitrogen fixation using heterocyst cells. Soda lakes also support abundant populations of small crustaceans, *Artemia* brine shrimp in particular (**COLE & BROWN 1967, SCHNEIDER ET AL. 2012**). The dead biomass is decomposed by a large variety of heterotrophic bacteria either aerobically or anaerobically. As **GRANT & SOROKIN (2011)** point out, the solubility of oxygen is reduced in hypersaline environments which creates a more favorable milieu for the establishment of anaerobic communities. The heterotrophic proteobacteria from *Halomonadaceae*, diverse *Bacillus* members along with actinobacteria species, are suspected to be among the most represented in saline soda lakes (**DUCKWORTH ET AL. 1996, GRANT & SOROKIN 2011**). Those microorganisms produce a spectrum of enzymes – proteases, cellulases, lipases, amylases, which break various polymers to the simpler compounds that in turn are utilized by anaerobic species. Complex nets of sediment anaerobic communities ferment simple substrates (mono- or disaccharides, amino acids) releasing hydrogen, carbon oxide, methanol, ethanol, lactate, butyrate, propionate, or acetate.

Saline alkaline lakes also hold an extensive diversity of archaeal organisms that among other metabolic lifestyles, may utilize methanol or methylamine to produce methane, which in turn is used by methylotrophic bacteria (e.g. *Methylomicrobium*). Some soda lakes may promote massive blooms of haloarchaea that colorize water red due to carotenoid accumulation (**GRANT & SOROKIN 2011**). It is believed that still much of the archaeal diversity is yet to be characterized, as inferred by the analyses of 16S rRNA clone libraries (**CATON ET AL. 2009, MWICHIA ET AL. 2010**). Reduced inorganic compounds as electron donors are utilized by different types of chemolithotrophic bacteria reoxidizing the environment. Sulfur oxidizers, nitrifiers, methanotrophs, carboxytrophs, hydrogenotrophs have all been shown to be present in several studied soda lakes as a part to the complex food web sustaining carbon, sulfur, nitrogen cycles (**SOROKIN & KUENEN 2005**).

Fungi occupy essentially every niche on earth and we recently showed that soda lakes are no exception. We found fungi that are not only capable of tolerating the harsh abiotic conditions

of soda soils, but actually require them for optimal growth. Our studies on the survey of such alkaline soils demonstrated the presence of polyphyletic fungal communities which show various degrees of adaptation to high pH, from weak alkalitolerants to obligate alkaliphiles (**GRUM-GRZHIMAYLO ET AL. 2013A, 2013B, 2015**). Fungi as chemoorganotrophic organisms obtain organic carbon compounds externally and use it as energy source. While many fungi are parasitic, either obligate or facultative, still a larger number are soil saprobes that thrive in different types of soil degrading organic matter using a powerful and versatile arsenal of enzymes. Fungi that populate a particular environment form intricately interacting communities, and their ecological assessment is often an ambiguous task requiring multiple approaches. Thorough recovery of fungi, morphological examinations, various growth tests coupled with enzyme bioassays are needed to draw conclusions on the role of fungi in a given habitat. Genome and transcriptome information is becoming increasingly valuable in the ecological characterizations of various fungi in view of rapidly rising numbers of genome sequencing projects over the last years (**GRIGORIEV ET AL. 2011, 2013**).

The current study attempts to assess the lifestyle of *Sodiomyces alkalinus* – an obligate alkaliphilic fungus recovered from saline soda soils in several locations on earth. Studying its morphology and performing growth tests with enzyme assays on various carbon sources at different pH values, we estimated the major enzymatic activities in comparison with the industrial fungus *Aspergillus oryzae*. In addition we performed *in silico* analysis of carbohydrate-active enzymes (CAZymes) encoded in the genome of *S. alkalinus*. We show that *S. alkalinus* grows better at pH 10 than at pH 8 on various carbon sources on plates. The fungus has alkaliphilic capabilities in liquid media as well, when grown on sugar beet pulp and wheat bran. A large number of cellulose-degrading enzymes are present in the genome of in *S. alkalinus*, and these enzymes are induced and active at very high pH as opposed to neutrophilic *A. oryzae*. Unexpectedly, we found rapidly induced high levels of proteases that were most active at pH 8–10. The detected alkaline enzymes from *S. alkalinus* might be promising for biomass conversion during alkaline pre-treatments. We speculate on the scenario of *Sodiomyces* invasion into densely populated saline soda soils environments through the evolved ability to quickly utilize easy and nitrogen-rich protein substrates.

Materials and methods

Strains, media, and growth

The wild ex-type strain (F11 = CBS 110278^{T}) of *S. alkalinus* isolated from alkaline soil (pH 10.7, Choibalsan area, north-east Mongolia) was used in the current study. Routine propagation of *S. alkalinus* was performed on alkaline agar (AA) medium (**GRUM-GRZHIMAYLO ET AL. 2013A**). To obtain metabolically active mycelium for subsequent enzymatic assays, we pre-grew *S. alkalinus* in alkaline liquid medium ($3.5 \cdot 10^{8}$ spores/ml, 28 °C, 200 rpm) of the same content as AA, omitting agar. The collected pre-grown mycelium was washed, filtered and inoculated in the liquid media

with different pH values containing 1 % carbon sources, in duplicates. The complete medium for enzyme production consisted of two components: the salt/buffer component **(1)** and the nutrient component **(2)**. 50 ml of component **(1)** was 0.2 M of suitable buffer supplemented with 5 g NaCl (Merck), 1 g KNO_3 (Merck), 1 g K_2HPO_4 (Merck). 50 ml of component **(2)** contained either 2 % (w/v in dH_20) wheat bran (obtained locally), or 2 % sugar beet pulp (obtained locally), or 2 % chitin (from crab shells, Sigma). **(1)** and **(2)** were autoclaved separately at 120 °C for 20 min, allowed to cool down to 55 °C and then mixed in 1:1 ratio. Finally, antibiotic rifampicin was added to the complete medium to a final concentration of 50 µg/ml. The fungus was incubated on an orbital shaker at 28 °C, 200 rpm. As a reference strain, we used Japanese industrial *A. oryzae* RIB40 pre-grown on complete medium (CM) (**DE VRIES ET AL. 1999**) at pH 6. For enzyme production in *A. oryzae*, we inoculated the fungus in 1 % wheat bran based on minimal medium (MM) at pH 6 (**DE VRIES ET AL. 1999**). We collected medium extracts on 24 h and 48 h of growth, put at -20 °C in single-use aliquots further analyzed by SDS-PAGE and enzymatic assays. We ensured that pH of the media was not changing significantly during growth. For illustration purposes, we backed-up our current results with previous growth experiments (**GRUM-GRZHIMAYLO ET AL. 2013A**).

SDS-PAGE and silver staining

We applied our media extracts on 12 % (w/v) SDS-PAGE topped with a 5 % stacking gel using a MiniProtean II system (BioRad), as described in **LAEMMLI (1970)**. The gels were further stained with silver nitrate (**BLUM ET AL. 1987**) to visualize the total proteins content.

CAZy genome screen

The genome of *S. alkalinus* was sequenced and obtained from the Joint Genome Institute (DOE JGI, Walnut Creek, CA, USA), available at **HTTP://GENOME.JGI.DOE.GOV/SODAL1/SODAL1.HOME.HTML**. Putative carbohydrate active enzymes (CAZymes, **HTTP://WWW.CAZY.ORG**): glycoside hydrolases (family GH), glycosyltransferases (family GT), polysaccharide lyases (family PL) and carbohydrate esterases (family CE), carbohydrate-binding modules (family CBM), and auxiliary activities (family AA) were screened in a total set of filtered ("best") protein models as determined by the JGI. Numbers for CAZy family members in *A. oryzae* RIB40 were used as references, and obtained from the curated on-line CAZy database (**LOMBARD ET AL. 2014**).

Enzymatic assays

Culture filtrates after 48 h of incubation were analyzed for selected enzyme activities involved in plant biomass degradation. All enzymatic activities were measured in technical triplicates at three pH values (0.1 M buffers with pH 6, 8, 10) and at 30 °C. Cellobiohydrolase (CBH), ß-1,4-glucosidase (BGL), ß-1,4-xylosidase (BXL), α-arabinofuranosidase (ABF), α-1,4-galactosidase (AGL), and ß-1,4-galactosidase (LAC) activities were measured using appropriate *p*-nitrophenyl (pNP) substrates (Sigma). A final mixture volume for the pNP-based enzyme assays was set to 100 µl containing: 50 µl of suitable buffer, 20 µl of 0.1 % pNP substrate, 10–30 µl of appropriately diluted crude culture filtrate and 0–20 µl of sterile water. The mixtures were

incubated for 24 h at 30 °C and stopped by the addition of 100 µl 0.5 M Na_2CO_3. The absorption was measured at 405 nm on a FLUOstar OPTIMA plate reader (BMG Labtech), and product release was calculated using a calibration curve. ß-1,4-endoglucanase (EGL), ß-1,4-endoxylanase (XLN) were measured by the amount of reducing sugars released, using dinitrosalicyclic acid (DNS) reagent. A reaction mixture (100 µl) for DNS-based enzyme assays contained: 50 ul of substrate (2 % carboxymethylcellulose or 2 % beechwood xylan), 30–50 µl of appropriately diluted crude culture filtrate, and 0–20 µl of sterile water. The reactions were kept for 2–24 h at 30 °C and stopped by the addition of 100 µl DNS, followed by the 30 min incubation at 95 °C. The DNS mixtures were measured at 560 nm on a plate reader, and activities were calculated using a calibration curve based on glucose. The optimal timing of the reaction was determined experimentally with the pilot reactions. The reaction mixtures for *A. oryzae* were incubated for 2 h, except for XLN activity (30 min). For above named activities, one unit of enzymatic activity (U) was defined as the amount of enzyme that liberated 1 mmol of the corresponding product per minute of reaction, under the assay condition used.

We measured total proteolytic activity with Pierce Fluorescent Protease Assay Kit (Thermo Scientific, USA) using a fluorescein isothiocyanate (FITC)-labeled casein assay according to the manufacturer's instructions. We tested the assay with the desired standard kit TBS buffer of pH 7.2, but also ensured the assay works with other buffers that yield pH 6, 8 and 10. We incubated the reaction mixes for 15 min, and for *S. alkalinus* we used 1 µl of crude culture extract (15 µl for *A. oryzae*) in a total reaction volume of 200 µl. One unit (U) of protease activity was defined as the amount of protein that has an equivalent activity of 1 µg of bovine pancreas trypsin (Thermo Scientific, USA) at pH 8.

Results

Growth on plant biomass-related carbon sources

Our previous results suggest that *S. alkalinus* is capable of growing on various mono-, di-, tri-saccharides at pH 10, better than at pH 7. However in general, *S. alkalinus* developed faint colonies on these pure sugars. Conversely, complex plant-derived substrates promoted the best growth showing full morphology and development of abundant sporulation with fruiting bodies (**Grum-Grzhimaylo et al. 2013a**). Notably, growth on pure crystalline cellulose was average, taking into account its extreme recalcitrance and no additional supplements in the medium. This growth profile led us to suspect *S. alkalinus* to be active in plant biomass degradation in natural environments of soda soils. To better address potential growth capabilities, we performed an *in silico* CAZome screen in *S. alkalinus* and compared its set of enzymes to the enzymes of the industirial neutrophilic fungus *Aspergillus oryzae* strain RIB40.

Comparison of CAZy profiles in *S. alkalinus* and *A. oryzae*

We first sought to estimate the capability of *S. alkalinus* to degrade plant-derived

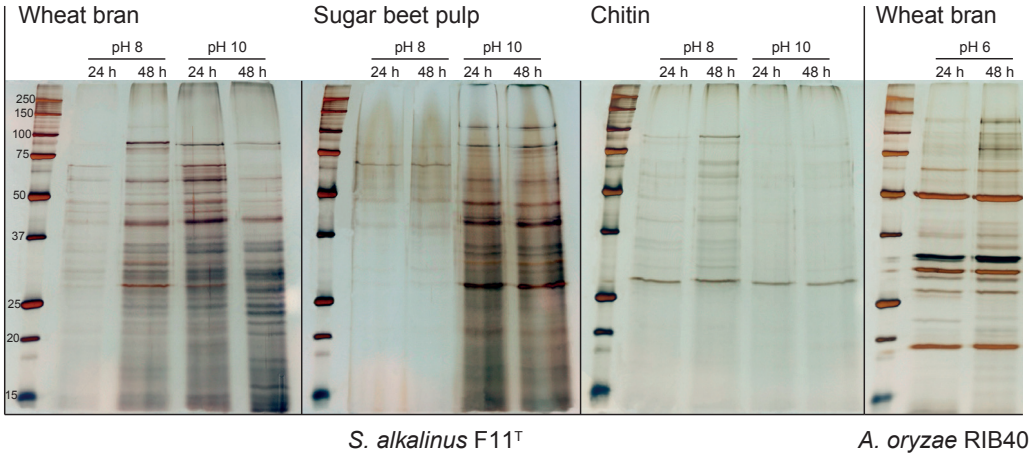

FIG. 1. Total protein sets (silver stained) from culture filtrates after growth of *S. alkalinus* and *A. oryzae* at various carbon sources and pH values.

carbohydrates in comparison to a well-studied reference fungus – *A. oryzae*. We identified 318 putative CAZymes encoded in the genome of *S. alkalinus* (501 CAZymes in *A. oryzae* in the CAZy database as of **13.05.15**). Cellulose, the principal and most abundant component of plant cell wall, is utilized by the concerted efforts of several enzyme types – lytic polysaccharide monooxygenases (LPMO), ß-1,4-endoglucananses (EGL), cellobiohydrolases (CBH) and ß-1,4-glucosidases (BGL) (**VAN DEN BRINK & DE VRIES 2011**). An eye-catching feature in the *S. alkalinus* genome is a large set of LPMOs – a novel family of Cu-dependent enzymes (now AA9, formerly GH61), some of which are believed to act on crystalline cellulose (**MORGENSTERN ET AL. 2014**). Twenty one enzymes of this family were identified in *S. alkalinus*, in contrast to 8 found in *A. oryzae* (**FIG. 2, C**). ß-1,4-endoglucanases are spread throughout four major CAZy families: GH5, GH7, GH12, and GH45. The numbers of enzymes in these families are similar as in *A. oryzae*, with the exception of family GH5, which possesses a large variety of enzymatic activity types. Cellobiohydrolases (CBH) act on both reducing and non-reducing ends of cellulose fibrils and are assigned to families GH6 and GH7. The GH6 family appears to be enriched in *S. alkalinus* compared to *A. oryzae* – 5 versus 1, respectively. ß-1,4-glucosidases (BGL) perform the final step in cellulose degradation splitting dimers into monomeric D-glucose units. The enzymes with this activity are grouped in families GH1 and GH3 (although probably not all act on cellulose), which *A. oryzae* has in bulk, if compared to *S. alkalinus*. There is another signature that may promote cellulolytic activity of an organism – cellulose-binding domains (designated as CBM1 in CAZy classification). In *S. alkalinus*, we found 17 CBM1 modules which are located within other GH enzyme families in a tandem manner, whereas only three of those modules were found in *A. oryzae*. Therefore, *in silico* genome analysis seem to provide evidence for efficient cellulolytic

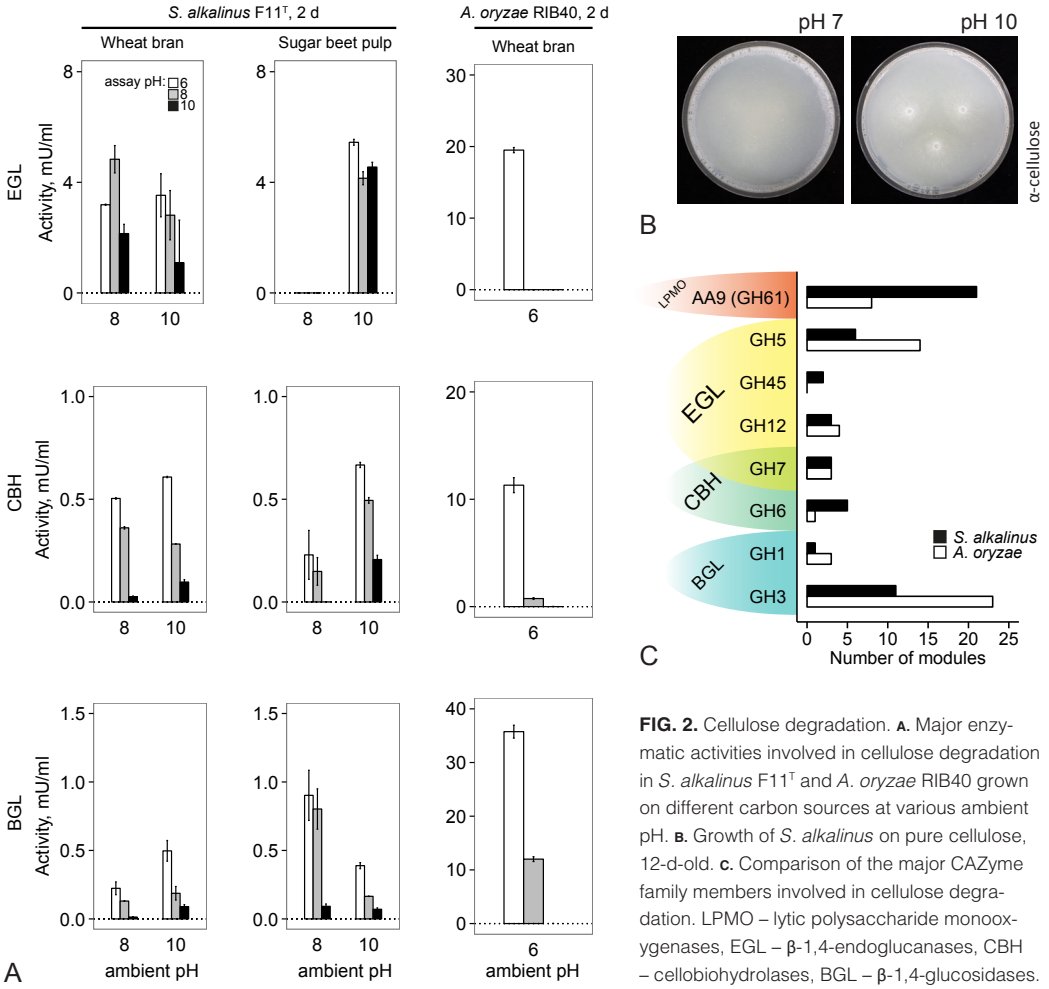

FIG. 2. Cellulose degradation. **A.** Major enzymatic activities involved in cellulose degradation in *S. alkalinus* F11^T and *A. oryzae* RIB40 grown on different carbon sources at various ambient pH. **B.** Growth of *S. alkalinus* on pure cellulose, 12-d-old. **C.** Comparison of the major CAZyme family members involved in cellulose degradation. LPMO – lytic polysaccharide monooxygenases, EGL – β-1,4-endoglucanases, CBH – cellobiohydrolases, BGL – β-1,4-glucosidases.

machinery in *S. alkalinus*.

Hemicellulose is the second most abundant plant polysaccharide with several varieties of sugar backbones and various types of side branches requiring a plethora of enzymes for its degradation. Xylan-backbone based hemicellulose is first digested by GH10 and GH11 families ß-1,4-endoxylanases (XLN), followed by ß-1,4-xylosidases (BXL, GH3 and GH43 families) that release D-xylose monomers. ß-1,4-endomannanases (MAN, GH5 and GH26 families) and ß-1,4-mannosidases (MND, GH2 family) are responsible for breaking the galacto(gluco)mannan-backbone hemicellulose. A third type of hemicellulose backbone includes xyloglucan, which is degraded by xyloglucan-active ß-1,4-endoglucanases (XEG, GH12 and GH74 families). All types

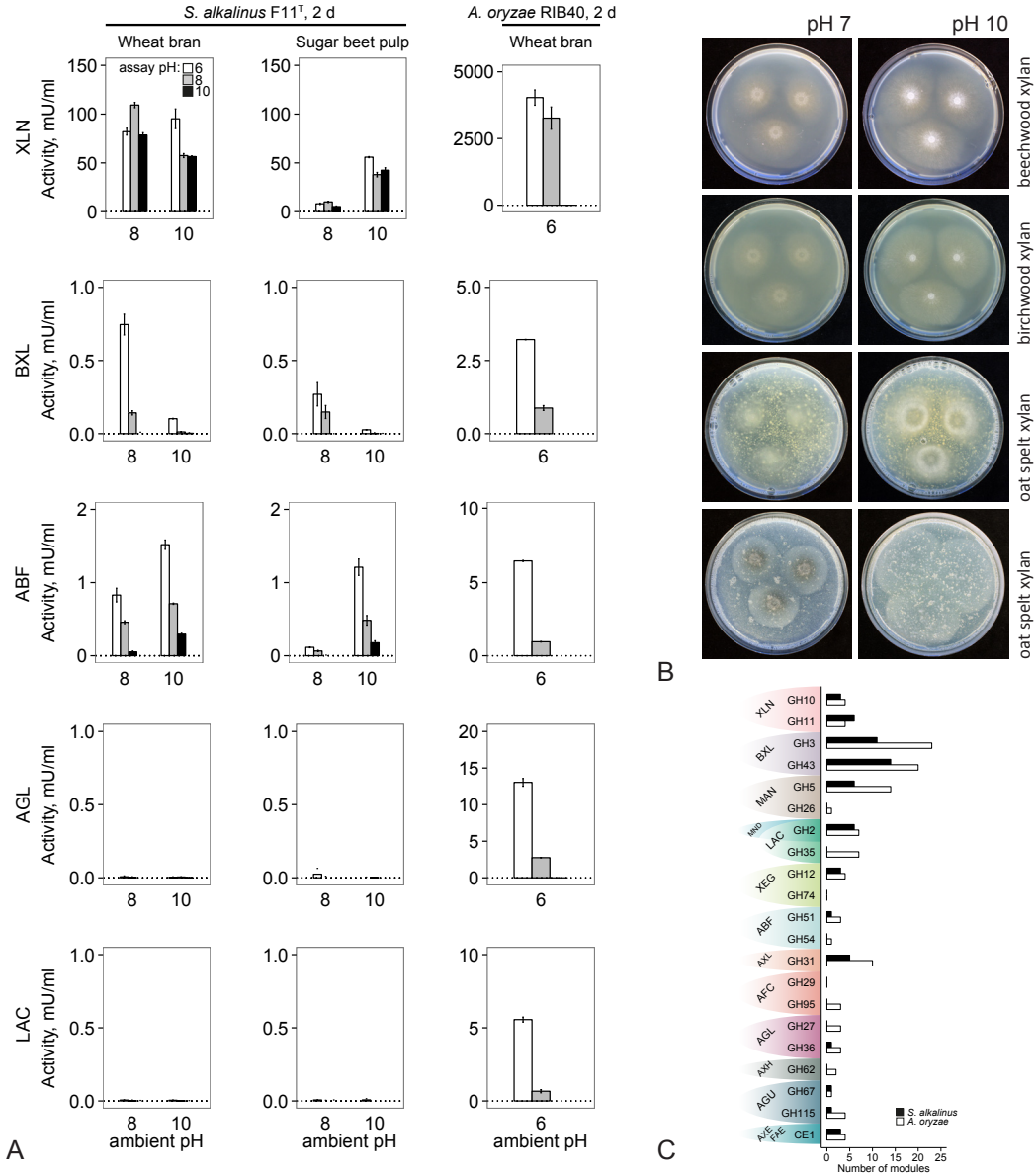

FIG. 3. Hemicellulose degradation. **A.** Few selected enzymatic activities involved in hemicellulose degradation in *S. alkalinus* F11^T and *A. oryzae* RIB40 grown on different carbon sources at various ambient pH. **B.** Growth of *S. alkalinus* on various xylans, 12-d-old. **C.** Comparison of the CAZyme family members involved in hemicellulose degradation. ABF – α-arabinofuranosidase, AFC – α-fucosidase, AGL – α-1,4-galactosidase, AGU – α-glucuronidase, AXE – acetyl(xylan) esterase, AXH – arabinoxylan α-arabinofuranohydrolase, AXL – α-xylosidase, BXL – β-1,4-xylosidase, FAE – feruloyl/p-coumaroyl estarase, LAC – β-1,4-galactosidase, MAN – β-1,4-endomannanase, MND – β-1,4-mannosidase, XEG – xyloglucan-active β-1,4-endoglucanase, XLN – β-1,4-endoxylanase.

of hemicelluloses carry side branches containing various types of sugar residues, which can be further modified by acetyl- and feruloyl-groups. These side branches have to be released for a complete degradation of hemicellulose, which is governed by a variety of enzymes spread over 16 CAZy families. In general, we found average numbers of hemicellulose-degrading genes in *S. alkalinus*. Notably, the *S. alkalinus* genome does not encode enzymes of GH27 and GH35 family members which possess AGL (α-1,4-galactosidase) and LAC (ß-1,4-galactosidase) activities.

Pectin is another plant polysaccharide with a complex architecture. Substantially reduced numbers of CAZy families known to target pectin in its genome, suggests low pectinolytic capabilities in *S. alkalinus*. The main CAZy family with pectin-degrading hydrolases is GH28. Two GH28 enzymes were found in *S. alkalinus* genome, as opposed to 21 found in *A. oryzae*. Pectin lyases of family PL1 are known to act optimally at acidic pH of around 5.5 (**MAYANS ET AL. 1997**), and are sparsely represented in alkaliphilic *S. alkalinus* – only two enzymes found in contrast to 12 located in the genome of *A. oryzae*. On the opposite side of the pH spectrum, pectate lyases preferentially act at alkaline pH (**MAYANS ET AL. 1997**) and spread over three CAZy families: PL1, PL3 and PL9. Numbers of PL3 and PL9 members in *S. alkalinus* are similar to *A. oryzae*.

As it seemed feasible to suspect *S. alkalinus* to utilize chitin, since crustacean carapaces abundant in soda lakes, we sought to *in silico* assess its chitinolytic capacity. The screen for chitinolytic enzymes encoded in *S. alkalinus* resulted in the detection of nine genes of the family GH18, which is a major family harboring different chitinase types. *A. oryzae* RIB40 has 18 chitinases from that family (**FIG. 5, B**). Another attribute that is believed to facilitate chitinolytic activity is chitin-binding domains – CBM18. We located 19 of those in *S. alkalinus*, as opposed to six in *A. oryzae*.

In addition, the CAZy screen revealed a low number of starch-degrading enzymes – GH13 family amylases. Five modules of this family were found in *S. alkalinus*, as opposed to 17 in *A. oryzae* RIB40.

Notably, no invertase and fructose degrading enzymes (CAZy family GH32) were found in the *S. alkalinus* genome.

Growth in liquid cultures confirms the alkaliphily of *S. alkalinus*

Here we confirm alkaliphilic capabilities of *S. alkalinus* when grown in liquid cultures. Visually, the most abundant growth of *S. alkalinus* was promoted on wheat bran at pH 10. Conversely, the chitin-based medium showed very poor biomass accumulation at both pH values tested. Growth on sugar beet pulp substrate was reduced compared to wheat bran. These observations were in line with pictures of our total protein extracts obtained after silver staining (**FIG. 1**). Sets of excreted proteins are time- and pH-dependent. For *S. alkalinus*, pH 10 induces strong protein excretion after 24 h of incubation, except for chitin medium. The first day of growth at pH 8 showed little protein accumulation in the extracts of all three media, indicating unfavorable growth conditions, as compared to pH 10. However, in wheat bran medium, by the second day of incubation, the total protein content appeared similar at both pH values. This result suggests more rapid induction of metabolic activity at pH 10, than at pH 8 on wheat bran, which

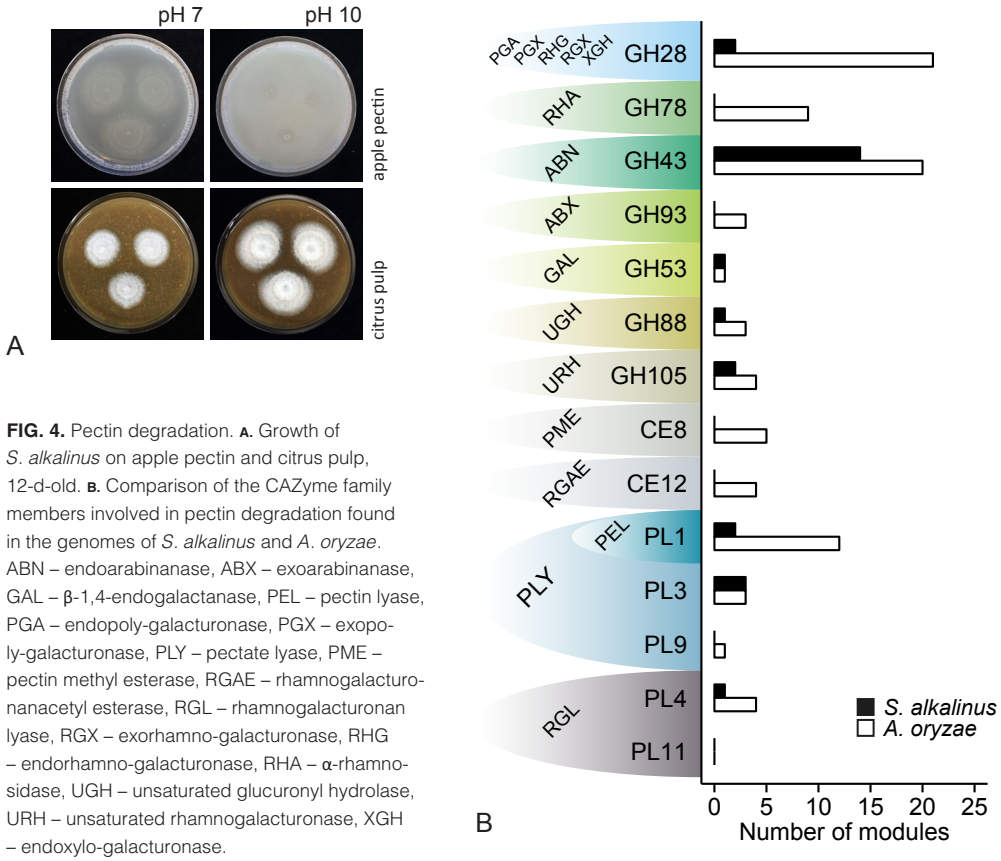

FIG. 4. Pectin degradation. **A.** Growth of *S. alkalinus* on apple pectin and citrus pulp, 12-d-old. **B.** Comparison of the CAZyme family members involved in pectin degradation found in the genomes of *S. alkalinus* and *A. oryzae*. ABN – endoarabinanase, ABX – exoarabinanase, GAL – β-1,4-endogalactanase, PEL – pectin lyase, PGA – endopoly-galacturonase, PGX – exopoly-galacturonase, PLY – pectate lyase, PME – pectin methyl esterase, RGAE – rhamnogalacturonanacetyl esterase, RGL – rhamnogalacturonan lyase, RGX – exorhamno-galacturonase, RHG – endorhamno-galacturonase, RHA – α-rhamnosidase, UGH – unsaturated glucuronyl hydrolase, URH – unsaturated rhamnogalacturonase, XGH – endoxylo-galacturonase.

then levels off as the incubation continues. On sugar beet pulp, pH 8 seems unfavorable for the whole duration of the experiment, displaying poor metabolic activity. Curiously, even though chitin-based medium in general showed very poor biomass accumulation, at pH 8 we see more metabolically active mycelium, if compared to pH 10 by the end of the second day incubation. *A. oryzae* showed very intense growth on wheat bran at pH 6, and strong metabolic induction from the first day of incubation. Notably, in *A. oryzae*, the sets of proteins do not change over time, as the same banding patterns occur after both days of growth, however, the intensity of several bands does vary. Especially evident for wheat bran medium, *S. alkalinus* produced different sets of enzymes depending both on pH and incubation time, possibly suggesting a succession of the preferred carbon source utilization over time in a multi-component wheat bran substrate.

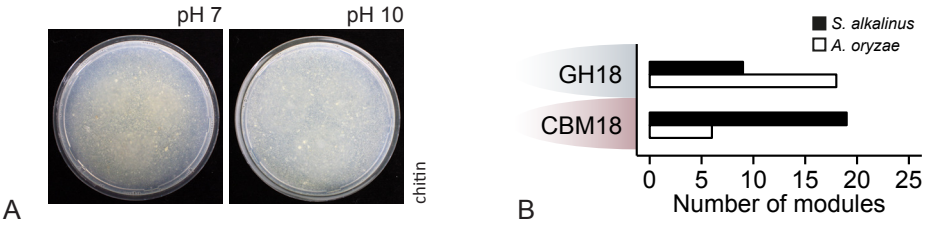

FIG. 5. Chitin degradation. **A.** Growth of *S. alkalinus* on pure chitin. **B.** Comparison of the CAZy modules encoded in the genomes of *S. alkalinus* and *A. oryzae* responsible for chitin degradation. GH18 - chitinase, CBM18 - chitin-binding domain.

S. alkalinus produces alkaline-active enzymes

To obtain direct evidence for the hydrolytic capabilities of *S. alkalinus* and draw biologically meaningful conclusions, we performed the enzyme assays on the crude media extracts after 2 d of growth. We need to point out that we did not include the results of the enzyme assays after growth on chitin (except for proteases), as this carbon source did not induce the secretion of the enzymes involved in the degradation of plant material. Also, as we measured the enzymatic activities in crude medium extract at a fixed time point for both *S. alkalinus* and *A. oryzae* – it was impossible to compare the enzymatic capacities of those fungi in absolute terms, as the activities were not corrected for the biomass accumulation rate or for the protein concentrations.

Our enzyme assays demonstrated cellulolytic activity for *S. alkalinus* after 2 d of growth on the carbon sources tested (**FIG. 2, A**). The EGL, CBH and BGL activities were about 4, 10, and 35 times lower than in *A. oryzae*, respectively. However in *S. alkalinus*, cellulases retained activity at pH 8 and 10, whereas cellulases of *A. oryzae* were only active at pH 6, except for BGL that retained about 30 % activity at pH 8. The relatively low cellulolytic activity after 2 d somewhat contradicts our previous growth experiments, where *S. alkalinus* showed decent growth on pure crystalline cellulose at pH 10 (**FIG. 2, B**). *In silico* CAZome screen suggests strong capacity of *S. alkalinus* for cellulases induction.

We measured several enzymatic activities involved in hemicellulose degradation. In general, our results show induction of hemicellulolytic enzymes in *S. alkalinus* after 2 d of growth (**FIG. 3, A**). The most prominent result of our assays is that endoxylanases activity of *S. alkalinus*, though about 40 times lower than in *A. oryzae*, remained active at pH 10 but not in *A. oryzae*. We measured three activities that remove side groups of hemicellulose: α-arabinofuranosidase (ABF, GH51, GH54 families), α-1,4-galactosidase (AGL, GH27, GH36 families) and ß-1,4-galactosidase (LAC, GH2, GH35 families). In *S. alkalinus*, they showed little or no induction on wheat bran or sugar beet pulp media, if compared to *A. oryzae* (**FIG. 3, A**). Our growth experiments on plates suggest average growth capabilities of *S. alkalinus* on various xylans (**FIG. 3, B**), which is in line with the CAZy genome content.

We did not measure pectinase activity by enzyme assays, however, poor growth on apple

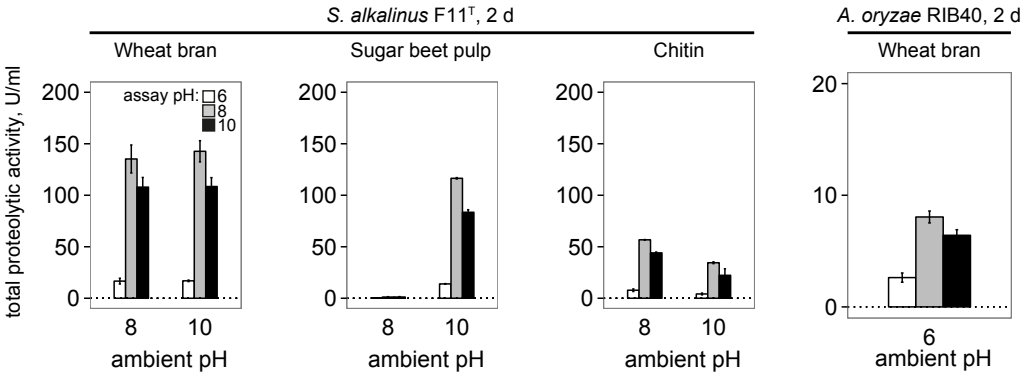

FIG. 6. Total proteolytic activity in culture filtrates of *S. alkalinus* F11^T and A. oryzae RIB40 grown on different carbon sources at various pH. Note protease induction on chitin medium.

pectin (**FIG. 4**) and low number of pectinolytic enzymes implies poor pectin-degrading capacity for *S. alkalinus*. Excellent growth on citrus pulp – a mixture of various carbohydrates enriched with pectins, suggests that *S. alkalinus* presumably avoids pectins and selectively utilizes alternative carbohydrates from a mixture. Notably, citrus pulp also contains cellulose, different vitamins, proteins and other compounds likely explaining a substantial boost to the growth of *S. alkalinus*.

Growth on pure chitin resulted in the development of extremely faint colonies of *S. alkalinus* (**FIG. 5, A**). Pure chitin is one of the most recalcitrant polysaccharides in nature and it is not surprising that the fungus was performing poorly, without extra nutrient complementation. From our current work which lacks bioassays for chitinases and dissonant CAZy sets in comparison to *A. oryzae*, it is hard to reliably assess chitinolytic capabilities of *S. alkalinus* (**FIG 5, B**).

Lack of invertase and fructose-degrading enzymes fits with the very poor growth capabilities of *S. alkalinus* on inulin and sucrose, confirming its inability to utilize those carbon sources.

Unexpectedly, we detected a very strong induction of proteases in all tested media extracts after 2 d of *S. alkalinus* incubation (**FIG. 6**). The greatest induction was observed on wheat bran medium at both pH values. Slightly less proteolytic activity was seen on sugar beet pulp at pH 10, whereas at pH 8 proteases were not induced, which corresponds to the overall poor metabolic activity on a gel shown in **FIG. 1**. Interestingly, the chitin medium induced proteolytic activity as well. All the proteases were most active at pH 8, and retained about 80 % of their activity at pH 10. *A. oryzae* too produced proteases that were active at high pH, however, the activity was 17 times lower than in *S. alkalinus* despite the superior growth rate of *A. oryzae* over *S. alkalinus*. This result is especially intriguing in light of the fact that the *A. oryzae* RIB40 strain is a Japanese industrial protease producer.

Discussion

S. alkalinus displays full morphology coupled with the fastest growth at aerobic conditions at pH 9–10.5 and total salinities at about 0.3–0.4 M Na^+, which leads us to assume it has capacity for full metabolic activity in natural saline soda soils and should not be treated it as a transient species in this habitat. The initial isolations of fungi from the oxic (1–5 cm) layers of soda soils from all the locations examined, consistently resulted in the recovery of *S. alkalinus*, either as a dominant or often the only fungal species for a given soil sample. We sought to estimate the capacity of *S. alkalinus* to degrade plant-derived polysaccharides at high pH. Low cellulolytic activity obtained in our enzyme assays suggests that either *S. alkalinus* has low intrinsic capacity to degrade cellulose, or cellulolytic activity is induced at later stages of incubation as compared to *A. oryzae*. There are several lines of evidence favoring the latter scenario. First, the biomass accumulation of *S. alkalinus* was slower than of *A. oryzae* at optimal growth conditions. Second, the genome analysis suggests strong potential capacity as exemplified by the large number of lytic polysaccharide monooxygenases (family AA9, formerly GH61) that are active against cellulose fibrils and average number of other enzymes that are involved in cellulose degradation. Third, *Acremonium alcalophilum* ex-type isolate CBS 114.92^T, a very close relative to *S. alkalinus* that was recovered from sludge of a pig feces compost, was shown to possess "cellulolytic physiological properties" (**OKADA ET AL. 1993**). Similar as for the cellulose-degrading enzymes, our results here indicate a potential for hemicellulose degradation in S. alkalinus, which however seems to be promoted at later stages of incubation.

An important finding is that measured enzymes of *S. alkalinus* are generally capable of performing at higher pH although not optimally, as compared to those of *A. oryzae*. In *A. oryzae*, none of the measured enzymes were capable of acting at pH 10 (except for proteases) and generally showed higher inhibition at pH 8, as opposed to *S. alkalinus*. We presume the evolved ability to act at high pH as a result of selection pressure acting on those enzymes at the natural alkaline conditions of soda soils. Very strong proteolytic activity and its quick induction in *S. alkalinus* as compared to *A. oryzae*, was not anticipated. This property may reflect the means by which *S. alkalinus* obtains nitrogen in natural environments. In soda soils, i.e. at alkaline conditions, NH_4^+ would turn into toxic volatile NH_3, making nitrogen extremely limited. High proteolytic activity may be necessary for obtaining nitrogen from amino acids. Proteins might also serve as alternative carbon source. Possible protein-rich substrates in soda lakes include eggs of micro-crustaceans that thrive in saline alkaline waters. Immense quantities of microscopic eggs lay down on the sediment building substantial biomass. During dry seasons the water body of soda lakes evaporates and protein biomass is retained to the ground on the edge of a soda lake – exactly the place where all *S. alkalinus* strains were recovered. Interestingly, decent proteolytic activity was detected in chitin medium (**FIG. 6**). Chitin, as a principal component of crustacean carapace, may signal *S. alkalinus* its potential proximity to the protein-rich eggs that trigger the induction of proteases. Evolution of strong proteases in *S. alkalinus* can be additionally governed by the strong competition with prokaryotes for the easily accessible nitrogen-rich protein substrate. Strong proteases for fungi and other heterotrophic organisms thus may be a essential

pre-requisite for accessing nitrogen-poor environments of soda soils and soda lakes.

As fungi are now added to the picture as a component of saline soda soils biota, it is interesting to speculate on how they interact with the rest of the biota and what ecological role they play and how they are maintained in these harsh environments. Our enzyme assays provide extra support that *S. alkalinus* is metabolically active in natural conditions of soda lakes and soils. An interesting question therefore is how then *S. alkalinus* is spread in natural environments. During rain seasons, saline alkaline water may provide efficient means for the dissemination of *S. alkalinus*, as its spore heads are arranged in tight clumps held together by hydrophilic mucous substance of yet unknown composition (**GRUM-GRZHIMAYLO ET AL. 2013**). Such an arrangement of conidiospores is in strong contrast with typical hydrophobic conidia chains of *Aspergillus* or *Penicillium* spread by air. Also, when grown on agar plate, the conidial sporulation of *S. alkalinus* occurs on the bottom layer of the colony, submerged in a network of hypha, as opposed to dry and exposed airborne conidia of *Aspergillus* or *Penicillium*. These observations support the idea that *S. alkalinus* is spread around by water, rather than air currents, as soda water efficiently 'dissolves' the hydrophilic conidial heads of *S. alkalinus*.

Despite slow induction of cellulases and hemicellulases, they seem to be active at alkaline pH – a property that is rarely observed in fungi, and is desirable in certain commercial processes, like alkaline pre-treatment of plant biomass for its conversion of several bio-products. It is unclear yet if *S. alkalinus* is capable of producing stronger cellulases or hemicellulases at longer incubation times. Slow growth rate of *S. alkalinus* coupled with our genome surveys make it a feasible scenario, which is worth testing in future experiments. Alkaline proteases are used in a plethora of commercial applications, such as food, leather, silk, detergent industries, waste management and others (**NIRMAL & LAXMAN 2014**). Although bacteria now constitute the source of most alkaline proteases, fungal producers are becoming increasingly utilized. *S. alkalinus* seems to be a strong alkaline protease producer. It is especially evident in view of the current study, which showed 17-fold increase in protease activity for the wild type *S. alkalinus* isolate, compared to industrial *A. oryzae* RIB40 strain. *S. alkalinus* thus appears to be a promising producer of alkaline-active enzymes and warrants further investigations.

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Chapter VI

PacC activation and up-regulation is triggered at higher pH in alkaliphilic *Sodiomyces alkalinus* as compared to neutrophiles

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Abstract

Environmental ambient pH is an important abiotic factor soil fungi have been facing with, inevitably imposing a constant driving force for adaptation. In some habitats, ambient pH is shifted up-scale from neutrality resulting in the emergence of an extreme alkaline ecotope, where the overall biodiversity is substantially hampered. Some filamentous fungi have been shown to thrive at high alkalinity. One of them is the alkaliphilic ascomycete *Sodiomyces alkalinus*, which should possess biochemical and molecular adaptations responsible for dealing with high pH stress. To address this, we sequenced the genome of *S. alkalinus*, and investigated a well-known transcription factor PacC, which in fungi is known to be a key player in the mediation of gene expression as a response to ambient pH fluctuations. We found that the *pacC* gene is represented by a single copy in the genome and has high similarity with homologues from other fungi, indicating that PacC is highly conserved between alkaliphilic and neutrophilic fungi. This observation provides extra evidence for a long evolutionary history of the PacC/Rim101 transduction system. The expression levels of *pacC* across a wide pH range showed strong up-regulation from pH 7 and peaking at pH 11. The expression pattern suggests that *S. alkalinus* has shifted up-regulation as compared to many neutrophilic fungi. In *S. alkalinus*, western analysis showed that PacC was most abundant at pH 8.7, whereas in *Aspergillus nidulans* the highest level of PacC was observed at pH 5.9. We hypothesize that one of the key difference in the regulation of the gene expression as a response to ambient pH in the diverse ecological groups of fungi might lie at the level of the pH-sensing complex located on the plasma membrane.

Key Words

PacC, alkaliphilic fungi, *Sodiomyces alkalinus*, pH sensing

Introduction

pH is one of the major abiotic effectors to which microorganisms are forced to adapt, including filamentous fungi as a ubiquitous component in diverse habitats. One peculiar type of natural environments is soda soils, habitats with stable elevated pH values over time usually maintained by soluble carbonate salts creating strong buffering capacity (**JONES ET AL. 1998**). Several studies have shown the presence of filamentous fungi in alkaline environments broadening the adaptation limits for fungal growth (**NAGAI ET AL. 1998, GRUM-GRZHIMAYLO ET AL. 2013**). Some of them develop the richest morphology and the fastest growth at elevated pH values, confirming their alkaliphilic nature (**GRUM-GRZHIMAYLO ET AL. 2015**). High ambient pH influence many physical properties of the environment vital to a fungal cell. Nutrient solubility, element availability, low water activity, high osmotic pressure, plasma membrane fluidity are few of many processes that are affected by the pH. Naturally, pH gradient across the plasma membrane is a stress factor as it is believed that intracellular pH remains more or less constant around or slightly below neutral to ensure proper functioning of evolutionary

conserved intracellular metabolism (**HESSE ET AL. 2002**). Alkaliphilic filamentous fungi therefore must have adapted strategies of dealing with the above noted factors, resulting in the successful invasion and prosper in hostile alkaline environments. Those adaptations should affect at least the following physiological aspects: maintaining intracellular pH homeostasis at elevated external pH; excretion of enzymes that can efficiently degrade substrate at high pH values; possession of transporters, which translocate nutrients at high ambient pH; a sensing system, which responds to external pH and regulates the gene expression accordingly. In our study, we focus on the property of gene expression regulation as a response to high pH.

Many studies have addressed the adaptation properties to high ambient pH in filamentous fungi. Cell wall mechanical sensors (**SERRANO ET AL. 2006, DUPRES ET AL. 2009**), sodium pumps (**CARACUEL ET AL. 2003A, LAMB & MITCHELL, 2003, BENITO ET AL. 2009**), alkaline enzymes (**CALCAGNO-PIZARELLI ET AL. 2009**), to name a few, seem to take part in the complex trait of alkaliphily. The pH-sensing system has been extensively investigated over the last decades in the filamentous fungus *Aspergillus nidulans* (**MINGOT ET AL. 2001, ARST JR & PEÑALVA 2003, PEÑALVA ET AL. 2008, GALINDO ET AL. 2012**). In *A. nidulans*, an essential transcription factor PacC (homologous to Rim101 in *Saccharomyces cerevisiae*) has been shown to act as a main switcher of 'alkaline'-related genes at elevated pH values. The molecular cascade initiates at the plasma membrane upon sensing high pH, where the proposed sensors, PalH and its helper PalI, pass the downstream signal to the complex network of players of Pal and ESCRT genes, resulting in the proteolytic activation of the initially inactive PacC transcription factor. Proteolytic cleavage of the PacC removes its C-terminus part, exposing its main regulatory domain – the zinc finger motif (**DÍEZ ET AL. 2002**). The truncated PacC then migrates to the nucleus and binds, via its zinc finger domain, to the promoters of the 'alkaline' genes up-regulating their expression. At high ambient pH, PacC also acts as a repressor of 'acid'-related genes by competing with other transcription factors for their induction, hence 'acid' genes are constitutively expressed when the PacC molecular cascade is not activated (**ESPESO ET AL. 2000**). The *pacC* gene itself has promoter sequences for the fully processed PacC, therefore creating a positive loop for self-expression at high ambient pH.

Recent studies showed that PacC mediates the expression of 1 % to up to 20.6 % of the fungal transcriptome, equivalent to hundreds of genes, which are involved in many essential cellular metabolic processes (**ALKAN ET AL. 2013, TRUSHINA ET AL. 2013, FRANCO-FRÍAS ET AL. 2014**). The role of PacC has also been studied as an important gene modulator upon the pH change during the pathogeneses of *Candida albicans* (**DAVIS 2003**), *Sclerotinia sclerotiorum* (**ROLLINS 2003**), *Clonostachys rosea* (**ZOU ET AL. 2010**), *Fusarium oxysporum* (**CARACUEL ET AL. 2003B**), *Magnaporthe oryzae* (**LANDRAUD ET AL. 2013**), *Penicillium digitatum* (**ZHANG ET AL. 2013**), and several others. As of now, dozens of PacC homologue sequences have been obtained in many filamentous fungi of various ecological groups, showing its similarity over many taxonomical lineages of the fungal kingdom, which indicates a long evolutionary history of the PacC molecular cascade.

In the present work, we investigate the PacC transcription factor in the alkaliphilic ascomycete *Sodiomyces alkalinus*. This fungus taxonomically stands close to neutrophilic, but also some alkalitolerant and other alkaliphilic species, indicating evolutionary flexibility for acquiring

alkaliphilic traits (**GRUM-GRZHIMAYLO ET AL. 2013**). Based on the parsimonious evolutionary principle one may assume no sudden major rearrangements in the PacC leading to alkaliphily trait. On the other hand, in case the alkaliphilic trait is caused by the evolutionary pressure onto the PacC, several adaptations to the gene may be hypothesized. Firstly, as 'alkaline' genes are activated by the fully processed PacC, we can anticipate an increased expression of *pacC* either due to the gene duplications or development of a stronger promoter in *pacC*. The latter solution in turn would require promoter changes in all 'alkaline' PacC target genes as well. Secondly, the alkaliphily trait might involve the translation of the truncated version of the PacC right away upon sensing high ambient pH, with the only functional zinc fingers domain left. By this, the final PacC activator would be generated quicker, by-passing a complex and biochemically costly pathway for its final activation. This seems less likely given that many studies point towards the evolutionary stability of the PacC transduction pathway in many distinct groups of fungi. Another possible solution would be changing the threshold for activation of the PacC cascade on the plasma membrane sensor complex. That hypothesis assumes no or minor changes in the intracellular PacC pathway while the key difference attributing to the sensitivity of the pH receptors PalH and PalI on the plasma membrane. Naturally, even single amino acid changes in the pH sensors may drastically change the properties of the protein leading to the threshold change, upon which the ambient pH would trigger the downstream response.

To address the above discussed hypotheses, we first obtained the genome sequence of *S. alkalinus* and located the *pacC* homologue. Next, we studied the full *pacC* message sequence from the transcription starting site downstream to its polyA site. The *pacC* gene was present in a single copy in the genome (also confirmed by quantitative PCR) and its expression was shown to be strongly up-regulated at pH above 7–8 with the highest increase (10-fold) at pH 11. Western analysis showed the PacC transcription factor levels in alkaliphilic *S. alkalinus* were the most abundant at pH 8.7, about three units higher than in neutrophilic *Aspergillus nidulans* control strain. Given the neutral cytoplasmic pH observed in both fungi, we suspect the retuned sensitivity of pH receptors may be pivotal in the evolution of the alkaliphilic trait in filamentous fungi.

Materials and methods

Strains and media

The natural ex-type strain of alkaliphilic *S. alkalinus* F11 (= CBS 110278^T) (*Plectosphaerellaceae*, *Hypocreomycetidae*, *Ascomycota*) isolated at Shar-Burdiyn Lake (Choibalsan area, North-East Mongolia) from soda soils with pH 10.7 and 49 g/kg of total salts was used for experiments in the current study. Routine sub-culturing of the strain was performed on the alkaline agar medium (AA) based on malt and yeast extracts buffered at pH 10 in the dark regime at 28 °C, as described previously (**GRUM-GRZHIMAYLO ET AL. 2013**). For gene-expression analysis and western analysis at various pHs, the strain was grown in liquid at pH 5.2, 5.9, 7, 7.8, 8.7, and 9.8 on malt

and yeast extract based media. Different types of buffers (final concentration of 0.1 M) were employed to generate specific pH value. Citric buffer was used to generate pH 5.2, phosphate buffers were used to generate pH 5.9, 7 and 7.8, while carbonate buffers were used for pH 8.7 and 9.8. Additionally, we included Na_2HPO_4/NaOH system, which produced pH 11.4 on the agar plates. Where necessary, the pH of the liquid medium in the end of the cultivation was verified to be stable.

DNA and RNA extraction

The mycelium for nucleic acid extraction was scraped from the cellophane membrane put on top of the AA medium after 5 days of growth. The mycelium in liquid cultures was collected by centrifugation after 2 days of growth. For RNA extraction, expression analysis and western analysis at various pHs, the mycelium was taken from the corresponding media and put at -80 °C immediately upon harvesting. Samples of approximately 0.1 g of wet mycelium were used for total genomic DNA and RNA extraction with DNeasy and RNeasy Plant Mini Kits (Qiagen Inc., CA, USA). Quantity and quality of the acquired nucleic acids were verified on a NanoDrop 2000 (Thermo Scientific Inc., Wilmington, DE, USA). Pure genomic DNA and RNA samples were stored at -20 °C and -80 °C, respectively, for further use.

Genome sequence

The genome of *S. alkalinus* CBS 110278^T was sequenced with Illumina technology by Joint Genome Institute (Walnut Creek, CA, USA), yielding a total size of 43.45 Mb with the average coverage of 113.5x. The assembly and annotation was performed with AllPathsLG version R44849 on a JGI pipeline and custom analyses. The genome was deposited online and publicly available at **HTTP://GENOME.JGI.DOE.GOV/SODAL1/SODAL1.HOME.HTML**. The *pacC* gene was located by the homology search, verified with the long PCR using *pacC_-1938-1915f*/*pacC_2982-3003r* primer pair and re-sequenced. Primers used in the current study are listed in **TABLE 1**.

RT-PCR

Two-step reverse transcriptase PCR (RT-PCR) was performed on mRNA to reveal introns of the *pacC* gene. Approximately 1 μg of total genomic RNA was random-primer converted into cDNA with iScript™ cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. The RT-PCR on the *pacC* gene was commenced with several primer combinations, which confirmed the presence of the introns. For example, one working primer pair is *pacC_24-48f*/*pacC_1114-1135r*. The RT-PCR mix ingredients concentrations were the same as for degenerate PCR described above, however, with following exceptions. Both primer concentrations in the mix were set to 0.4 μM and 0.6 ng of cDNA template was used in the RT-PCR. The PCR conditions were as follows: 3 min at 94 °C; 32 cycles of 1 min at 94 °C, 1 min at 59 °C, 80 s at 72 °C, followed by the final extension step for 5 min at 72 °C. The products were visualized on a 1 % agarose gel, sequenced in both directions and compared to the genomic DNA sequence of the *pacC* gene.

3' and 5'RACE

Both 3' and 5' rapid amplification of cDNA ends (3' and 5'RACE systems, Invitrogen) techniques were employed to obtain a full-length message of the *pacC* gene. As for 3'RACE, 2.5 µg of genomic RNA was used as an initial template for the subsequent manipulations. cDNA synthesis was performed as described in the manual. For the first 3'RACE PCR we used target specific primer *TspC* and a kit-provided *AUAP* primer with 2.5 U of SuperTaq (HT Biotechnology) in the reaction mix. Annealing temperature for the first 3'RACE PCR reaction was set to 61 °C. To specify the products of the first 3'RACE PCR, the subsequent nested PCR was performed with the *TSP_C/AUAP* primer pair with PCR annealing step at 61 °C. The products of the nested PCR were checked on a 1 % agarose gel and sequenced directly with *TSP_C* primer in 3' end direction of the *pacC* message. As for 5'RACE, 1 µg of genomic RNA was taken for the following cDNA synthesis at 47 °C with the *pacC_351-367r* primer. After TdT tailing of the synthesized cDNA, the following 5'RACE PCR was performed using *pacC_174-197r* primer. The PCR program was set as follows: 2 min at 94 °C; 32 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, followed by the final extension step for 7 min at 72 °C. The third PCR with *pacC_120-141r* primer was run to specify the products amplified in second PCR. Several dilutions (10^1 to 10^4 fold) of the second PCR products were used as templates for the third PCR. The third PCR program was set as follows: 2 min at 94 °C; 33 cycles of 1 min at 94 °C, 1 min at 58 °C, 1 min at 72 °C, and final extension step for 7 min at 72 °C. The acquired products were verified on a gel and directly sequenced with *pacC_120-141r* revealing the position of the transcription starting site of the *pacC*.

TABLE 1. Primers used in the current study.

Primer name	Sequence (5' -> 3')	Purpose	T_m
pacC_-1938-1915f	AAC CCA TCT GCA CGC GAA TAC TTA	*pacC* gene sequence verification	61.0
pacC_2982-3003r	TGA TGC ATG CCA GAC ACC ACA A	*pacC* gene sequence verification	60.3
pacC_27-48f	TTC GGG AAG TGC GTC GTC AAA C	Intron check of *pacC*	62.1
pacC_1114-1135r	GAG GGC ATA TGG GCG GGA GTC T	Intron check of *pacC*	65.8
TspC	CCC ACA TGG CCT CTC CTC TCA	3'RACE	63.7
TSP_C	CCG AGA GCG CGA GGA GAA GT	3'RACE	63.5
pacC_351-367r	AGG TTG TTG GTG CTC TT	5'RACE	50.4
pacC_174-197r	TTG AGA TTG CAC TGA TTC CAC TTG	5'RACE	59.3
pacC_120-141r	ACT GGC AGC CGT TGA AGA AGA C	5'RACE	62.1
pacC_336-357f	CGT CAT GTA GGC CGC AAG AGC A	qPCR/qRT-PCR of *pacC*	64.0
TSP3	ACG CGG ATG TGG GAG GTG ATG T	qPCR/qRT-PCR of *pacC*	64.0
TEF1alpha_f	CGG CTT CAA CGG CGA CAA CAT	qPCR of *TEF1-α*	61.8
TEF1alpha(2)_r	GTG GGA CGG GTG GGA GGA AT	qPCR of *TEF1-α*	63.5
act1_f	CCC AAG TCC AAC CGT GAG AAG ATG	qPCR/qRT-PCR of *act1*	64.4
act1_r	CGA CCA GAC GCG TAC AGA GAC AGG	qPCR/qRT-PCR of *act1*	67.8
RPB2_f	ACA GGC CTG CGG TCT CGT CAA G	qPCR of *RPB2*	65.8
RPB2_r	TGT CGC GTT CGG ATA CCT CAG TGG	qPCR of *RPB2*	66.1

qPCR and qRT-PCR

Gene copy number of the *pacC* in the genome of *S. alkalinus* was asserted by the quantitative PCR (qPCR) on the genomic DNA. Three reference genes were utilized for signal normalization, which are known to be normally single copy genes in the ascomycetous fungi genomes (**HELGASON ET AL. 2003, SCHMITT ET AL. 2009**). Those are elongation factor 1 alpha (*TEF1-α*), gamma-actin (*act1*) and second largest subunit of RNA polymerase II (*RPB2*). Primers for *TEF1-α* (accession **JX158383**) and *RPB2* (accession **JX158449**) were designed based on the previously acquired sequences from **GRUM-GRZHIMAYLO ET AL. 2013**. The primers for the *act1* gene were designed based on the genome sequence. For primers sequences, see **TABLE 1**. The qPCR final mix volume was 8 µl and contained 2x iQ SYBR Green SuperMix (Bio-Rad), 0.2 µM of each primer and 0.35–3.5 ng of gDNA. The reaction was run in technical triplicates and biological duplicates, in a temperature gradient of 58–64 °C in Bio-Rad CFX96 thermocycler. The most-efficient amplification curves were taken into account and averaged for calculating C_t difference, subsequently used for $^{\Delta\Delta}C_t$ method for gene copy number quantification. Baseline threshold line was set automatically by the Bio-Rad CFX Manager v.2.0 software and results were normalized to the *RPB2* gene signal. To track the expression profile of the *pacC* across pH scale, we used RNA samples acquired from the mycelium grown in liquid (2 days old) and on agar plates (5 days old) at pH from 6 to 11.4 in duplicates. 1 µg of genomic RNA was converted into cDNA as described above. *act1* gene was employed as a reference gene and annealing temperature for the qRT-PCRs was set to 61 °C. The analysis of the raw data was performed as described above for gDNA, normalized to the *act1* gene and signal of *pacC* gene at the lowest pH tested.

Protein extraction

0.5 g of wet 2 days old mycelium grown at different pH in liquid media was used for protein extraction. The mycelium was disrupted by the glass beads (2 mm diam) in a bead beater machine. 200 ml of extraction buffer containing 50 mM of potassium phosphate buffer (pH 7), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.5 mM EDTA and proteinase inhibitor cocktail (Roche) was added to the mycelium powder and gently mixed. The solution was clarified by centrifugation for 5 min at 13000 g at 4 °C and supernatant was taken for protein concentration estimation by Bio-Rad Quick Bradford assay with BSA calibration. Protein extracts were stored at -80 °C as single-use aliquots.

Western analysis

A total of 20 µg of protein extracts were run on a 12 % SDS-PAGE gel and further transferred onto the nitrocellulose membrane (Bio-Rad), which was then stained with Ponceau S to verify the successful transfer. The primary polyclonal antibody against the conserved PacC zinc finger region epitope 137–150 aa (CLKKHVKTHADDSVL) was raised in rabbits and purified by GeneScript (Piscataway, NJ, USA). We used 1/5000 dilution of the primary antibody and further incubation with 1/100000 secondary GAR-HRP antibody. Immunodetection was performed using Immun-Star WesternC Chemiluminescent Kit (Bio-Rad) according to manufacturer's

instructions. We included pre-immune serum control throughout our western analysis to check for unspecific antibody binding. In addition, we included *A. nidulans* W6 wild-type strain protein extracts as a neutrophilic fungus reference.

pH probe fluorescene measurements

For the *in vivo* measurements of the cytoplasmic pH, we used a ratiometric fluorescence probe (LysoSensorTM Yellow/Blue DND-160, Invitrogen) on the 4 d mycelium grown on thin agar layer at pH 4 (except for *S. alkalinus*), 7, and 10 placed on a microscope slide. The fluorescence was measured after 20 min after loading of the probe using Leica DM5500 Flu microscope. The color calibration for the corresponding pH values was performed on blank agar slices.

Phylogenetic inference of the PacC homologues

The sequences for the phylogenetic analysis of the PacC homologues were obtained in GenBank or publicly available genome databases. The sequences were aligned with online MAFFT v. 7 (**KATOH & STADLEY 2013**), and corrected manually using BioEdit v. 7.1.3.0 (**HALL 1999**). PacC phylogeny was estimated both with Bayesian inference and Maximum likelihood bootstrap. For Bayesian inference we used MrBayes v. 3.1.2 (**HUELSENBECK & RONQUIST 2001**) set at mixed amino acid model run for 1 M generation sampling each 100th tree. First 50 % trees were discarded as burn-in and the rest were summarized to produce consensus tree with the posterior probabilities (PP) recovery at the nodes. Maximum likelihood inference was made using Dayhoff model for amino acid substitution as implemented in GARLI v. 2.0 (**ZWICKL 2006**) set to 200 bootstraps with five searches for each bootstrap. The bootstraps were summarized with Sumtrees v. 3.3.1. application within DendroPy v. 3.11.0 package (**SUKUMARAN & HOLDER 2010**) run on Python v. 2.6. The branches were displayed thickened if they were strongly supported, i.e. received joint ML > 90 and PP > 0.94 scores.

Results

pacC gene

The genomic sequence of the *pacC* area was confirmed by the long PCR and re-sequencing. The analysis of the nucleic acid sequence revealed open reading frame (ORF) of 1932 bp length encoding PacC protein of 643 amino acids. The ORF was predicted to have three introns of 98, 80 and 100 bp. The presence of introns and their size was later confirmed by the cDNA sequencing. Subsequent RACE experiments allowed us to obtain the full length messenger of *pacC*, from 5'UTR starting site (position -499 from the ATG start codon) to 3'UTR (position 2868). The 5'RACE experiment resulted in a single PCR product on a gel, suggesting a single message type of the *pacC* with a single transcription initiation site at position -499. Quantitative PCR on genomic DNA displayed *pacC* as a single copy gene in the *S. alkalinus* genome (**FIG. 1, C**). Upstream of 5'UTR, we located three putative binding sites for the PacC transcription factor (5'-GCCAAG-3')

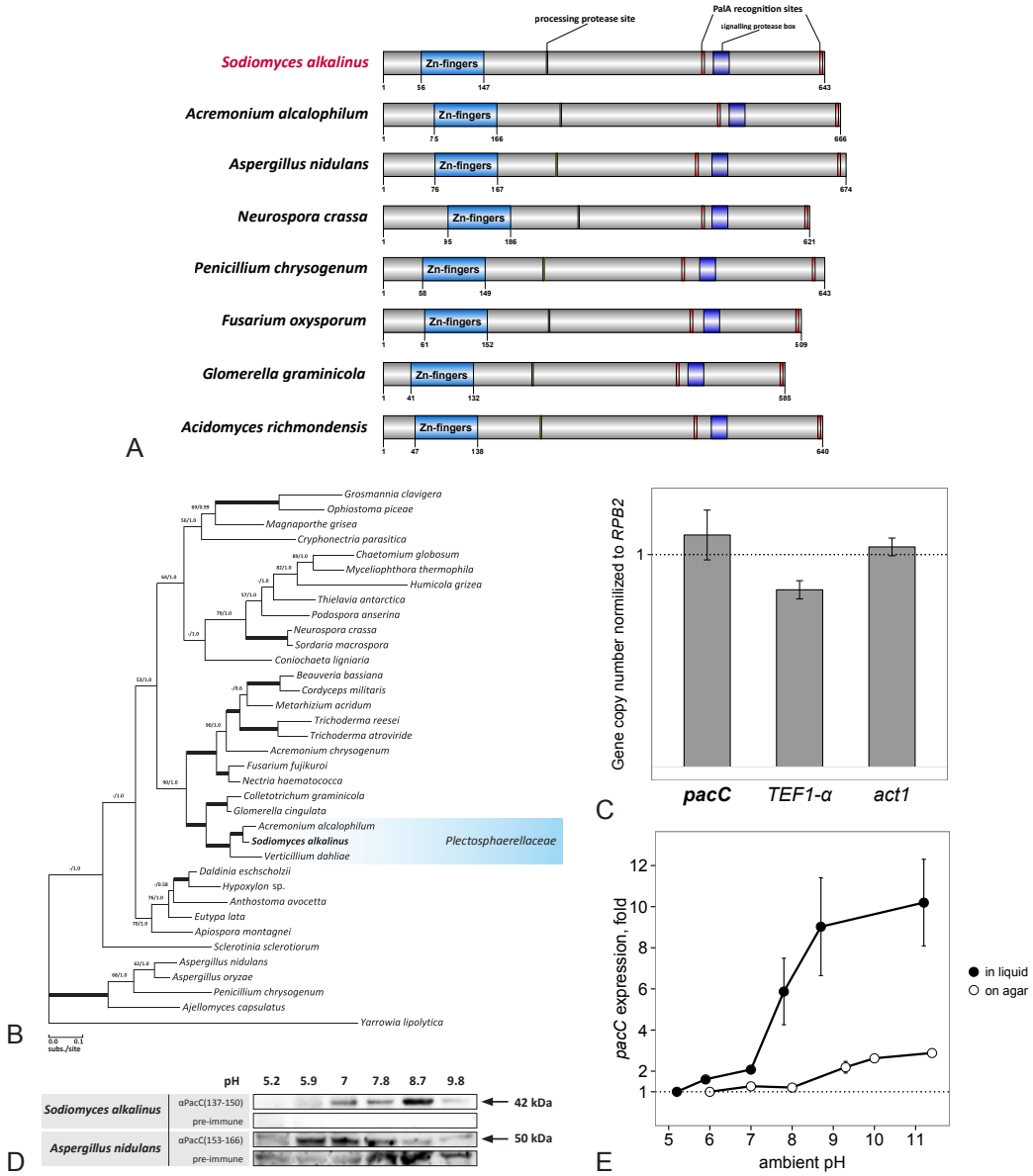

FIG. 1. PacC homologue study in *S. alkalinus*. **A.** PacC structure in comparison to the few selected ascomycetous fungi: *Acremonium alcalophilum* (JGI genome data), *Aspergillus nidulans* (XP_660459), *Neurospora crassa* (XP_957214), *Penicillium chrysogenum* (AAC36492), *Fusarium oxysporum* (AAM95700), *Glomerella graminicola* (EFQ28428), *Acidomyces richmondensis* (JGI genome data). **B.** Phylogenetic position of PacC in alkaliphiles is in compliance with neutral evolution rates. Thick branches indicate strong support. **C.** *pacC* gene copy number in the genome. **D.** Western analysis of the PacC processing in *S. alkalinus* and *A. nidulans* across the pH range. **E.** *pacC* expression analysis on the mycelium grown in liquid (2-d-old) or solid agar medium (5-d-old) at different pH values.

at positions -606, -596 and -584, and one inverted, 5'-GCCAGG-3' at -1274 (**TILBURN ET AL. 1995**). Curiously, the sense binding sites are arranged in a tandem manner separated by the ATAT sequence motifs. Beyond the coding sequence, there are two long (10 bp) mononucleotide stretches, one at position -226 and another one at 2222, both caused polymerase slippage during the PCR, resulting in unreadable sequence chromatograms. We later verified those problematic areas by sequencing after proof-reading PCR amplification.

PacC protein

The predicted PacC protein sequence in *S. alkalinus* is conserved with homologues throughout the *Ascomycota* fungal kingdom. The alignment of the PacC in *S. alkalinus* with its homologues studied in many other fungi showed 35 % identity in *Penicillium digitatum* (**ZHANG ET AL. 2013**), 50 % in *Sclerotinia sclerotiorum* (**ROLLINS & DICKMAN 2001**), 58 % in *Acremonium chrysogenum* (**SCHMITT ET AL. 2001**), 61 % in *Fusarium oxysporum* (**CARACUEL ET AL. 2003B**), 43 % in *Wangiella dermatitis* (**WANG & SZANISZLO 2009**), 45 % in *Aspergillus nidulans* (**TILBURN ET AL. 1995**), 38 % in *Penicillium chrysogenum* (**SUÁREZ & PEÑALVA 1996**). The reconstructed phylogenetic tree, as inferred from PacC homologues, demonstrates that the variation can be explained by the neutral evolution, as *S. alkalinus* unambiguously (100/1.0) groups with other members of *Plectosphaerellaceae*, like *Acremonium alcalophilum* and *Verticillium dahliae* (**FIG. 1, B**). The size of the PacC homologue in *S. alkalinus* is also similar to that of other fungi, and comprises 643 amino acids (68.8 kDa) containing all functional domains necessary for its proper putative processing. The major functional domain is a highly conserved zinc finger DNA-binding regulatory motif located on the N-terminus spanning from 56 through 147 aa. On the C-terminus, two short YPXL motifs required for the putative first pH-dependent cleavage, serving as recognition sites for PalA protein bound to PalB protease, which cleaves the PacC upon the so-called 'signalling protease box' at approximately 499 aa position. After the first cleavage, the shorter PacC (52.7 kDa) undergoes a second proteolytic (pH independent) step upon the 'processing protease site', as shown in *A. nidulans*. In *S. alkalinus*, the 'processing protease site' is located at the position 238 (RKR motif). The shortest, processed form of PacC is predicted to be 25.7 kDa in size and exclusively localized to the nucleus, as predicted by the cNLS Mapper (**KOSUGI ET AL. 2009**). A comparative scheme for the few selected PacC homologues with the outlined major motifs is shown in **FIG. 1, A**. In summary, our *in silico* PacC analysis in alkaliphilic *S. alkalinus* provides extra evidence for the highly conserved molecular pH signaling cascade across various physiological groups of fungi.

pacC expression analysis

We found different expression levels of *pacC* in *S. alkalinus* grown for 2 days in liquid cultures or for 5 days old on solid agar plates (**FIG. 1, E**). However, at both growth modes, *pacC* was up-regulated at high pH levels, starting form pH 7 in liquid and from pH 8 on agar plates. In liquid cultures, the mRNA levels at high pH were about 9–10 times more abundant than at low pH. On the agar plates, we observed only 2–3 fold induction in expression at high pH as compared to the levels at lower (6–8) pH. At both growth regimes *pacC* expression was peaking at the highest

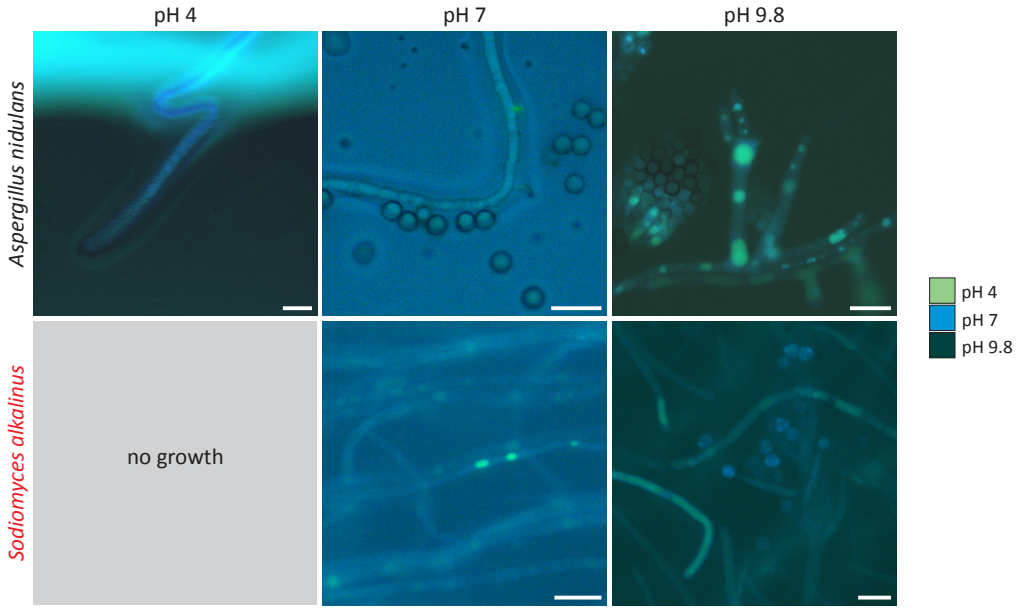

FIG. 2. Intracellular pH of the *A. nidulans* and *S. alkalinus* grown at different ambient pH, as inferred with a ratiometric fluorescence probe. *Scale bars:* 10 µm.

pH 11 value tested. The *pacC* induction at alkaline pH is in line with previous studies, however, it seems that in *S. alkalinus* the *pacC* starts to be strongly up-regulated at higher pH values (by 1–2 pH units) than in the neutrophilic filamentous fungi studied so far.

PacC activation

Up-regulation of expression of *pacC* at elevated pH levels in *Sodiomyces* does not necessarily coincide with its proteolytic activation at higher pH values. To show which pH leads to the abundant formation of the processed PacC, we performed western analysis on the protein extracts from the mycelium grown at pH from 5.2 to 11.2. Pre-immune serum control did produce unspecific binding on the protein extracts from *S. alkalinus* (data not shown), so we did not take ambiguous areas of low-molecular weight on the blot into consideration. Also, we verified the secondary antibody alone would not give a signal. We detected a band that displays different intensity across the pH range (**FIG. 1, D**). It has maximum intensity at pH 8.7, suggesting maximal levels of the processed PacC at that particular pH. The product seems to have larger apparent molecular weight than predicted from the amino acid sequence, which might be explained by the posttranslational processing of the PacC. We took advantage of the fact that the epitope sequence against which the primary antibody was raised completely matches both in *S. alkalinus*

and *A. nidulans*. We included western-blot analysis for protein extract obtained from *A. nidulans* strain W6 as a neutrophilic fungus reference. As seen in **FIG. 1 (D)**, corresponding PacC product has the highest levels at pH 5.9, about three units lower than in *S. alkalinus*.

Cytoplasmic pH measurements

The cytoplasmic pH of the fungi is thought to be around neutral, and was experimentally shown to be so in *Aspergillus niger* and *Neurospora crassa* (**SANDERS & SLAYMAN 1982, HESSE ET AL. 2002, BAGAR ET AL. 2009**). We sought to confirm this notion for the alkaliphilic *S. alkalinus* species. **FIG. 2** depicts the fluorescence of the pH probe for the *A. nidulans* and *S. alkalinus* grown at different pH values. Grown at the pH 4, *A. nidulans* hyphae stained blue indicating higher pH, as compared to the acidic (greenish) environment. At pH 7 we could not see markedly different colours of cytoplasm for both fungi. Finally, at pH 10, where the dye is non-sensitive, both species produce blue interior coloration. Cellular vacuoles gave greenish coloration with respect for the rest of the cytoplasm, indicating more acidic pH (4–5). Our results show that despite the preferred differences in external pH for *A. nidulans* and *S. alkalinus*, both of them are maintaining internal pH around neutral.

Discussion

Filamentous fungi show a large array of preferences with respect to growth at different environmental conitions. A growing number of available genomes of fungi displaying various physiologies provide an excellent opportunity to study the adaptations to the environmental effectors, such as pH. In the current study, we raised a question about the pH cascade response in two distinct physiological groups of fungi – neutrophiles and alkaliphiles, using *A. nidulans* and *S. alkalinus* as model organisms, respectively. To assess this question we sequenced the genome of alkaliphilic *S. alkalinus* and studied the key transcriptional factor PacC, which in fungi is known to be the major activator of the 'alkaline'-related genes. Notably, the genome of *S. alkalinus* is a second alkaliphilic genome sequenced so far, with the first being from sister species *Acremonium alcalophilum*. The sequence variation of the *pacC* in *S. alkalinus* seems to be explained by the neutral evolution that is in line with taxonomic distances to other species within the family (**FIG. 1, B**). Our *pacC* message analysis have not revealed mutations that would create premature stop codon resulting in the translation of the short/active variant of PacC, thereby by-passing the need for the upstream activation cascade. This would be expected if the fungus would only grow at high pH and never encountered lower pHs. In a pH constant milieu, pH sensing would become irrelevant, promoting constitutive expression of the alkaline genes. It appears that pH sensing is still important and that 'gene-expression' decisions based on ambient pH must be made by the fungus. Such conclusion seems puzzling though, as *S. alkalinus* thrives in soda soils, which are thought to have stable high pH. On the other hand, at the isolation site of the *S. alkalinus* F11^T isolate, during the rainfall seasons the environmental pH may decrease for a short while. This short-term pH decrease may promote the selection force to keep the pH-

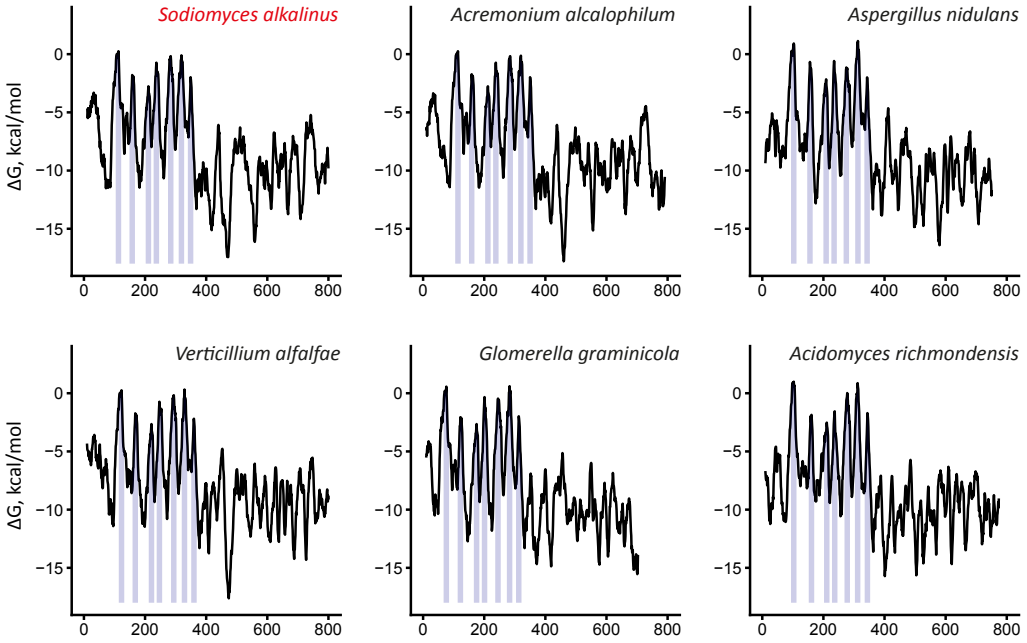

FIG. 3. Transmembrane domain (TMD) predictions of the pH receptor PalH in *S. alkalinus* and other reference species, showing similarity in global topology across fungi having different physiologies. The predicted TMDs spans are highlighted. Sequences data of PalH comes from JGI database, except for *Verticillium alfalfae* (**SHI-KUNNE ET AL. UNPUBLISHED**).

responsive molecular cascade in *S. alkalinus*.

We detected only one translation initiation site for the *pacC* gene, indicating the presence of a single transcript type for the *pacC* in *S. alkalinus*. Also, RT-PCR analysis did not reveal signatures for the alternative splice variants of the *pacC* message. Similarities to the neutrophilic *pacC* system extend further to the protein level. *In silico* analysis of the PacC in *S. alkalinus* revealed all the necessary domains required for its putative proteolytic processing (**FIG. 1, A**), after which it migrates to the nucleus. Adding the fact that the *pacC* gene was shown to be present in one copy in the *S. alkalinus* genome (**FIG. 1, C**), all above described observations led us to conclude that the *pacC* gene is not under strong positive selection in *S. alkalinus*, which would produce a highly altered PacC structure.

The expression levels of the *pacC* measured across the pH gradient, were starting to rise at pH above 7 in liquid shaking growth regime and at higher pH in the mycelium grown on a solid medium (**FIG. 1, E**). In both cases the strongest up-regulation observed at the maximum pH tested. Such a general expression pattern, down-regulation at low pH, and up-regulation at high pH is in line with previous studies on the *pacC* expression. From our observations, the

middle point on the growth pH range could be used as a point at which the *pacC* starts to get strongly up-regulated, likely switching to 'alkaline'-expressed phenotype. In *S. alkalinus*, such threshold happens at pH above 7 as grown in liquid. The difference in expression pattern of *pacC* between the two growth regimes in our experiments could be explained by the fact that the shaken liquid environment creates a more stressful environment for the mycelium since washing intensifies its exposure to the pH of the native medium. However, on the agar surface, the fungus is potentially capable of creating a more favorable local environment as it colonizes the substrate, possibly by producing lower pH surroundings from the rest of the medium. We were not able to detect the pH change of the agar medium utilizing pH indicator dyes (data not shown), implying that such a presumed lower pH barrier is very close to the hyphae and not dissipating through the solid medium across large distance. Such potentially created favorable barrier effect would be mitigated in the shaking regime. The pH drop in liquid medium by one unit in alkaline media after several days of *S. alkalinus* growth supports that idea. Therefore, in a liquid regime, the pH stress was more prominent and induced the *pacC* up-regulation at a lower pH than when grown on solid, where the pH stress had been diminished. We noticed that the observed *pacC* up-regulation point in *S. alkalinus* is higher that of neutrophilic fungal species. For example, in *Sclerotinia sclerotiorum*, northern analysis showed strong *pacC* up-regulation starting at pH about 6 (**ROLLINS & DICKMAN 2001**), in *Clonostachys rose*a at approximately pH 4–5 (**ZOU ET AL. 2010**), in *Penicillium digitatum* starting at pH 6 (**ZHANG ET AL. 2013**), in *Coniothyrium minitans* at pH 4 (**HAN ET AL. 2011**). The difference of pH values at which the *pacC* starts to be strongly up-regulated in various species might indicate that there is a certain threshold for ambient pH at which the PacC is triggered for downstream processing in different fungi.

To get extra support to that idea, we performed western analysis with an antibody against the zinc-finger domain of PacC both in *A. nidulans* and *S. alkalinus* grown in pH range (**FIG. 1, D**). In addition to a number of unspecific signals, we detected a band, which responded to external pH. The dynamics, at which the processed PacC levels are accumulated in two distinct physiological groups of fungi, strongly supports our hypothesis about the different ambient pH triggering thresholds for the downstream PacC proteolytic processing. The pH at which we observed maximal PacC levels differed by approximately three pH units between the two studied species, which corresponds to a 1000-fold difference in H^+ concentration. Remarkably, *S. alkalinus* requires higher ambient pH values for optimal growth, at the same time investing energy to keep the internal pH about neutral (**FIG. 2**). It seems that such costly investment have benefited *S. alkalinus* in occupying extremophilic niche regardless the obtained trade-offs for the alkaliphilic trait.

Our result provide evidence that extremely alkaline soda soil habitat may have shaped the pH sensing ability in *S. alkalinus*, which ensures PacC processing at higher pH than in neutrophiles. Earlier studied in *A. nidulans*, the pH sensors on the plasma membrane include PalH possessing 7-TMD and its 3-TMD helper PalI (**CALCAGNO-PIZARELLI ET AL. 2007**). Maximum parsimony principle implies that even minor amino acid change in the pH sensor may alter its folding pattern resulting in a shifted pH triggering threshold for the downstream signal transmission

in alkaliphilic fungi. This solution would by-pass or minimize the need for the rearrangements of the intracellular part of the pH transduction pathway. We did not detect major structural rearrangements in the PalH pH sensor in alkaliphiles *S. alkalinus* and *A. alcalophilum* as compared to other fungi (**FIG. 3**). As predicted with MPEx v. 3.2.6 (**SNIDER & JAYASINGHE, UNIV. CALIF., IRVINE**), PalHs from fungi of different ecologies have similar seven TMDs on the N-terminus with a long cytoplasmic hydrophilic C-terminus tail. Unfortunately, our preliminary analyses did not reveal signs of positive selection acting on PalH pH-sensor within the alkaliphilic clade occupied by *S. alkalinus* and *A. alcalophium*.

By no means, we are denying the need for the evolved target structural genes, encoding the exo-cellular enzymes and metabolites, which would operate optimally at high environmental pH, nor the other pH-induced molecular cascades. However, we propose the shifted pH-sensing trait to be an important prerequisite in the adaptive evolution of alkaliphily in filamentous fungi. Further studies should focus on the target genes that are under control of the PacC transcription factor, as well as a detailed study of the membrane pH sensor in *S. alkalinus*. That data could provide important clues towards understanding the evolution of alkaliphilic phenotype in filamentous fungi.

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Chapter VII

Cryptic double-stranded RNA mycoviruses in *Sodiomyces alkalinus*

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Abstract

Double-stranded RNA (dsRNA) mycoviruses are present in many lineages of filamentous fungi. Here, we report the detection of virus-like icosahedral particles of 35 nm size in alkaliphilic fungus *Sodiomyces alkalinus*, family *Plectosphaerellaceae*. Sister alkaliphilic species, *Acremonium alcalophilum, Sodiomyces magadii* and *Sodiomyces tronii* were free of enigmatic dsRNA in the nucleic acids profile on a gel. At least four isolates of *S. alkalinus* out of eighteen contained dsRNA fragments. DsRNA-bearing isolates were symptomless as compared to dsRNA-free strains, thus are not likely to be associated with alkaliphilic trait in the host. The banding pattern of the dsRNAs was different among the isolates, however, they all shared the largest dsRNA fragment of about 6 kb size. This observation might indicate different types of viruses present in *S. alkalinus*, including the possibility of mixed infections in a single host. The dsRNA fragments were transmitted vertically through spores, but not horizontally through anastomoses, giving the evidence for a long and persistent isolate-specific fungus-virus association. The fungal strains harboring most similar dsRNA banding patterns matched their geographical isolation pointing at the possible origin sites for the virus acquisition. The detected dsRNA of *S. alkalinus* in the present study is the first instance of a viral presence in an alkaliphilic filamentous fungus.

Key words

mycovirus, dsRNA, *Sodiomyces*

Introduction

Mycoviruses are common in fungi (**REFOS ET AL. 2013**). Although most of them do not constitute easily detectable phenotypic alterations of a host, there are cases when the presence of a virus affects host's growth, sporulation and virulence. A well-known example includes a killer phenotype of *Saccharomyces cerevisiae* yeast governed by the presence of the toxin-producing cytoplasmic dsRNA virus (**SCHMITT & BREINIG 2006**). Important virus-induced phenotypes are those causing significant economic losses, like oyster mushroom spherical virus (OMSV) affecting fruit bodies in cultivated *Pleurotus ostreatus* (**YU ET AL. 2003**). Several mycoviruses were found to be associated with the diseases in *Agaricus bisporus* and other cultivated mushrooms (**QIU ET AL. 2010**). Sometimes a virus can modulate the interactions of a fungus and a plant it infects, like in a well-studied case of hypovirulence phenomenon of the chestnut blight fungus, *Cryphonectria parasitica* (**DAWE & NUSS 2001**). The property of some mycoviruses to cause hypovirulence is currently under consideration for using it as biological agents against human fungal infections (**VAN DE SANDE ET AL. 2010, REFOS ET AL. 2013**). A remarkable example of the three-level interaction between a virus, a fungus, and a plant was reported by **MÁRQUEZ ET AL. (2007)**, where heat tolerance of a plant was conferred by a mycovirus present in a fungus infecting that plant.

The genetic material of a mycovirus usually constitutes single or several dsRNA molecules packed into a symmetric icosahedron-shaped protein coat within a size range of about 20–50

nm. However, about a third of the described mycoviruses possess single-stranded RNA (ssRNA) (**PEARSON ET AL. 2009**). A unique case of a single-stranded DNA (ssDNA) mycovirus was recently shown to confer hypovirulence in *Sclerotinia sclerotiorum* (**YU ET AL. 2010**). The classification of the dsRNA mycoviruses is based on the number and size of the genome fragments they constitute, and are assigned to *Totiviridae, Partitiviridae, Reoviridae,* or *Chrysoviridae* families (**POTGIETER ET AL. 2013**).

In the current study, we report the detection of virus-like particles in the alkaliphilic ascomycete *S. alkalinus* (family *Plectosphaerellaceae*). The bands of suspected viral origin appeared on a total nucleic acid gel in four isolates of *S. alkalinus.* After DNase I and RNase A treatments we proved these bands to be dsRNA. The banding pattern was different in three isolates; however, all four virus-containing isolates shared the largest dsRNA fragment of approximately 6 kb size. We did not detect obvious phenotypic alterations of the isolates carrying dsRNA fragments, as compared to viral-free strains. These dsRNA fragments have been demonstrated to transmit vertically through both asexual and sexual spores. In our experiments, we showed the dsRNA fragments to be isolate-specific and non-infective between the isolates within the species. The presence of the viruses was confirmed by a transmission electron microscope, which visualized the icosahedral-shaped virus-like particles of approximately 35 nm size. We speculate on the origin of the virus-containing strains, as there seems to be a geographical and phylogenetic relation between the viral-bearing strains of *S. alkalinus*. The current study present the first instance of the virus detected in an alkaliphilic filamentous fungus.

Materials and Methods

Strain manipulations and media

All known 18 strains of *S. alkalinus* isolated earlier were used in the current study (**GRUM-GRZHIMAYLO ET AL. 2013**). Routine sub-culturing was performed using the alkaline agar (AA) medium, propagating in the dark at 28 °C. Where necessary, single conidiospore colonies were obtained by washing the conidia off from a parental strain with 0.9 % saline/0.005 % Tween-80. The conidia suspension was surface-plated on a fresh agar plate with several dilutions. Single colony initials were picked up and transferred on a fresh medium. To obtain the colonies derived from single ascospores, we manually picked cleistothecia and wiped out the outer surface on a water agar plate to clean the fruiting body from mycelial remnants. Clean fruiting bodies were put in saline/tween solution and gently crushed. The released ascospores were plated out on the AA medium and young colonies were transferred to fresh AA plates.

Nucleic acid extraction

Total nucleic acids extraction was performed on the mycelium grown on a cellophane membrane put on top of the AA medium. About 0.1 g of frozen mycelium was ground to a fine powder with glass beads in a bead beater machine, followed by the conventional phenol/

chloroform nucleic acid extraction. We used 0.6 % agarose gels pre-casted with either GelRed™ (Biotium, CA, USA) or ethidium bromide to visualize the nucleic acids. For the long term storage, nucleic acids were put at -20 °C.

DNase I treatment

The total nucleic acids were treated with DNase I in 20 µl reaction volume contained 5 µl of the sample, 2 µl of 10x Reaction Buffer (New England Biolabs, UK), 12.5 µl of MQ-water, and 0.5 µl (1 Unit) of DNase I (New England Biolabs, UK). The reaction was incubated for 10 min at 37 °C, and then terminated by the addition of 1 µl of 0.1 M EDTA. After 10 min inactivation at 75 °C, nucleic acid integrity was determined by gel electrophoresis.

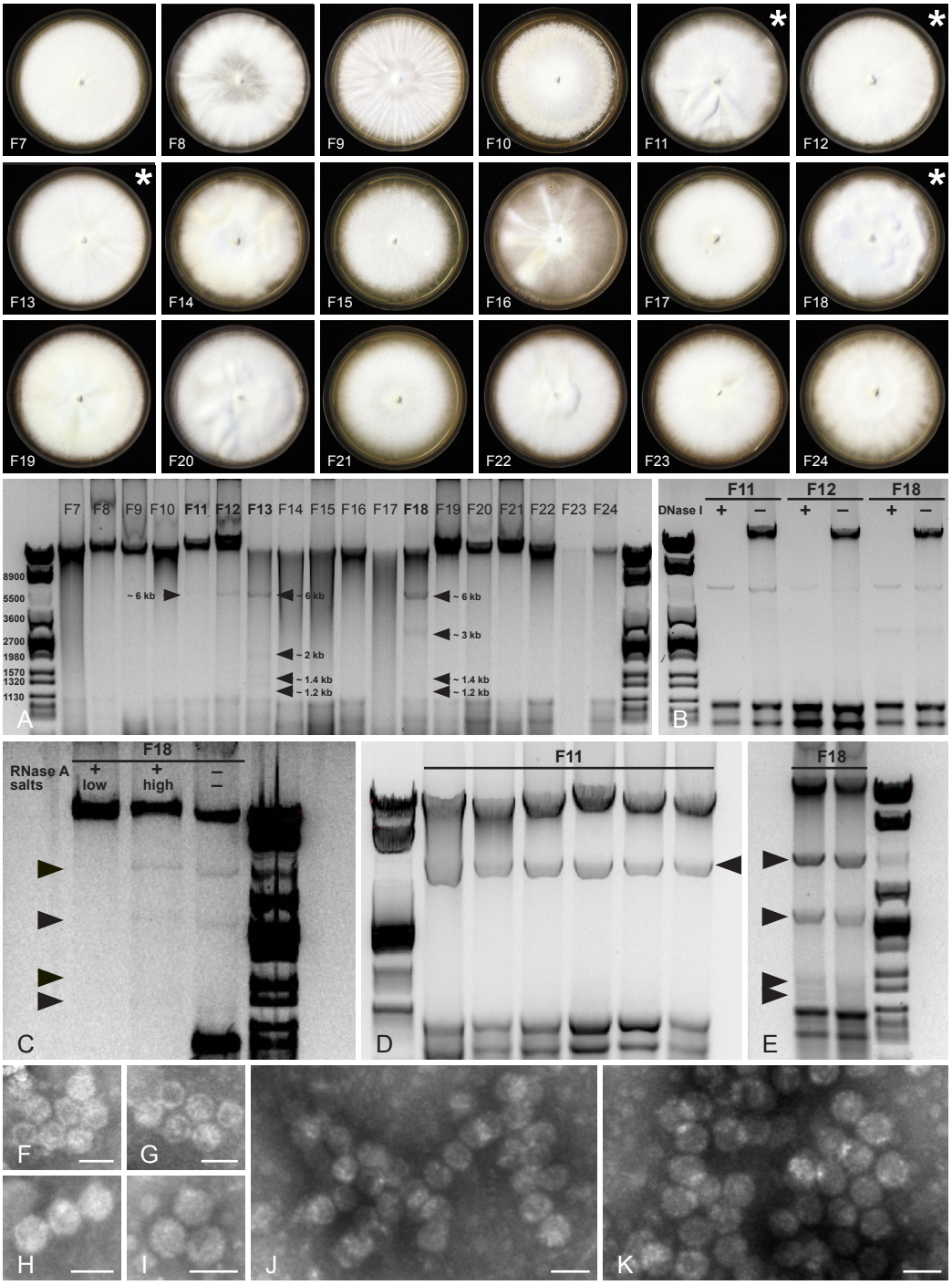
RNase A treatment

To figure out the nature of the RNA molecules, we performed RNase A treatment at different salt concentrations in a total volume of 10 µl. 5 µl of the sample was mixed with either 0.1x SSC (low salt) or 3.2x SSC (high salt), followed by the addition of RNase A (Qiagen, Chatsworth, CA, USA) to a final concentration of 10 µg/ml. After 30 min incubation at 37 °C, the samples were loaded on an agarose gel, along with a non-treated control.

Negative staining and transmission electron microscopy

The mycelium was ground with a pestle and a mortar with liquid nitrogen. The powder was resuspended in 0.1 M phosphate buffer and clarified by centrifugation for 10 min at 10000 rpm. The supernatant was taken for further ultracentrifugation at 30000 rpm for 3 h. The pellet was resuspended in 100 µl of 0.1 M phosphate buffer; the obtained solution was used for negative staining as follows. A droplet of the sample was placed on a nickel grid for 1 min and drained off by a filter paper, then washed by a droplet of water. Then a drop of uranyl acetate was put on the grid for 15 sec and then drained off. In case negative stain was performed using phosphotungstic acid, we did not wash the grid with a water droplet. The grids were then analyzed with a JEM1011 (JEOL, Japan) transmission electron microscope.

FIG. 1. Viruses in *Sodiomyces alkalinus*. The top panel shows 18 known isolates of *S. alkalinus* grown on the alkaline agar (AA) medium after 20 d incubation (9 cm Petri dish, dark, 28° C). Asterisks mark dsRNA-containing strains. **A.** Total nucleic acids profile of *S. alkalinus* isolates. DsRNAs are arrowed with approximate sizes. **B.** DNase I treatment on the total nucleic acids. **C.** RNase A treatment at high and low salt conditions, proving the dsRNA nature of the enigmatic extra bands. **D.** Asexual spores (conidia) progeny nucleic acids profile of the F11^{T} strain. **E.** Sexual spores (ascospores) progeny nucleic acids profile of the F18 strain. All gels had a Lambda marker restricted with EcoRI, BamHI, and HindIII. **F–H.** Transmission electron micrograph of the isometric virus-like particles in the strain F13 extracts, after negative staining with phosphotungstic acid. **I–K.** Transmission electron micrograph of the isometric virus-like particles in the strain F13 extracts, after negative staining with uranyl acetate. *Scale bars:* **F–K** = 50 nm.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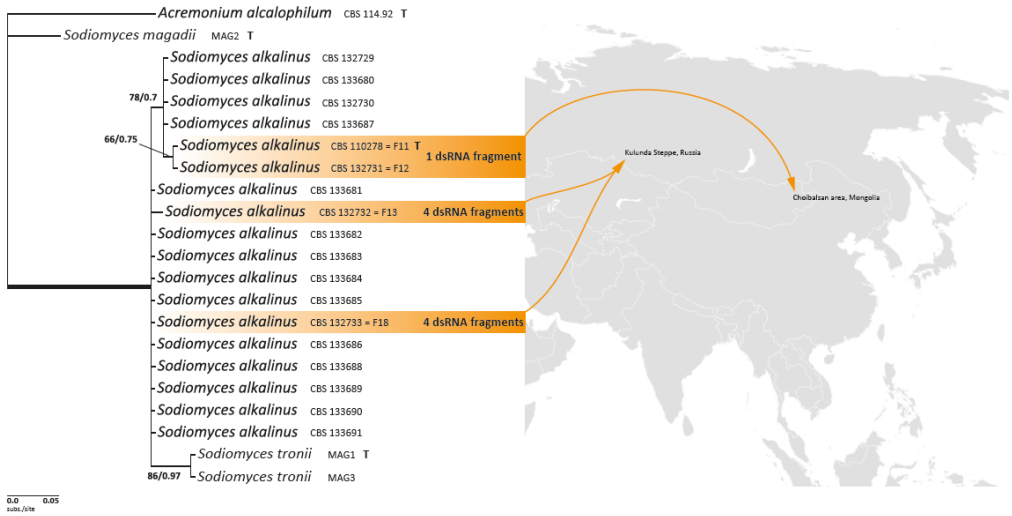

FIG. 2. Phylogeny of the *Sodiomyces* species group based on the ITS rDNA sequences (from **GRUM-GRZHIMAYLO ET AL. 2015**) with the origin locations of dsRNA-containing strains. Thickened branch indicate strong combined support of ML > 90 and PP > 0.94.

Results

Total nucleic acid profiles on the gels included enigmatic extra bands, which appeared in at least four strains (F11^T, F12, F13, and F18) of *S. alkalinus* out of 18 described thus far (**FIG. 1, A**). After the DNase I treatment, a major high-molecular weight genomic DNA band disappeared, however, the suspected viral bands stayed in place, as well as ribosomal RNA bands of lower size on the bottom edge of a gel (**FIG. 1, B**). The RNase A treatment at high and low salt contents revealed the nature of a RNA band of interest. At high salts, RNase A selectively degrades only single-stranded RNA, whereas at low salts, both RNA types. In our RNAse A experiment (**FIG. 1, C**), a combination of treatments suggest these bands are double-stranded RNA, which strongly supports a viral origin of those bands. Curiously, the banding pattern in four presumably viral-containing strains differed from one another, except for F11^T and F12 isolates. All four strains share the largest dsRNA fragment of approximately 6 kb size, but F13 and F18 isolates contain extra dsRNA molecules of smaller size. F11^T and F12 seem to possess a single large dsRNA molecule, however we cannot exclude the possibility of the existence of yet undetectable low-titer bands, as we are limited by the sensitivity of ethidium bromide staining. F13 isolate has at least four dsRNA molecules (ca. 6, 2, 1.4, and 1.2 kb size), which suggests either a fractionated genome of a single virus, or the presence of several virus types in one host. Isolate F18 also has at least four dsRNA bands, the two smallest seem to be shared with those of F13 strain. Extra 3 kb band is only associated with the F18 isolate. In **FIG. 1 (A)**, the smallest F18's bands are not clearly distinguishable, however, we detected them at a higher titers from alternative

extraction samples (data not shown). It seems that the relative abundance of the dsRNA bands may be associated with the certain time-points within a life cycle of the fungus.

We sought to estimate how stable the dsRNA association with the fungus is by bottlenecking the host to a colony derived for a single spore. We checked the vertical transmission both from sexual (ascospore) and asexual (conidia) progenies. The association seems to be vertically stable, as the bands in all replicates persisted after single cell bottlenecking (**FIG. 1, D, E**). The stable isolate specific viral association is supported by our horizontal transmission experiments (data not shown). We attempted to "infect" the dsRNA-free isolates by anastomoses contacts with the dsRNA-containing isolates growing next to it. Also, in another approach, the dsRNA-containing cell extracts were poured on top of the damaged dsRNA-free strains – to let the dsRNA fragments enter in a more invasive manner. However, both approaches failed to achieve a detectable transfer of dsRNAs to dsRNA-free isolates. We did not detect any phenotypic manifestations for the strains containing dsRNA bands (**FIG. 1, TOP PANEL**). There is a variation in colony morphology across all of our 18 strains of *S. alkalinus,* however, they do not seem to be associated with the presence of dsRNA in the cell.

Using a transmission electron microscope, we detected virus-like particles in strain F13 (CBS 132732). Since we did not analyze other dsRNA-containing strains with TEM, we cannot claim the virus-like particles would be identical to those in other isolates having dsRNA. In F13, we found particles of icosahedral shape in fungal cell extracts, stained both with phosphotungstic acid (**FIG. 1, F–H**) and uranyl acetate (**FIG. 1, I–K**). The average size of a virus-like particle was about 35 nm. The nucleic acid type, the shape and the size of our virus-like particles fit into the range of the described common mycoviruses (**MERTENS 2004**).

Our previous phylogenetic analysis of the *Sodiomyces* based on the ITS sequence showed isolates F11^{T} and F12 to be segregated from the rest. Curiously, they originated from the same geographical area (Choibalsan area, North-East Mongolia), and harbored similar single dsRNA band (**FIG. 2**). On the other hand, strains F13 and F18, both containing four dsRNA fragments, were more similar genetically, however, those isolates also came from the same geographical area (Kulunda Steppe, Altai, Russia). The correlation of the dsRNA-containing strains with their geographical origin might reflect sites where the fungus-virus association arose.

Discussion

The present study reports a first case of virus-like particles detected in an alkaliphilic filamentous fungus, *Sodiomyces alkalinus. Acremonium alcalophilum,* another closely related alkaliphile, does not have detectable titers of viral bands on an agarose gel. Recently described *Sodiomyces magadii* and *Sodiomyces tronii* (**GRUM-GRZHIMAYLO ET AL. 2015**) also seem to be free of viral bands, as well as an unrelated facultative alkaliphile – type strain of *Emericellopsis alkalina* (data not shown). This makes *S. alkalinus* thus far the only filamentous alkaliphilic fungus known to harbour dsRNA viruses.

Inability of intraspecies transfection both through hyphal anastomoses and damaged

mycelium led us to suspect a high specificity level of the viral-containing strains. An evident lack of horizontal spread of the viral dsRNA seems to be counteracted by the efficient vertical transmission, as we found that all single spore colony (both sexual and asexual) descendants did contain dsRNA fragments (**FIG. 1, D, E**). As with many described mycoviruses, we did not detect phenotypic alterations associated with the presence of dsRNA in the strains of *S. alkalinus* (**FIG. 1, TOP PANEL**). That observation led us to suspect that alkaliphilic trait in the host is not likely to be conferred by the presence of the viruses. Symptomless phenotype of viral-containing stains, efficient vertical heredity, and inability of horizontal transmission of dsRNA fragments, provides evidence for a persistent strain-specific fungus-virus association, which may enhance survival of viruses in *S. alkalinus* (**LIPSITCH ET AL. 1996**).

Four isolates of *S. alkalinus* out of 18 contain dsRNA fragments, however, the banding pattern varies among them (**FIG. 1, A**). In two strains, F11^T and F12, we detected only a single dsRNA fragment of about 6 kb size, while the other two, F13 and F18, have at least four dsRNA fragments of various sizes. And yet, the largest dsRNA fragment of about 6 kb size seems to be shared among all virus-containing strains. It should be noted that we used non-denaturing gels, therefore the migration rate will likely change with denaturing setup accounting for secondary structures of dsRNAs that may form. Difference in banding pattern may indicate the presence of different viruses among isolates, or co-existence of several types of viruses resulting in mixed infections in a single host. Another possibility includes the presence of defective/satellite dsRNAs (**GHABRIAL 1998**). Our direct transmission electron microscope examination of strain F13 extract, possessing several dsRNA bands, revealed the presence of a single type of icosahedral-shaped virus-like particles of about 35 nm size (**FIG. 1, F–K**); we did not detect capsids of another size in the same extract. Whether the dsRNA fragments are encapsidated in a single virion, or are packed in several identical capsids remains unclear.

Notably, we attempted to clone and sequence the viral fragments several times without success. Therefore without sequence information, the taxonomic affinity of the viruses in *S. alkalinus* is purely speculative. However, based on the number of nucleic fragments, nature of genetic material, isometric shape and size of the viral capsid, we may suspect they belong to either *Totiviridae* (monopartite dsRNA genome) or *Chrysoviridae* (multipartite dsRNA genome) family. In case mixed infections occur, viruses from *Partitividae* may be present as well (**GHABRIAL & SUZUKI 2009**).

Our recent phylogenetic analysis performed on the *Sodiomyces* species group has shown strains F11^T and F12 to be segregated from the rest (**FIG. 2**). Coincidentally, those two isolates were recovered from the same locality, and both bear a single dsRNA fragment, which may point to a single origin of fungus-viral association for these two isolates. The other two dsRNA-containing strains, F13 and F18, also originate from the same area, however, the presence of the dsRNA fragments does not correlate with the phylogenetic distance, as other dsRNA-free isolates are genetically identical for the ITS locus of rDNA region. The strains F11^T and F12 bearing one dsRNA molecule have larger phylogenetic distance than F13 and F18 possessing four dsRNA fragment. If we assume a single origin for the fungus-virus association in *Sodiomyces,* then we

may be seeing the loss of dsRNA fragments over time for the isolates F11^T and F12, conformed to their evolutionary distance.

Most of the research on mycoviruses has been carried out on the viruses that infect plant pathogenic fungi. Observed strong effects on the host, which are associated with the viral presence, fuel exploration in the field. Since *S. alkalinus* has not been shown to be a plant pathogenic fungus, the discovery of the cryptic viruses in it raises more general biological questions. It is intriguing to speculate on the origin of the viruses in harsh soda soils where the fungus thrives, and yet undetected effects of the viral presence on the host. Present results suggest a long fungus-virus association history, however, conversely, evidence coming from our sequence data of several loci, indicate relatively recent evolutionary history of this species (**GRUM-GRZHIMAYLO ET AL. 2013**). More convincing evidence may come from the sequence of the viral dsRNA and search for its closest homologues across available references, possibly helping to assess the evolution of the viruses in *S. alkalinus.*

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Chapter VIII

Horizontal transfer of a bacterial DD-peptidase into alkaliphilic filamentous fungi

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Abstract

A genome sequence screen of alkaliphilic filamentous fungi, *Sodiomyces alkalinus* and *Acremonium alcalophilum*, revealed the presence of an enigmatic gene. The closest homologues search list contains hits for the low molecular weight D-Alanyl-D-alanine carboxypeptidase of class C ('DD-peptidase') from various bacterial species. Our phylogenetic analysis of this gene shows that DD-peptidases from these alkaliphilic fungi group close to the homologues found in halophilic bacteria isolated from terrestrial saline or marine habitats. Fungal DD-peptidases display a high degree of divergence from bacterial homologues, however, the functional amino acids are shared among all known isolates, except for one mutation, which is unique for alkaliphilic fungi. In bacteria, the DD-peptidases are involved in the cell wall biosynthesis, facilitating cross-linking of peptidoglycan units. The detected DD-peptidase in alkaliphilic fungi had a strong prediction for possessing a signal peptide sequence, which is evidence for this protein to act exo-cellularly, or to be integrated into the plasma membrane or the cell wall. The expression of the DD-peptidase in *S. alkalinus* was both pH and culturing-mode dependent. Strong up-regulation was induced in young mycelium in a shaken liquid medium peaking at pH 8, suggesting that the gene is functional. By contrast, older mycelium grown on solid agar medium showed residual expression at every pH tested. In bacteria, the DD-peptidase is inhibited by beta-lactam antibiotics causing cell wall disruption, which was not the case for the filamentous fungus *S. alkalinus* having no effect of penicillin on the growth, possibly but not exclusively suggesting an alternative substrate for the newly acquired gene. We hypothesize a horizontal transfer event of the DD-peptidase gene from bacteria thriving in soda soils to the fungus, in which it may have been fixed due to advantageous effects in dealing with alkaline/saline conditions or degrading the cell wall of prokaryotic competitors.

Key words

Horizontal gene transfer, DD-peptidase, prokaryotes, extreme habitats, *Sodiomyces alkalinus*, *Acremonium alcalophilum*

Introduction

Horizontal gene transfer (HGT; also known as lateral gene transfer) is termed as a relocation and stable integration of genetic material from one genome to another in the course of evolutionary history (**DOOLITTLE 1999**). Contrary to vertical gene transmission, as a typical way of gene transfer from an ancestor to a descendant, HGT is not foreseeable and appears to be spontaneous through mechanisms which are not fully understood. In addition, there are difficulties associated with the confident detection of such events, especially if the HGT occurred between closely related eukaryotic organisms (**FITZPATRICK 2012**).

There is a growing body of evidence that fungi are capable of acquiring genes from both prokaryotic and eukaryotic donors (**ROS & HURST 2009, SLOT & ROKAS 2011**). Detection of these events

in fungi is largely based on an increasing number of whole-genome sequencing projects and a cautious bioinformatics pipeline involved. Among confirmed cases of HGT into fungi, many involve a transfer from a prokaryotic donor (**GARCIA-VALLVE ET AL. 2000, FITZPATRICK ET AL. 2008, DUAN ET AL. 2009**). Not only the detection of prokaryotic genetic signatures is computationally easier on the eukaryotic genome background, but also there have been biological reasons postulated for what makes a prokaryote-to-eukaryote HGT likelier rather than eukaryote-to-eukaryote (**FITZPATRICK 2012**). First, the presence of introns in eukaryotic genes may act as a barrier for transfer, as incorrect splicing in a new recipient would result in a non-functional product and be selected against. Second, the taxa richness of prokaryotes gives more possibilities for HRT and selection to work upon. Lastly, the operon-based genome structure in bacteria may promote HRTs which result in the acquisition of the whole metabolic pathways by a single transfer event (**KEELING & PALMER 2008**).

It remains controversial how frequent and important HGT events are in fungi. However, there is a growing realization that HRTs are actually happening at a higher magnitude than originally thought (**BRUTO ET AL. 2014**). Expression and functionality of newly acquired genes remain important issues before conclusions on the evolutionary impact can be drawn, as some horizontally transferred genes show residual expression and may represent a background noise (**ROS & HURST 2009**). Others though have been demonstrated to have conferred beneficial functional capabilities for a fungus and be essential for niche adaptation (**KEELING & PALMER 2008**). For example, as demonstrated by **DUAN ET AL. (2009)**, a HGT of the phosphoketolase (Mpk1) of bacterial origin into the entomopathogenic fungus *Metarhizium anisopliae* is a necessary constituent for its virulence against insects. The authors show that the enzyme is adapted to insect haemolymph and required for the fungus to fully function as an insect pathogen. Furthermore, after acquiring from bacteria, the *mpk1* gene has been diverged into two superfamilies showing patchy distribution in other fungal lineages. Another study highlights several genes of bacterial origin in *Saccharomyces cerevisiae* that enable facilitated anaerobic growth and an ability to utilize sulfate from several organic sources previously not available for the *S. cerevisiae* lineage (**HALL ET AL. 2005**). Another noteworthy example includes HGT of many (if not all) glycosyl hydrolases from bacteria into rumen fungi, a peculiar group of anaerobic chytridiomycetes (**GARCIA-VALLVÉ ET AL. 2000**). Acquired enzymes are believed to allow these fungi to flourish in a new environmental niche – the anoxic rumen of herbivorous mammals with abundant cellulose and hemicellulose content, a primary target for those hydrolases.

Above noted examples display the belief that some ecologically specialized organisms are rich in horizontally transferred genes (e.g., **SCHÖNKNECHT ET AL. 2013**). This suggests that genes that allow such adaptations are among the most commonly acquired. As stated before, the exact mechanisms of HGT are poorly understood, however, it is clear that physical intimate and continuous association of microorganisms greatly enhances the chances for gene transfer (**KEELING & PALMER 2008**). The case we describe in the present study echoes the given above notions. The genomes of two sister alkaliphilic filamentous fungi, *S. alkalinus* and *A. alcalophilum*, contain a gene encoding for a DD-peptidase of bacterial origin, as its taxonomic reconstruction results

in a strong phylogenetic incongruence with a species phylogeny. The located DD-peptidase is more related to the homologues coming from halophilic bacteria isolates from marine and salty habitats. In *S. alkalinus*, we showed this DD-peptidase to be differentially expressed depending on the pH of the medium and mode of incubation. The maximum expression was observed at pH 8 at shaking regime in liquid medium, whereas only residual expression was shown on agar plates. We speculate on the possible functions of this DD-peptidase including the protection against highly alkalinity and salt stress or contributing to the defense ability against dense prokaryotic communities thriving in soda lakes.

Materials and methods

Strain, media, and growth

In the current work we used the wild ex-type strain F11 (= CBS 110278^T) of *S. alkalinus*, an alkaliphilic filamentous fungus. Routine propagation was done on alkaline agar (AA) medium, composition of which is given in **GRUM-GRZHIMAYLO ET AL. (2013)**. For gene expression analysis at various pH, the strain was grown both in liquid shaking regime and on agar (20 g/l) plates at 28 °C in the dark. The media were based on different 0.1 M buffers generating the following pH values: 5.2 (citric buffer); 5.9–7.8 (phosphate buffer); 8.7–10.5 (carbonate buffer); and, finally, Na_2HPO_4/NaOH buffer was used to create pH 11.4 for the agar plates. All the media were based on malt and yeast extracts: malt extract (Merck) – 17 g/l, yeast extract (BBL) – 1 g/l. The mycelium was collected on 2 d from liquid medium or after 5 d from agar plates for subsequent RNA extraction. For the penicillin effect experiment, we added penicillin G potassium salt (Sigma) to the liquid media at pH 7, 7.8 and 8.7 to a final concentration of 100 µg/ml. The dry biomass of *S. alkalinus* was measured after 40 h of growth on an orbital shaker (220 rpm) at 28 °C. The penicillin G effect experiment was performed in ten biological replicates.

Genome screening

We located the DD-peptidase gene in the genome of both *S. alkalinus* and *A. alcalophilum*, sequenced, assembled and annotated by the Joint Genome Institute (DOE JGI, Walnut Creek, CA, USA). Both genomes are publicly available at **HTTP://GENOME.JGI.DOE.GOV/SODAL1/SODAL1.HOME.HTML** and **HTTP://GENOME.JGI.DOE.GOV/ACRAL2/ACRAL2.HOME.HTML**.

RNA extraction, cDNA synthesis and qRT-PCR

For the total genomic RNA extraction we used RNeasy Plant Mini Kit (Qiagen Inc., CA, USA). Quantity and quality of the obtained RNA was verified on a NanoDrop 2000 (Thermo Scientific Inc., DE, USA). Approximately 1 µg of RNA was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. Quantitative reverse transcription PCR (qRT-PCR) reactions were performed in a CFX96 thermocycler (Bio-Rad) and contained 2x SYBR Green SuperMix (Bio-Rad), 0.2 M of each primer, and 1.5–15 ng of cDNA in a total volume of 8

µl in technical triplicates. The annealing/extension temperature was set to 61 °C, and the most efficient reaction profiles were used to calculate the relative expression with $^{\Delta\Delta}C_t$ approach. Baseline threshold level was set automatically by the CFX Manager v. 2. 0 (Bio-Rad). The results were normalized to the expression of the house-keeping gene, actin (*act1*), at pH 5.2. We used the following primers to assess the expression of the DD-peptidase gene and *act1* genes (5' -> 3'): *S13_1f* GGG TGG AGA CAG TGG ATG GA (T_m = 63.1 °C), *S13_1r* TGC GGT TAT GTA GGA GAA GGT TG (T_m = 62.5 °C), *act1_f* CCC AAG TCC AAC CGT GAG AAG ATG (T_m = 64.4 °C), *act1_r* CGA CCA GAC GCG TAC AGA GAC AGG (T_m = 67.8 °C).

In silico analysis of the DD-peptidase

In silico sequence analyses were done with FGENESH HMM-based gene structure prediction (**SOLOVYEV ET AL. 2006**). For signal peptide prediction we used online SignalP 4.1 Server (**PETERSEN ET AL. 2011**).

Phylogenetic inference of the DD-peptidase

Closest homologues of the DD-peptidase were obtained from GenBank via BLAST search. The collected set of amino acid sequences was aligned with online MAFFT v. 7 (**KATOH & STADLEY 2013**) using the automatic alignment strategy. The resultant matrix was inspected manually and ambiguously aligned areas and large gaps were cut using BioEdit v. 7.1.3.0 (**HALL 1999**). Bayesian phylogenetic inference was done using MrBayes v. 3.1.2. (**HUELSENBECK & RONQUIST 2001**) with a mixed-model setting, set to run for 1 M generations sampling every 100th tree. First 50 % of the unstable set of trees was discarded as burn-in, and the rest were summarized to produce a 50 % majority rule consensus tree. Maximum likelihood analysis was performed in GARLI v. 2.0. (**ZWICKL 2006**) with WAG+I+G amino acid substitution model. Each of the ten bootstraps was set to have five searches. The best bootstraps were summarized with Sumtrees v. 3.3.1 application in DendroPy v. 3.11.0 package (**SUKUMARAN & HOLDER 2010**). The branches were considered strong and displayed thickened if they received joint supports ML > 90 and PP > 0.94.

Results

In *Sodiomyces alkalinus*, the DD-peptidase spans the positions 17729–19186 in scaffold 6, as assembled by the JGI, at the time of writing. This gene was also located spanning at 162840–164276 of scaffold 11 in a closely related alkaliphile, *Acremonium alcalophilum*. We identified a single ORF 1458 nucleotides long (**FIG. 1, A**), encoding a 485 aa protein in *S. alkalinus*, which shares 80 % identity with its homologue from *A. alcalophilum*. Curiously, the next best homology hit (56 %) comes from the soil-borne opportunistic human pathogen ascomycete *Scedosporium apiospermum*, specifically strain IHEM 14462 sequenced recently, isolated from a human sputum (**VANDEPUTTE ET AL. 2014**). There are no more known homologues of this protein coming from other fungi, making these three species unique holders among all sequenced and annotated fungi thus far. Our phylogenetic analysis firmly (100/1.0) groups three fungal DD-peptidases together

(**FIG. 1, B**). On the phylogenetic tree, sister bacterial species are spread across many families, the majority of which belong to actinomycetous bacteria (genera *Actinomadura*, *Amycolatopsis*, *Kutzneria*, *Kribbella*, *Nocardiopsis*, *Streptomyces*, and others). Notably, DD-peptidases from fungi are more related to the homologues found in halophilic bacteria, which were recovered from marine or terrestrial salty habitats (**FIG. 1, B**). A strong phylogenetic incongruence between the DD-peptidase and species phylogenies (**GRUM-GRZHIMAYLO ET AL. 2013**) leads us to treat the DD-peptidase as a result of HGT into *S. alkalinus*, *A. alcalophilum* and *Sc. apiospermum* from bacteria.

The alignment of the bacterial DD-peptidases to its homologues in alkaliphilic fungi showed a complete match for all three known groups of functional amino acids, except for one substitution mutation (**FIG. 1, C**). The substitution of serine to threonine comes exclusively from two alkaliphilic fungi at the position 315 for *S. alkalinus* (position 317 for *A. alcalophilum*), and might lead to the formation of the modified catalytic cavity altering the function of the enzyme. *In silico* analysis of the fungal DD-peptidases revealed the presence of a signal peptide, indicating that the protein is likely to be destined into the secretory pathway, ultimately secreted from the cell or incorporated into the plasma membrane (**RAPOPORT 2007**). The cleavage site for the signaling proteolysis was predicted to be the VAG|QA, at the N-terminal positions 19–20 of the DD-peptidase in *S. alkalinus* (**FIG. 1, D**). Our expression analysis shows the gene to be functional in a fungal host, yet only strongly expressed in shaking incubation regime. As seen in **FIG. 1 (E)**, the up-regulation of the DD-peptidase gene starts at pH 7, and rapidly increases culminating at pH around 8 with the 27-fold increase, as compared to the actin house-keeping gene reference. The expression of the DD-peptidase gene subsides as the pH further goes up. Strikingly, the gene shows only residual expression in *S. alkalinus* growing on agar plates, across all the pH range tested. The expression pattern of the DD-peptidase gene was shown to be similar using an alternative primer pair (data not shown). Coupling above mentioned results together, we suspect the DD-peptidase to be involved in creating a local favorable milieu just outside the fungal cell effectively washed away by shaking, which in turn promotes compensatory over-expression.

Fungi do not possess peptidoglycan cell-wall architecture, an ultimate target for bacterial DD-peptidases. As the bacterial DD-peptidases can be inhibited by beta-lactam antibiotics, we sought to see the possible inhibition effect of penicillin G on the growth of *S. alkalinus*. However, incubation in liquid with 100 μg/ml of penicillin G at the pH, which induces different levels of the DD-peptidase gene expression, did not detect a distinct effect on biomass accumulation, as compared to penicillin-free control (**FIG. 2**). Although there is a marginal statistically significant difference at pH 7.8 ($p=0.045$, Student's t test, $n=10$), we do not consider this result biologically meaningful. No penicillin effect was also observed on agar plates (data not shown). This experimental setup does not allow us to draw a firm conclusion on the penicillin-enzyme interaction, as several scenarios are still possible which we address in the discussion section.

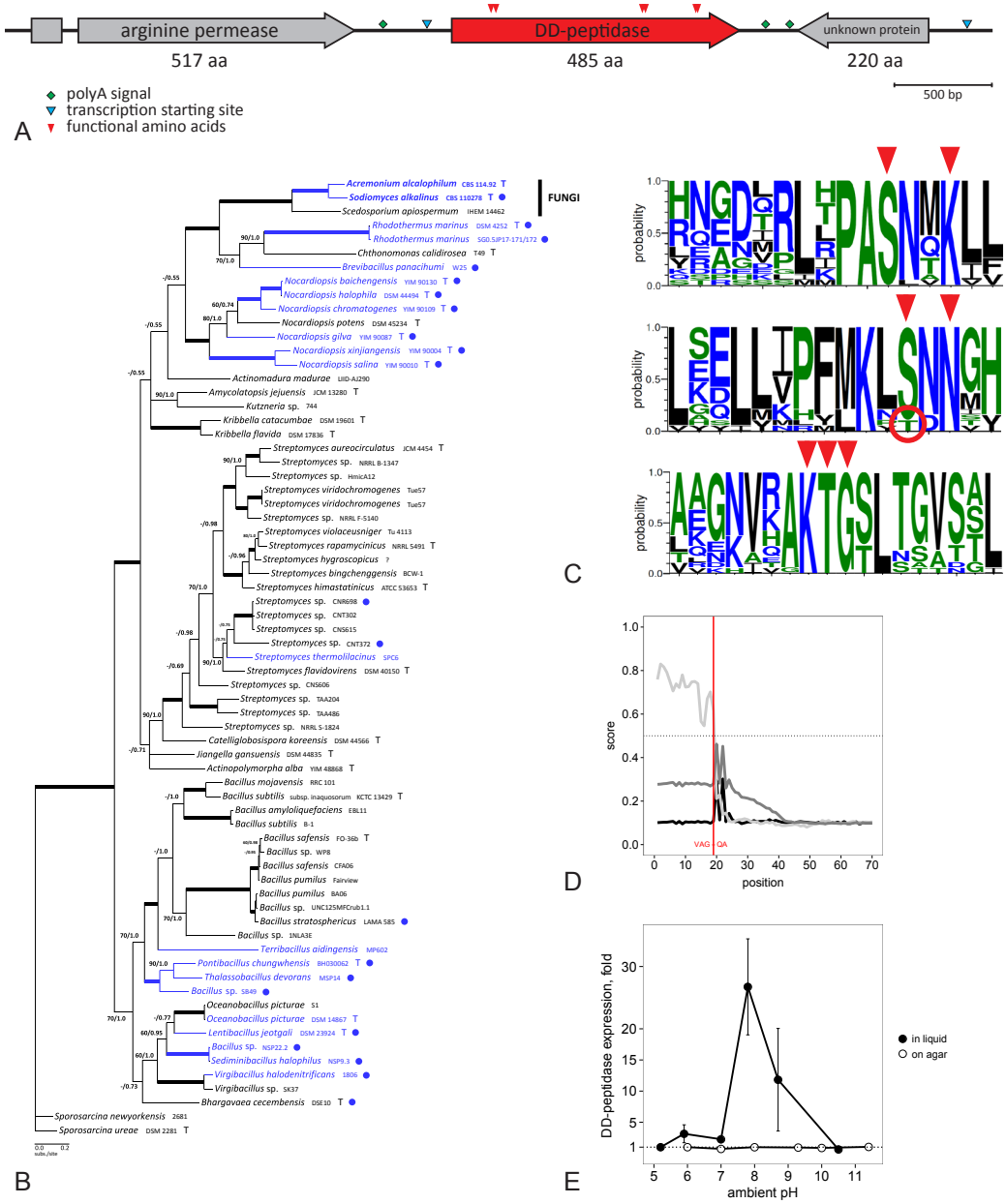

FIG. 1. Bacterial DD-peptidase in alkaliphilic fungi. **A.** Genomic map of the DD-peptidase gene area in *S. alkalinus*. **B.** Phylogenetic position of the DD-peptidase in alkaliphilic fungi (bold) within the closest bacterial homologues available. Halophilic/alkaliphilic isolates are marked in blue. Blue dots indicate isolates recovered from marine/saline habitats. **C.** Conservation of the DD-peptidase catalytic sites (arrowed). Circled amino acid comes from alkaliphilic fungi. **D.** Signal peptide prediction on the N-terminus and its cleavage site. **E.** Expression of the DD-peptidase in mycelium grown in liquid or agar cultures.

Discussion

The genome screens resulted in the detection of what appears to be a horizontal transfer of a gene encoding a low-molecular mass class C DD-peptidase homologue from bacteria into two closely-related alkaliphilic fungi, *Sodiomyces alkalinus* and *Acremonium alcalophilum* (**FIG. 1, A**). In bacteria, this DD-peptidase is a member of a larger set of enzymes, called penicillin-binding proteins (PBPs). PBPs are a family of enzymes of common evolutionary origin that catalyze various reactions of the cell wall biosynthesis in bacteria (**VOLLMER & BERTSCHE 2008**), and usually are inhibited by beta-lactam antibiotics (**ZAPUN ET AL. 2008**). Based on the functions and domain structure, the PBPs are divided in two classes, the high-molecular mass (HMM) and low-molecular mass (LMM). The HMM PBPs are multi-modular enzymes catalyzing peptidoglycan polymerization and essential for growth, while the LMM PBPs are non-essential and act as DD-transpeptidases, DD-carboxypeptidases or endopeptidases, promoting crosslinking between the peptidoglycan polymers (**VAN HEIJENOORT 2011**). The LMM PBPs are further subdivided into classes A, B, and C by the clustering as inquired from the evolutionary trees and may have functional significance (**PRATT 2008**). It is believed that LMM PBPs of class C catalyze DD-carboxypeptidase or endopeptidase reactions *in vivo* (**NEMMARA ET AL. 2011**). At least five HMM and eight LMM PBPs (PBP4, PBP4b, PBP5, PBP6, PBP6b, PBP7/8, AmpC, AmpH) have been found in *E. coli*, with deviations existing in other bacteria (**SAUVAGE ET AL. 2007**). Strikingly, the deletion of all LMM PBPs results in still viable cells, however with detectable morphology alterations. Various deletion combinations point out particular importance of PBP5 in determining cell shape (**VAN HEIJENOORT 2011**). In similar fashion, the PBP4 homologues in *Bacillus subtilis* (PBP4a) and *Neisseria gonorrhoea* (PBP3) also are dispensable (**SAUVAGE ET AL. 2005**). Bacteria that lack obvious DD-peptidase homologues (**GHOSH ET AL. 2008**) strengthen the evidence for LMM PBPs not to appear as essential determinants for cell growth. There is a considerable variation in the modes of action and spatial locations where these enzymes work, therefore it is purely a speculation about the function of DD-peptidase in a new fungal host, as enzymes of this type were not detected in fungi before. Previous studies report LMM PBPs of class C to be either soluble enzymes, or loosely attached to the plasma membrane by the C-terminal amphiphilic domain or by electrostatic interactions (**PRATT 2008**). There is evidence that PBP4 in *E. coli* is bound to the membrane through a protein intermediate, possibly MtlB lytic transglycosylase (**VON RECHENBERG ET AL. 1996**). The DD-peptidase of *S. alkalinus* has a signal peptide on the N-terminus (**FIG. 1, D**), however the hydrophobicity analysis indicates no signs for the transmembrane domain (data not shown), providing similar evidence for this protein to be either soluble exo-cellularly, or loosely bound to the outside of the plasma membrane, or anchored via a protein intermediate.

A truly exciting puzzle is to assign the function of the DD-peptidase in alkaliphilic fungi. HGT events naturally raise a question about whether the gene is functional or not in a new host. Our qRT-PCR analysis confirmed that the gene is differentially expressed at different ambient conditions (**FIG. 1, E**). *Sodiomyces alkalinus* growing in liquid culture on an orbital shaker has a strong up-regulation (27-fold) of the DD-peptidase at pH 8. The expression fades away as the pH further increases. Strikingly, the incubation mode of the fungus radically affects the expression

profile of the DD-peptidase, as stationary mode of incubation on solid agar plates induced only residual expression. It appears that growth in a liquid shaken culture, being more stressful, reinforces the need for the DD-peptidase. It is tempting to speculate that this DD-peptidase may be involved in the creation of the local favorable milieu just outside the fungal hyphae, protecting the fungal mycelium from the harsh environmental conditions. Thus, growth of the fungus at high pH in a liquid shaken culture, which promotes washing-off of the DD-peptidase from the hypha surface, may have induced strong expression in a compensatory manner. The favorable milieu might be a local drop in pH, as compared to the bulk of ambient environment. In growth experiments, we detected pH to decrease by about 1 unit at the end of the longer liquid incubation (for several days), while on plates with an indicator dye we did not see pH change (data not shown). These results support the idea about the local favorable environment being synthesized by *S. alkalinus*. The mucous substance developed around the spore heads or in the fruiting bodies of *S. alkalinus* (**GRUM-GRZHIMAYLO ET AL. 2013**) might be accounted by the DD-peptidase activity. However, the homology principle fails to explain the function, as the cell-wall components of fungi markedly differ from those of bacteria. The fungal cell wall lacks the D-ala-D-ala substrate for utilizing the DD-carboxypeptidase or DD-transpeptidase enzymatic reactions. However, endopeptidase activity seems probable, as some bacterial LMM PBP DD-peptidases have been shown to catalyze such reactions (**SAUVAGE ET AL. 2005**). Although we should not exclude the possibility of shifting the DD-peptidase mode of action to execute an alternative function, known as the exaptation event (**GOULD & VRBA 1982**). Evidence for a possible shift in a function for the fungal DD-peptidase comes from the alignment of its catalytic sites. As seen in **FIG. 1, C**, the functional amino acids match those from bacteria, except for the S/T substitution (circled), which is found only in *S. alkalinus* and *A. alcalophilum*. This mutation might change the way the catalytic cavity of the enzyme is folded, resulting in preference towards an alternative substrate.

The uniqueness of the DD-peptidase gene found in alkaliphilic fungi biases us to anticipate this enzyme to be involved in tolerating stressful environment encountered in soda soils. The phylogenetic reconstruction of the fungal DD-peptidases and their closest homologues available thus far, gives us more compelling reasoning to suspect that. The phylogenetic tree in **FIG. 1, B** shows the fungal DD-peptidases to be more related to its homologues from the bacteria isolated from saline or marine environment (marked by blue dots), which often coincides with the halophilic phenotype of those organisms (taxa marked in blue). Recently, a third fungal DD-peptidase appeared coming from *Scedosporium apiospermum* IHEM 14462, as seen on a tree grouped with alkaliphilic fungi. Strikingly, this fungal strain is a human pathogen and was recovered from a sputum sample of a cystic fibrosis patient (**VANDEPUTTE ET AL. 2014**). *Scedosporium apiospermum* is a rather common soil-borne fungus, but to our knowledge, was never recovered from saline/alkaline environments. Such unexpected relatedness of bacterial DD-peptidases of alkaliphilic fungi and a human pathogen might indicate a broader and yet undiscovered distribution of this gene in fungi occupying various ecological niches.

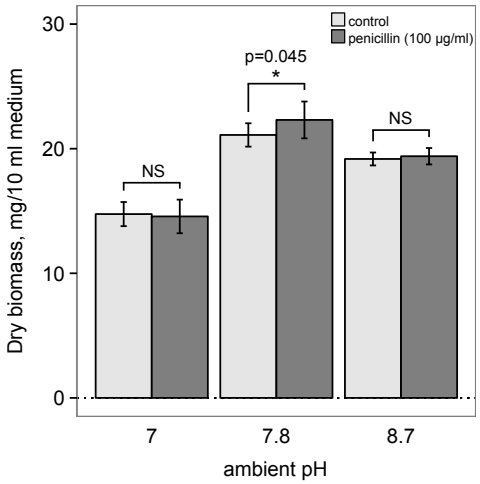

FIG. 2. Penicillin G effect on the biomass accumulation of *S. alkalinus* grown at different pH levels corresponding to various expression levels of the DD-peptidase. Mean comparison with Student's *t* test (n=10). * – $p < 0.05$, NS – nonsignificant.

Based on the assumption that beta-lactam antibiotics can inhibit the action of the DD-peptidases, we sought to test whether we can see the detrimental effects for the fungal growth caused by penicillin G, potentially effectively mimicking a phenocopy of the DD-peptidase knockout in *S. alkalinus*. We tested the penicillin G effect in the conditions at which the DD-peptidase gene expresses at various levels in liquid culturing, from low at pH 7 to high at pH 8–9. However, even though marginal statistically significant difference in the biomass accumulation was observed (**FIG. 2**), we conclude that penicillin G has very little effect (if any) on the growth of *S. alkalinus*. This biomass accumulation experiment by all means does not account for all the possible outcomes of the penicillin-enzyme interactions. If we assume the inhibition of the DD-peptidase by the penicillin, it is possible that the enzyme is non-essential for the growth, which is an unlikely scenario as it turns the horizontal gene transfer event unimportant for the fungus in the first place. Alternatively, the inhibition of the enzyme might cause a compensatory over-expression of the DD-peptidase, which results in unnoticeable phenotype. One of the other explanations may also be a low affinity of the penicillin to the active site cavity, leaving the enzyme largely unaffected. Surely, a gene knockout would be the best alternative to study the DD-peptidase gene function. As noted before, we suspect this gene to be involved in the building of some sort of external protection to the fungus against salt or alkaline stress. In fact, a study by **PALOMINO ET AL. (2009)** showed that PBP4 contributes to the halotolerance in *Bacillus subtilis*. And our hypothesis is additionally supported by the recent study showing that one of the LMM PBPs is required for halotolerance of the *Vibrio cholera*, a pathogen that causes cholera, and was particularly sensitive to Na^+, rather than other osmotic challenges (**MÖLL ET AL. 2015**). This discovery makes the DD-peptidase a very promising attribute potentially involved in salt or pH tolerance in the studied filamentous alkaliphilic fungi. Although the function of the detected DD-peptidase in alkaliphilic fungi can turn up in another way. As **EICHINGER**

ET AL. (2005) report in their study, a social amoeba *Dictyostelium discoideum* has acquired a DD-peptidase homologue (encoded by the *pscA* gene), presumably to help degrading bacterial cell walls. Acquisition of such a tool for *S. alkalinus* can be extremely advantageous against massive prokaryotic communities known to harbor harsh soda soils, where the fungus was recovered. In the future experiments, the obtained DD-peptidase deletion mutant should be tested not only for the pH and salt tolerance, but also for the alternative phenotypic alterations like defects in mucous production around the spore heads, or possible effects targeting the cell wall structure. Insight on the function of the DD-peptidase may be provided by the competition experiments with bacteria.

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Chapter IX

Discussion

The biota of saline soda lakes includes various functional groups of bacteria and archaea that are addressed in a number of studies. However, the fungal component of the soda lakes biota has not gained attention probably because fungi for a long time were not suspected to thrive at highly alkaline pH. In addition, isolation methodology in favour of recovery of prokaryotes over eukaryotes may have introduced a bias. Although fungi were isolated from alkaline habitats, such as limestone caves (**NAGAI ET AL. 1998**), no research has been performed on the fungal diversity of saline soda lakes. In the recent study by **ANTONY ET AL. (2013)**, the authors detected the presence of fungal 18S rRNA sequences obtained from the Lonar Lake of India. However, this finding has not yet been followed up with the recovery of these fungi. To my knowledge, there is no more information on the filamentous fungi living at the alkaline lakes. My thesis aimed to search for fungi in saline soda soils and validate the notion that fungi are capable of surviving high ambient pH.

Alkaliphilic fungi do exist

I was at first interested in the diversity of fungi from soda soils that are adapted to this harsh environment, i.e. display (optimal) growth at high pH. To address this, we sampled alkaline soils at the edge of soda lakes throughout Middle Asia and Africa. For initial isolation we used alkaline selective medium buffered at pH about 10 that favored the recovery of alkaliphilic and alkalitolerant species. Our first recovery attempts were hampered by a strong bacterial domination on the agar plates, which led us to use an exceptionally high amount (2 g/l) of the antibiotic rifampicin to restrict abundant prokaryotic growth and let tentative fungi to appear. A surprising array of filamentous fungi emerged which we then purified and deposited in public collections for long-term preservation. **CHAPTER 2** assesses a fungus that appeared from all our soda soils samples across Middle Asia and Africa – *Sodiomyces alkalinus*. Morphological details of its life cycle were studied thoroughly and reconstructed as shown in **FIG. 1**. I tested its linear growth rates at various pH values and it displayed a rare obligate alkaliphilic phenotype. Less stringent – alkalitolerant – type of physiology appears to be far more common and can be encountered in various neutral types of soil (**VAN DE GOOR & DEN HARDER 2013**). To better illustrate and put a unique obligate alkaliphilic phenotype into perspective, I later performed the same growth test on agar plates, with the neutrophilic *Aspergillus nidulans* as a reference (**FIG. 2**). On the top panel, the neutrophilic phenotype of the *A. nidulans* is strongly contrasted to that of *S. alkalinus* on the bottom panel, which no longer grew at pH 5 and had optimum at pH around 9–10. Aberrant yellow sporulation of the *A. nidulans* is due to the inaccessibility of copper required for laccase activity during the development of the green conidial pigment (**CLUTTERBUCK 1972**). This deficiency is a result of low solubility of certain metals at high pH. As pH decreases, the green colour of the spores is restored because more metals are chemically accessible in the medium. An extra trace of copper added to the medium at pH 7 produces normal green phenotype, as shown in the inset (**FIG. 2**).

Initially, *S. alkalinus* was described as *Heleococcum alkalinum* (*Hypocreales*) based on morphological characters such as acremonium-type sporulation and details of the sexual stage

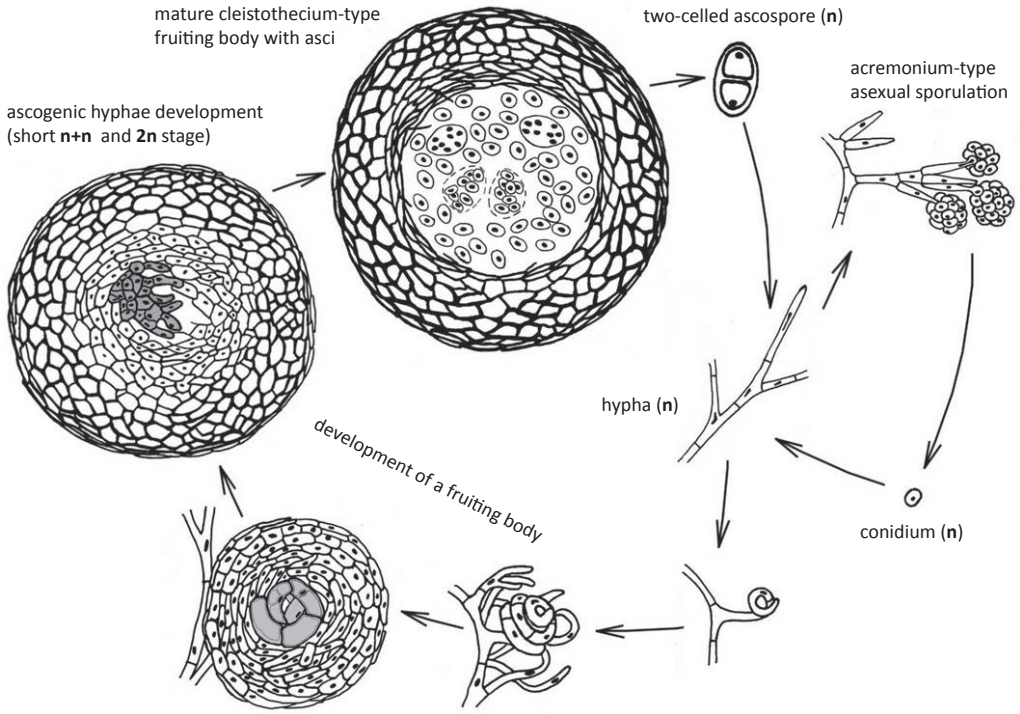

FIG. 1. Life cycle of *Sodiomyces alkalinus*, adapted from **KOZLOVA (2006)**.

(**FIG. 1**). The new isolates were added to the genus *Heleococcum* in a rank of new species, which seemed appropriate given the morphological criteria for this genus (**BILANENKO ET AL. 2005**). However, this affiliation was reconsidered after the employment of DNA-based information. I used several loci often used in phylogenetic reconstructions to show its relation to a known asexual alkaliphilic filamentous fungus – *Acremonium alcalophilum*. The only known isolate of this fungus (CBS 114.92^{T}) was recovered from sludge of pig feces compost (presumably alkaline) in Japan, and described by **OKADA ET AL. (1993)** as a new species in the asexual *Acremonium* genus – one of the most troublesome genera from a fungal taxonomy perspective. Acremonium-type morphology was established by **GAMS (1968)** to accommodate one of the simplest morphological types of sporulation. Many species have been described over the last decades, and they had subtle species-specific differences in the morphological details, which pose a substantial problem for proper identification for a non-experienced researcher. DNA-based information proved that acremonium-type asexual sporulation is characteristic to many fungal lineages, i.e. is polyphyletic. The latest phylogenetic overview of the acremonia fungi performed by **SUMMERBELL ET AL. (2011)** illustrates the problem well. Our taxonomic research on the *S. alkalinus*

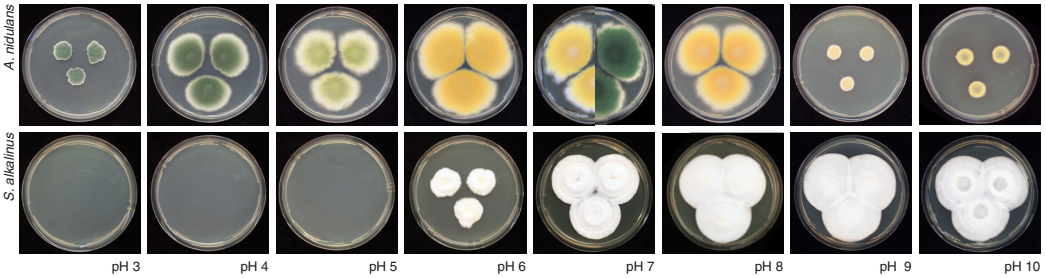

FIG. 2. Growth profiles of the neutrophilic *A. nidulans* and alkaliphilic *S. alkalinus* at various pH. The inset on the right of pH 7 demonstrates the phenotype of *A. nidulans* on the medium supplemented with traces of heavy metals.

is a clear example of how convergent acremonium-type morphology (even coupled with the presence of a sexual stage) may mislead and result in false identification. DNA information becomes essential not only for proper identification but additionally for assessing the evolution of a given isolate.

Alkaliphily in fungi is polyphyletic

Our phylogenetic analyses placed *S. alkalinus* in the family *Plectosphaerellaceae*, which was introduced by **ZARE ET AL. (2007)** and accommodates well-known plant pathogens such as *Plectosphaerella* and verticillia along with several acremonia species. It is interesting to speculate on the evolution of the alkaliphilic trait in this family. On the one hand, within the *Plectosphaerellaceae*, there is a single clade of obligate alkaliphilic species, *Acremonium alcalophilum* and *Sodiomyces*, surrounded by the species that have not been tested for the alkaliphilic phenotype yet. If we assume those reference species do not display alkaliphilic capabilities, this would mean alkaliphily could evolve rather quickly in fungi. On the other hand, I have evidence for many members of this family to show at least alkalitolerant abilities, while some genera such as *Acrostalagmus* or *Chordomyces* even displayed a facultative alkaliphilic phenotype, as I describe in **CHAPTER 4**. Thus it is possible that the entire *Plectosphaerellaceae* have a predisposition to alkaliphily. In addition, every phylogenetic analysis in my thesis and other studies that deals with this family portrays it as a long-branched monophyletic clade. A long branch on a clade could reflect taxa under-sampling, which means we simply have not discovered species that would fall within this branch. On the other hand, this might also suggest a long evolutionary history of the *Plectosphaerellaceae*, perhaps concomitant with the origin of the alkaliphilic phenotype. Systematic growth experiments elucidating the pH preference of the *Plectosphaerellaceae* members would provide stronger arguments for choosing between the two scenarios. For example, our study in **CHAPTER 4** provides extra support for the hypothesis that the *Plectosphaerellaceae* is a prominent reservoir for alkaliphilic fungi – two extra species of *Sodiomyces* recovered from extremely alkaline Magadi Lake displayed obligate alkaliphilic phenotype in the lab. And several other species that display facultative alkaliphily and strong

alkalitolerance showed up in the sister clades within the *Plectosphaerellaceae*.

Alkaliphilic isolates are not restricted to the *Plectosphaerellaceae*, as I show a second prominent source ć the *Emericellopsis*-clade of hyporealean fungi (*Hypocreales*). In **CHAPTER 3**, I treated this group in more detail and showed that alkaliphilic isolates from soda soils are genetically related to the sea-borne species, such as *Emericellopsis maritima*, *E. pallida* and some others. The established genetic link between the fungi of two ecologies, marine habitats and soda soils, provides insights on the evolution of the alkaliphilic trait in the *Emericellopsis* group of fungi. It is evident that sea habitats and soda soils share abiotic conditions, suggesting that alkaliphilic trait in this group seems to have evolved from the alkalitolerant sea-borne species. The cause-and-effect relationship in the evolution is often hard to assess, we therefore should be cautious in stating that the direction of the phenotypic change was from alkalitolerant marine to soda soil alkaliphilic. In any case, we may be seeing a recent evolutionary transition, as mainly highly variable parts (introns) of the ß-tubulin sequences contributed to the delineation of the clades. **CHAPTER 4** detected alternative taxonomic locations of alkaliphilic fungi which points towards one of the most pronounced results in this thesis – alkaliphily in fungi is a polyphyletic trait. How species evolve alkaliphilic capabilities is an intriguing question, which can be addressed after the traits that govern this phenotype will have been asserted.

Mechanisms of adaptation

GOSTINČAR ET AL. (2009) argued that extremophily in fungi has evolved from a generalist physiology exerted through strong selection pressures acting on the extreme end of a phenotypic range that a given genotype can produce through plasticity. Fungi, similarly to bacteria, have to adapt and solve the similar hurdles of stress conditions posed by the environment. Therefore, a good starting point would be to presume a polygenic nature of the alkaliphilic phenotype in fungi, as it was shown for bacteria. Those assumptions coupled with the polyphyletic nature of the alkaliphilic trait suggest that there are likely several ways to alkaliphily from various starting genotypes. An intriguing question is what genes would be under strong positive selection pressure and which mutations would be fixed first and how those mutations would affect subsequent evolutionary routes on the way to alkaliphily. How stringent would these changes be, what is the degree of epistasis and what is the magnitude of mutation effects – all are interesting questions that may be addressed using experimental evolution setup and genome analyses.

Following the strategies that bacteria adopt to thrive in alkali, I could speculate on the types of adaptations that fungi may require for surviving under similar conditions. The outer surface of the hyphae is the first barrier that external factors act upon. Similarly to bacteria, I may expect the enrichment of acidic polymers in the cell wall that would contribute to the proton retention closer to the surface (**JANTO ET AL. 2011**). We noted another possible solution that might be adaptive to high pH – the mucous matrix that envelops conidia and hyphae in *S. alkalinus*. This mucous structure may act as a buffering layer and contribute to the acidification of the immediate surroundings of the hyphae. Indirect evidence supporting this hypothesis

was obtained after I used a pH indicator to reveal the acidification around the fungal colony growing on an agar plate. I could not detect any pH change outside the colony, suggesting that acidification might be very local just surrounding the hyphae without diffusion into the medium. This scenario seems reasonable if one would view the alkaliphilic fungus living in a dense community with bacteria that might benefit from the fungal acidification activity, which spreads too far outside the hyphae. Evidence for the local acidification by the exo-cellular mucous matrix was obtained when I grew *S. alkalinus* in liquid medium and detected a pH drop by about one unit after several days of incubation. This mucous matrix thus appears to be a promising factor that passively contributes to the alkaliphilic phenotype of the host.

As for active means of salt and pH homeostasis in the cell, I expect the P-type Na^+-ATPases to be highly expressed and perhaps the genome to be enriched with genes encoding those pumps. Located on the plasma membrane, these enzymes are important for the salt homeostasis, pumping toxic amounts of sodium out of the cell. Conversely, I may expect down-regulation of the P-type H^+-ATPases, to prevent leakage of vital protons out of the cell. In fungi, proton pumps are believed to be important players that extrude protons out generating a $\Delta\Psi$ across the plasma membrane, which in turn drives an array of secondary transport systems for nutrient uptake (**MORTH ET AL. 2011**). How alkaliphiles build sufficient membrane potential under alkaline pH, assuming down-regulated proton pumps, is an intriguing aspect of the bioenergetics of alkaliphilic fungi.

Fungi excrete enzymes that break various complex organic compounds and uptake simpler molecules translocating them across the cell wall and the plasma membrane using dedicated transporters. Hundreds of enzymes that degrade and modify complex carbohydrate substrates include hydrolases, transferases, lyases and some others (CAZy database, **HTTP://WWW.CAZY.ORG**). They should perform optimally at exo-cellular conditions the fungal cell is exposed to at a given moment. For example, in the genome of *S. alkalinus* we detected 318 putative carbohydrate-active enzymes. It is reasonable to suspect that functional exo-cellular enzymes involved in substrate degradation would have evolved to perform optimally at higher pH than in neutrophiles – the aspect I addressed in **CHAPTER 5**. For alkaliphilic *S. alkalinus*, I sought to show that major enzyme classes involved in cellulose and hemicellulose degradation would perform optimally at high pH. Indeed, most of the measures cellulases and hemicellulases retained activity at pH 10 for *S. alkalinus*, if compared to the neutrophilic generalist *Aspergillus oryzae* that showed no activity. Those enzymes were not performing optimally at elevated pH, but the detected increase in performance at alkaline pH might be sufficient to meet the nutrient requirements of *S. alkalinus*. Overall magnitude of the cellulolytic and hemicellulolytic activity for *S. alkalinus* was substantially lower than of *A. oryzae* after two days of incubation. This result suggests either low intrinsic capacity to degrade plant material, or slow induction of those enzymes in *S. alkalinus* possibly due to slower growth rate when compared to *A. oryzae*. Genome screening of *S. alkalinus* showed a reasonably high number of genes encoding cellulose-degrading enzymes (**CHAPTER 5**). Good growth on pure cellulose (**CHAPTER 2**), and the fact that the closest species, *Acremonium alcalophilum*, was demonstrated as cellulolytic (**OKADA ET AL. 1993**) supports the hypothesis that *S.*

alkalinus potentially is capable of exhibiting potent cellulases. Longer incubation times followed by the enzyme assays are needed to substantiate this claim. Quite unexpectedly, *S. alkalinus* is capable of quick and strong induction of proteases that are active at pH 8–10 (**CHAPTER 5**). Previous studies suggest that it is an intrinsic property of proteases to be more active at higher pH than other enzymes, even in neutrophilic species. This aspect may help to explain the evolution of strong proteases in *S. alkalinus*, as they may have had no physiological constraints to prevent functioning at high pH. Given suspected slow induction of cellulolytic and hemicellulolytic activity and strong induction of proteases, proteins might stand out as quick carbon and nitrogen sources when available. Notably, at high pH, nitrogen is extremely limited as it turns into toxic and volatile ammonia, therefore efficient protein degradation might reflect a strong need for nitrogen in the form of amino acids. This interesting property of *S. alkalinus* raises the question what natural substrate the proteases act upon. As noted in the introductory **CHAPTER 1**, soda soils may harbour substantial amounts of small crustaceans that lay ample eggs that sink down on the sediment from which larvae develop later on. *Sodiomyces alkalinus* might efficiently utilize these protein-rich microscopic eggs to obtain nitrogen and carbon.

The idea of the traits that have pre-requisites to function well at high pH brings me to point out another peculiar observation that might help to explain the evolution of alkaliphily in fungi. As already noted before, alkaliphilic bacteria exhibit an interesting property for its exo-cellular and cell-wall proteins to have a lower isoelectric point (pI) than in neutrophilic counterparts. This is believed to be adaptive, as low pI means strong negative charge on the proteins that are exposed to high pH. Negative charge on the proteins supposedly serves to retain H^+ and Na^+ around the cell – the ions that are crucial for pH homeostasis in bacteria (**JANTO ET AL. 2011**). That idea led us to check whether something similar is going on in alkaliphilic fungi. I selected the genomes of three taxonomically unrelated fungi that exhibit different physiologies with respect to the pH factor – an acidophile, a neutrophile, and an alkaliphile. Among all the protein models predicted to be encoded in the genomes, I selected those that have a signal peptide, and therefore putatively are directed into the endosomal pathway and ultimately excreted out from the cell or, alternatively, incorporated into the plasma membrane or cell wall. I estimated isoelectric points of both subsets of proteins and plotted them as histograms (**FIG. 3**). The panel on the left shows the pI distribution of a total set of proteins encoded in the genomes, whereas the red histograms on the right depict only the fraction that possesses a signal peptide. Interestingly, in all cases there is enrichment for the signal peptide protein to have a lower isoelectric point of about 4.5. As I checked several other genomes and obtained similar distributions (data not shown), this observation seems to be a common property of filamentous fungi. Assuming that the low isoelectric point of the exo-cellular proteins is beneficial for alkaliphilic fungi, then this intrinsic property might be considered a pre-adaptation favoring the evolution of alkaliphily. Therefore, it may also suggest easier 'evolvability' of an alkaliphilic phenotype than an acidophilic phenotype in fungi.

Another way of adapting to high pH is assessed in **CHAPTER 6** where I looked into the conserved cascade that mediates a global expression pattern under different ambient pH. This

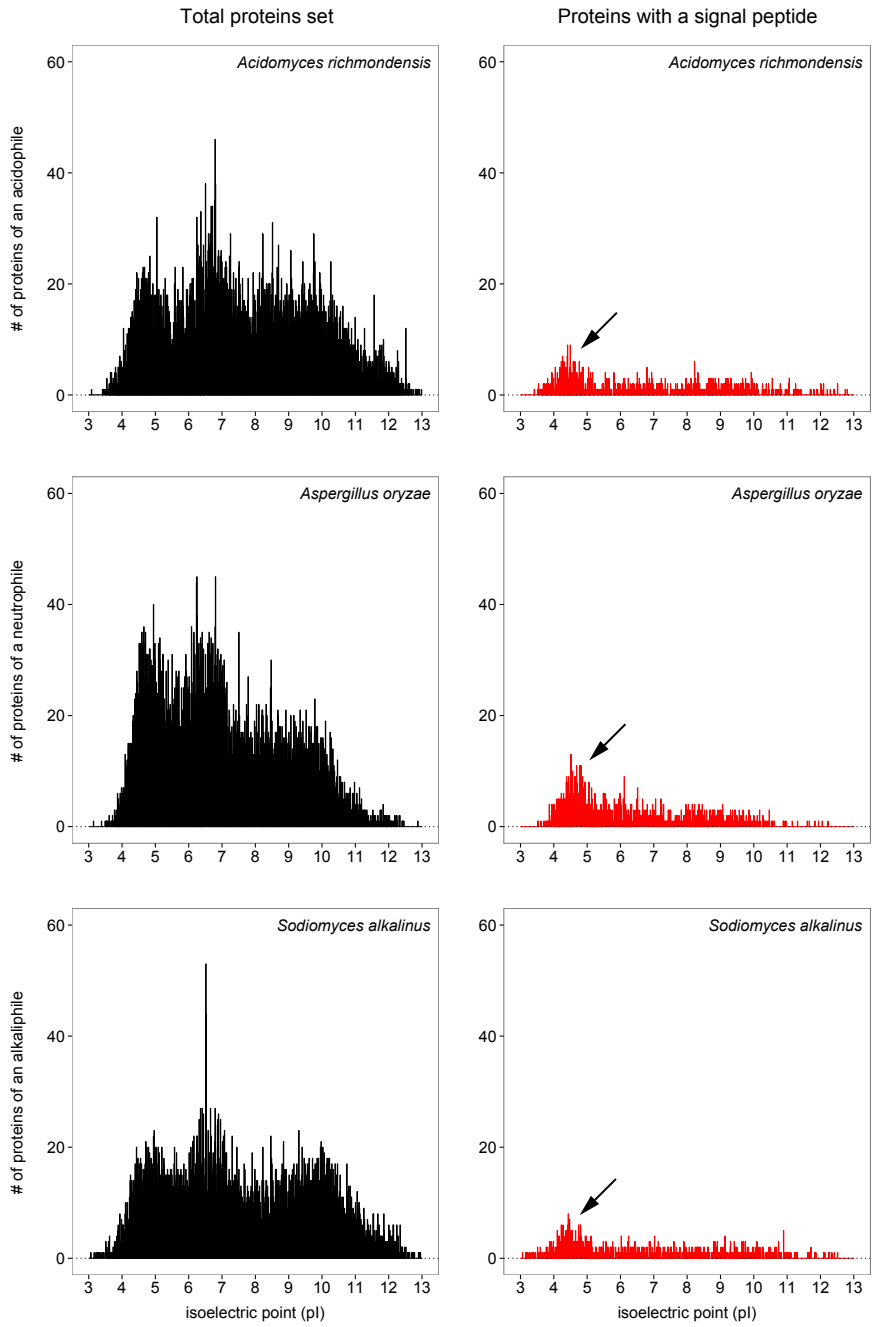

FIG. 3. Distribution of total and signal peptide proteins with respect to their isoelectric point (pI) of the three physiologically and taxonomically different filamentous fungi. Enrichments for low pI signal peptide proteins are arrowed.

cascade centers on the major transcription factor PacC that is proteolytically activated upon high ambient pH. The active PacC switches on the genes required at alkaline pH and repressed 'acid-related' genes (**PEÑALVA ET AL. 2008**). Given that *S. alkalinus* cannot grow at acidic pH (< 5.2), I sought to detect structural differences of PacC that would reinforce the alkaliphilic phenotype of the fungus. I expected to find *pacC* present in multiple copies in the genome, the hypothesis that was proven wrong. Another idea regarded the aspects of the PacC translation. Given optimal performance of the *S. alkalinus* at high pH, I sought to detect a mutation in the *pacC* gene resulting in the emergence of a premature stop-codon that would yield translation of the short/activated form of PacC. This would by-pass the need for proteolytic activation and quicker delivery of the PacC factors under extremely high pH conditions. Yet I could not detect a stop-codon and showed full translation of the PacC, in similar fashion as in neutrophilic fungi. Moreover, PacC of *S. alkalinus* has all the putative functional domains that are required for its proper processing. A global role and importance of this transcription factor may be a constraint that is maintained by purifying selection – any changes introduced in the PacC would be detrimental and selected against. Our western-blot experiment provided evidence for an alternative way of adapting to high pH without major rearrangements in the intracellular molecular cascade. I noted that the proteolytic activation of PacC takes place at higher pH values than in a neutrophilic homologue, which might be a result of the shifted threshold of the pH-receptors that trigger the cascade. Shifted sensitivity of the pH-receptors can result from a single mutation that would change the conformation of the sensor. This can be an elegant and evolutionary parsimonious solution towards regulation of gene expression under higher pH without affecting intra-cellular parts of the cascade. pH-sensors with a shifted threshold at which it triggers the downstream response seems a plausible scenario that deserves attention. Future experiments need to be done to confirm this idea. Development of the transformation protocol for *S. alkalinus* would be of great help, as then pH-sensors of neutrophilic fungi can be introduced into an alkaliphile to observe the response, and vice versa – an alkaliphilic pH-receptor inserted in a neutrophile may provide interesting insights, too.

A phenotypic trait of an organism may be affected by the presence of a closely interacting species, often genetically distant. There are many documented cases where algae, fungi, bacteria, or viruses alter the host's traits resulting in parasitism, intimate mutualistic interactions, or commensalism – a way of interaction with no evident benefit for one of the two parties. Even though sometimes phenotypic manifestations can be explained by the species interaction, I do not expect that *S. alkalinus* benefits or is compromised from having intracellular viruses I pictured in **CHAPTER 7**. Only four strains of *S. alkalinus* out of 18 we recovered from various soda lakes, harboured mycoviruses. Neither of the other closest alkaliphilic species had detectable titers of the mycoviruses, nor did the virus-containing isolates of *S. alkalinus* have detectable alterations when grown on plates. These observations suggest that alkaliphily in that lineage is not likely to be explained by the presence of intracellular viruses. However, it would of interest to determine the sequences of dsRNA these viruses possess and address their origin and evolution. Reconstructing the phylogenetics of the dsRNA may help to trace the dynamics of the

spatial distribution of the fungal host.

Another way to quickly achieve an environmental adaptation is through horizontal gene transfers (HGTs). It is postulated that prokaryote-to-eukaryote HGTs are more likely to happen for a number of reasons (**FITZPATRICK 2012**). In addition, large amounts of bacteria in physical proximity – a situation exemplified in saline soda lakes – is believed to facilitate HGTs. These are attractive arguments for finding and studying these events in *S. alkalinus*, as HGTs may contribute to its alkaliphilic capabilities. In **CHAPTER 8**, I touched upon the study of putative HGT events in *S. alkalinus* and found one promising candidate that could contribute to the host's alkaliphily. A gene that is likely introduced into the *S. alkalinus* genome from bacteria by HGT, encodes a putative DD-peptidase that is highly expressed at pH 8 in a shaking-culture regime. However, its expression trails off as the pH goes further up. This is somewhat puzzling if we are to assign this gene as one of the determinants of the host's alkaliphilic phenotype, suggesting that there are probably other means for the fungus to adapt to very high pH. The fact that this gene was located in another alkaliphile, sister species *Acremonium alcalophilum* and in one more fungus (*Scedosporium apiospermum* IHEM 14462) thus far, strengthens our hypothesis about its putative role in extreme physiological adaptations that these two fungi display. As we recovered two more species of *Sodiomyces* – *S. magadii* and *S. tronii*, it would be exciting to show whether the gene for the bacterial DD-peptidase is present in those species as well. This could be done with a PCR, however, this approach may run into difficulties because the gene is extremely variable with no conservative areas. Even stronger evidence could come if this gene would be found in other alkaliphiles, such as *Emericellopsis alkalina*. Surely, to robustly display this DD-peptidase as an alkaliphilic trait determinant, we need to construct a knockout and test it for the growth at high pH. Functional gene studies in *S. alkalinus*, as a model system for fungal alkaliphily, depend on the development of a transformation protocol for this fungus. I attempted to transform *S. alkalinus* through a protoplast/PEG-mediated transformation technique, unfortunately without success. *Agrobacterium*-mediated transformation seems a promising alternative, as various verticillia species (close relatives to *Sodiomyces*) are being routinely transformed using this technique (**JAN VAN KAN, PERSONAL COMMUNICATION**).

Future directions

Although I demonstrated the comprehensive diversity of fungi recovered from saline soda soils, the taxonomic composition is far from complete. Our sampling distribution was rather sparse and did not include many other known soda lakes across the globe. In fact, a large part of Africa, the American continents, Australia and Western Europe were not sampled at all, which are likely to contain other fungal species. Conversely, showing how the fungal taxonomic diversity is conserved throughout the continents seems an interesting endeavor. Now, with a diversity of alkaliphiles that are deposited in open public collections, we can start addressing the evolution of the alkaliphilic trait more systematically. The ongoing cost reduction of the high-throughput sequencing technologies opens up a broad array of possibilities to address many pertinent questions systematically. We made the first necessary step – high-quality sequencing of the

genome and transcriptome of *S. alkalinus*, providing an immense amount of data we have not yet fully investigated. Aspects of the genomics I portrayed in several chapters throughout this thesis are just a tip of the iceberg. To continue with the genomics approach, it seems logical to run comparative genomics analysis with a closest neutrophile or, conversely, a distant alkaliphile to study the evolution and, possibly, convergence of alkaliphilic trait. Transcriptomics analysis on *S. alkalinus* grown at various pH levels would be a good approach for detecting putative genes governing the alkaliphilic phenotype. Candidate genes can be further functionally studied with the knockouts and subsequent validation. A couple of times throughout this thesis I have pointed out the importance of the development of the transformation protocol for *S. alkalinus*. The ability to transform *S. alkalinus* would stimulate research and bring us closer towards understanding the molecular mechanics of the alkaliphilic phenotype. Another intriguing approach appears to be an experimental evolution setup in attempt to evolve the alkaliphilic/alkalitolerant capacity of a neutrophilic fungus throughout serial transfers on the alkaline medium. Conversely, can an alkaliphilic fungus lose its capacity to withstand high pH throughout the experimental evolution in a laboratory setting? Given a likely complex nature of the alkaliphilic phenotype in fungi, a combination of above noted approaches have to be utilized to render a better understanding of alkaliphily in fungi. The results in this thesis may potentially lead to the more applied direction of the future work that uses alkaliphilic fungi. I showed that enzymes of *S. alkalinus* can function at very alkaline pH – a rare property of enzymes that is of interest in certain industrial processes. The unexpected discoveries driven by scientific curiosity that were portrayed throughout this thesis reflect the power and need for fundamental research in the future.

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Summary

English summary

The presented thesis addresses aspects of biology and evolution of fungi that were recovered from saline soda soils. The work highlights the fact that saline soda soils are populated by a large diversity of fungi capable of withstanding high salts content and high pH. Some of these fungi have been shown to require exceptionally high pH and salts to grow optimally and therefore are called alkaliphiles.

Introductory **CHAPTER 1** provides examples of seemingly inhabitable environments and some of its most prominent tenants, with the emphasis on soda lakes ecosystem and alkaliphilic organisms. Aspects of physiology and major adaptive strategies to high pH and salts found in bacteria are portrayed. To our knowledge, there are no studies devoted to the fungi inhabiting saline soda lakes making this work a starting point towards further explorations in the field.

In **CHAPTER 2**, I show that fungi are actually present in saline soils and focus closely on the fungus that dominated across all our soda soils samples. This fungus displayed a rare obligate alkaliphilic phenotype – it was capable of growing at pH 11.4 on agar plates, with the optimum of around 9–10 and no ability to grow at pH 5.2. Using a combination of morphological and phylogenetic approaches, I describe it as a new name *Sodiomyces alkalinus* (previously known as *Heleococcum alkalinum*). We looked at the morphological details of its life cycle and tested for the capacity of utilizing various carbon sources. Given its unique extreme physiology, dominance across the soil samples, and partly for historical reasons, *S. alkalinus* has become our model organism that found considerable attention across this thesis.

Inspired by the fact that saline soda soils harbour new fungi, I moved on to the investigation of another set of isolates we obtained from soda soils, which belong to the *Emericellopsis* group (*Hypocreales*). **CHAPTER 3** presents an investigation of the *Emericellopsis* isolates that showed a much broader pH preference tagging them as facultative alkaliphiles. Here again, combined morphological, phylogenetic, and physiological data allowed us to set this group apart from the rest and described it as a new species – *Emericellopsis alkalina*. This species is genetically unrelated to *S. alkalinus*, which provides evidence for the alkaliphilic trait to be polyphyletic, i.e. arisen several times throughout evolutionary history. I showed *E. alkalina* to be genetically closer to marine-bourne isolates than typical terrestrial species. Such a result provides evidence for the origin of alkaliphilic trait in this group from the marine-bourne fungi, as sea and soda soils environmental factors coincide.

CHAPTER 4 is devoted to a systematic study of our whole collection of fungi recovered from saline soda soils across the world. I investigate over a hundred isolates morphologically, phylogenetically, and test them for growth pH preference. These data confirms the notion that alkaliphily is polyphyletic and has emerged in several lineages of the fungal phylogenetic tree. Detailed morphological descriptions and phylogenetic reconstructions gave me confidence in describing several more new species. A prominent finding is the discovery of two additional *Sodiomyces* species that also show the obligate alkaliphilic adaptation. Systematic approaches let me to link certain morphological characters of the species to the alkaliphilic phenotype they possess. Although a substantial part of fungi from soda soils indeed displayed alkaliphilic

capabilities, we detected typical neutrophilic species that presumably are transient or reside in a dormant state as spores or survival structures.

The next chapters of the thesis are focused on *S. alkalinus*, chosen as a model organism for studying alkaliphily that we sequenced in collaboration with Joint Genome Institute (Walnut Creek, USA). **CHAPTER 5** looks into the aspects of the hydrolytic capabilities of *S. alkalinus*. The genome and transcriptome provide immense body of data that gave insight on the enzyme sets encoded in the genome involved in the degradation of carbohydrate compounds (so-called CAZymes). Such *in silico* analysis was backed-up by the enzyme bioassays carried out at various pH and substrates. In *S. alkalinus*, I found cellulolytic and hemicellulolytic enzymes that act at high pH, as opposed to neutrophilic *A. oryzae* enzymes that were active mostly at pH 6. Another prominent finding was the detection of strong proteolytic enzymes acting optimally at pH 8. Based on the genome data and bioassays patterns, I speculate on the ecological role of *S. alkalinus* in soda soils.

CHAPTER 6 addresses the aspects of the PacC transcription factor, a key player in mediating the gene expression under different ambient pH. I sought to find differences in the primary structure of PacC or detecting the multicopiness of the *pacC* gene, given its function under extreme alkaline conditions. It turned out that the primary structure of the PacC was the same as in other fungi and the *pacC* gene is presented in a single copy in *S. alkalinus* genome. However, I noted a shifted expression and proteolytic activation pattern of PacC if compared to neutrophilic fungi. This results provides evidence for the re-tuned pH-sensors on the plasma membrane, however we could not convincingly detect signs of positive selection affecting the PalH sensors that would change its threshold to trigger the downstream molecular cascade.

CHAPTER 7 gives insights into a quite unexpected finding – the presence of viruses in several of the *S. alkalinus* isolates. I show their effective vertical but not horizontal transmission. Possession of dsRNA as genetic material, icosahedral shapes, and symptomless phenotypes are common characters for a mycovirus. The virus I studied in *S. alkalinus* exhibits these exact same features. Curiously, no other alkaliphiles from our collection nor known sister species harboured mycoviruses, making this the first instance of mycoviruses detected in an alkaliphilic filamentous fungus.

CHAPTER 8 focuses on another peculiar finding – a bacterial gene in the genome of *S. alkalinus*. Presumably introduced by a horizontal gene transfer event, this gene encodes for a DD-peptidase homologue commonly found in bacteria, but only in very few eukaryotes. I found only three fungi that possess this gene; two are alkaliphilic – *S. alkalinus* and its sister species *Acremonium alcalophilum*. This suggests the importance of this gene for alkaliphily in those species. The DD-peptidase gene appears to be functional and its peak expression was observed at pH 8. Comparative analysis showed this fungal DD-peptidase to be closely related to the homologues derived from halophilic and alkaliphilic bacteria, rather than from neutrophilic ones. I speculate on the putative function of this unusual gene, including the role in the build-up of exo-cellular matrix or defense against dense communities of prokaryotes in soda soils.

The discussion in **CHAPTER 9** contemplates on the results obtained throughout the thesis and

provides future perspectives on the topic.

Nederlandse samenvatting

Dit proefschrift behandelt aspecten van de biologie en evolutie van schimmels die zijn geïsoleerd uit soda-zout bodems. Het werk benadrukt dat soda-zout bodems een grote diversiteit schimmels bevatten die bestand zijn tegen een hoog zoutgehalte en een hoge pH. Sommige van deze schimmels bleken zelfs een uitzonderlijk hoge pH en zoutgehalte nodig te hebben voor optimale groei en worden daarom alkalifielen (houden van hoge pH waardes) genoemd.

Het inleidende **HOOFDSTUK 1** geeft voorbeelden van op het eerste gezicht onbewoonbaar ogende omgevingen en een aantal van haar meest prominente bewoners, met de nadruk op zoutmeren en alkalifile organismen. De fysiologie en de belangrijkste aanpassingen van bacteriën voor hoge pH en zouten worden beschreven. Voor zover wij weten zijn er nog geen studies gewijd aan de schimmels die voorkomen in zoute sodameren, waardoor dit werk de weg opent voor meer schimmel-gerelateerd onderzoek in het veld.

In **HOOFDSTUK 2** laat ik zien dat schimmels inderdaad in zoute gronden aanwezig zijn en beschrijf ik de schimmel die domineert in al onze sodagrond monsters. Deze schimmel toonde een zeldzaam obligaat alkalifiel fenotype – hij kan groeien tot een pH van 11.4 op agarplaten met het optimum bij ongeveer 9–10 terwijl hij niet meer kan groeien bij pH 5.2. Op basis van een combinatie van morfologische en fylogenetische kenmerken geef ik deze schimmel de nieuwe naam *Sodiomyces alkalinus* (voorheen bekend als *Heleococcum alkalinum*). We beschrijven de morfologische details van zijn levenscyclus en de groeicapaciteit op verschillende koolstofbronnen. Gezien de unieke en extreme fysiologie, zijn dominantie in de bodemmonsters, en deels om historische redenen, is *S. alkalinus* ons modelorganisme geworden waaraan veel aandacht wordt besteedt in dit proefschrift.

Geïnspireerd door het feit dat zoute sodabodems tot de ontdekking van onbeschreven schimmelsoorten leidt, ben ik een andere set isolaten uit sodagrond, die behoort tot de *Emericellopsis* groep (*Hypocreales*), gaan onderzoeken. **HOOFDSTUK 3** beschrijft het onderzoek waarin we laten zien dat de *Emericellopsis* isolaten een veel bredere pH voorkeur hebben en daarom tot de facultatieve alkalifielen behoort. Ook hier konden we door de combinatie van morfologische, fylogenetische en fysiologische kenmerken deze groep onderscheiden en beschrijven als een nieuwe soort: *Emericellopsis alkalina*. Deze soort is niet nauw verwant aan *S. alkalinus*, en toont dus aan dat de alkalifile eigenschap polyfyletisch is, ofwel meerdere keren is ontstaan tijdens de evolutie. Ik heb laten zien dat *E. alkalina* nauwer verwant is aan marine soorten dan typische soorten die op het land leven. Dit geeft bewijs dat de alkalifile eigenschap zijn oorsprong vindt in de groep van de marine schimmels, doordat de zee en sodagronden overeenkomsten in fysiologische omstandigheden hebben.

HOOFDSTUK 4 is gewijd aan een systematische studie van onze wereldwijde collectie schimmel-isolaten uit zoute sodagronden. Ik onderzoek de morfologie, fylogenie en pH voorkeur van meer dan honderd isolaten. De data die hieruit volgen bevestigen dat alkalifilie

polyfyletisch is en ontstaan is in meerdere takken van de schimmelfylogenie. Gedetailleerde morfologische en fylogenetische studies zorgden ervoor dat ik met vol vertrouwen verschillende nieuw ontdekte soorten kon beschrijven. Een interessante bevinding is de ontdekking van nog twee *Sodiomyces* soorten met obligate alkalifile eigenschappen. Door een systematische aanpak kon ik de link maken tussen bepaalde morfologische eigenschappen van deze soorten en hun alkalifile fenotype. Hoewel een behoorlijk deel van de schimmels uit sodagronden inderdaad alkalifilische eigenschappen vertoonde, vonden we ook typische neutrofiele soorten die waarschijnlijk vanuit andere plekken daar zijn beland of zich er in rust bevinden als sporen of andere overlevingsstructuren.

De volgende hoofdstukken richten zich op *S. alkalinus*, Dat was gekozen als en model organisme voor onderzoek naar alkalifilie, en waarvan we de genoomsequentie bepaald hebben in samenwerking met het Joint Genome Institute (Walnut Creek, USA). **HOOFDSTUK 5** behandelt aspecten van het hydrolytische vermogen van *S. alkalinus*. De genoom- en transcriptiesequenties geven een grote schat aan gegevens die inzicht gaven in de door het genoom gecodeerde enzymen die van belang zijn bij de afbraak van diverse koolhydraten (de zogenoemde CAZymes). Deze bioinformatica analyse was opgevolgd met enzym bioessays op verschillende substraten bij een reeks van verschillende pH waardes. Ik vond lytische enzymen voor cellulose en hemicellulose die werken bij een hoge pH in *S. alkalinus*, in tegenstelling tot de neutrofile schimmel *A. oryzae* waarvan de enzymen vooral actief zijn bij pH 6. Een andere opvallende bevinding was de detectie van proteolytische enzymen die hun optimale werking bij pH 8 hadden. Op basis van de genoomdata en de bioassays speculeer ik over de ecologisch rol die *S. alkalinus* heeft in sodagronden.

HOOFDSTUK 6 beschrijft de PacC transcriptiefactor, een belangrijke schakel in de regulatie van genexpressie in reactie op verschillende pH waardes van de omgeving. Gezien de belangrijke functie van PacC onder extreem alkaline omstandigheden, was ik gericht op het vinden van verschillen in de primaire structuur van het PacC enzym of het aantal kopieën van het gen in het genoom. Het bleek dat de primaire structuur van PacC hetzelfde is als in andere schimmelsoorten en er slechts één kopie van het gen aanwezig is in het genoom van *S. alkalinus*. Echter, vond ik wel een verschuiving in de expressie en proteolytisch activatiepatroon van PacC vergeleken met neutrofiele schimmels. Deze resultaten geven aanwijzingen dat de pH-sensoren op het plasma membraan anders zijn afgesteld. Ondanks dat vonden we geen tekenen van positieve selectie op de PalH sensors, waardoor de drempelwaarde om de moleculaire machinerie in werking te stelling zou kunnen veranderen.

HOOFDSTUK 7 geeft inzicht in een onverwachte bevinding: de aanwezigheid van virussen in verscheidene *S. alkalinus* isolaten. Ik laat zien dat de virussen zich effectief verticaal van ouder op nageslacht verspreidt maar niet horizontaal naar soortgenoten kan verspreiden. Typische mycovirussen bezitten dsRNA als genetisch materiaal, zijn icosaëdrisch van vorm en veroorzaken geen symptomen. Het virus dat ik bestudeerd heb in *S. alkalinus* heeft precies deze eigenschappen. Merkwaardig is dat geen van de andere alkalifielen uit onze collectie of bekende verwante soorten ervan mycovirussen bevatten, waardoor dit het eerste beschreven geval is van

mycovirussen in een alkalifiele filamenteuze schimmel.

HOOFDSTUK 8 richt zich op een andere interessante ontdekking: de aanwezigheid van een bacterieel gen in het genoom van *S. alkalinus*. Dit gen is waarschijnlijk binnengekomen via horizontale genoverdracht en codeert voor een homoloog van DD-peptidase dat veel voorkomt in bacteriën, maar in slechts heel weinig eukaryoten. Ik heb dit gen in slechts drie schimmelsoorten gevonden, twee ervan zijn de alkalifielen *S. alkalinus* en zijn verwante soort *Acremonium alcalophilum*. Dit suggereert dat dit gen een functie heeft voor alkalifilie in deze soorten. Het DD-peptidase gen lijkt functioneel te zijn en heeft zijn expressiepiek bij pH 8. Uit een vergelijkende studie blijkt dat de DD-peptidase uit de schimmels het meest overeenkomt met de homologe genen uit halofiele en alkalifiele bacteriën en niet met neutrofiele bacteriën. Ik speculeer over de mogelijke functie van dit vreemde gen, waaronder een rol in het maken van een extracellulaire matrix of in de verdediging tegen de vele prokaryoten die de sodagronden bewonen.

De discussie in **HOOFDSTUK 9** beschouwt de resultaten van dit proefschrift voor toekomstig onderzoek aan dit onderwerp.

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The booklet you are holding wouldn't see the light of the day without influence and contribution of many people that I encountered throughout my PhD-life. My PhD-research at Lomonosov Moscow State University (MSU) had initiated by the collaborative efforts of Elena Bilanenko and Elena Efremenko, who had agreed to assess the enzymatic potential of alkaliphilic fungi, for which I am grateful. The starting year of my Russian PhD was tightly associated with educational and teaching activities. I would like to express my gratitude to the Mycology and Phycology chair group at MSU for giving a solid knowledge of many aspects of fungi and algae. Details of fungal ecology and diversity you taught me have been continuously recurring over these years. Days passed and nothing meant trouble until one day...

"Alexey, take a look at this, you should do it" – I heard in the corridor a commanding tone of the dean, Galina Belyakova, handing over a piece of paper to me. A list contained several universities across Europe bound together by the Erasmus Mundus consortium program that allowed foreign students to jump into 'unknown' labs, do research, soak up foreign experience and bring it back to the home lab. I chose Laboratory of Genetics at Wageningen University and wrote an e-mail to Fons Debets (a person chosen purely by the charming smiling photo on the group's website) with a proposition to investigate our collection of fungi from soda soils. A theme not really being in line of the chair group research, I had little expectations for the positive response, but I got it. And that was the pivotal point that had determined my scientific direction. That said, I warmly thank Galina Belyakova, Alla Gavrina, Petra Krop for initiation and coordination of my mobility program within Erasmus Mundus consortium, and of course Fons for your open-mindedness and giving a chance to a suspicious Russian student to conduct research at Genetics.

A year of Erasmus program was perhaps the most productive year in my scientific life due to the motivating environment at the Genetics group. I thank the 'old group' Genetics led by Rolf Hoekstra. Rolf, when I invaded Genetics, you were leaving the group and, unfortunately, we did not have much time for proper discussions. However, I'll never forget the impression of you after my first presentation at the group: "That tall professor carries a lot of wisdom". After the talk, I clearly remember you asked me a single question: "What is the major stress for fungi in alkaline habitats?" That question I have been asking myself for several years to come and I'm still unsure. Thank you, Rolf, for the stimulating atmosphere at your research group and for fantastic Christmas dinners every year at your lovely house!

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Anna, Arjan, Bart, Bart, Bas, Duur, Fons, Gabriella, Hans, Klaas, Mark, Mark, Martijn, Merijn,

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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- On the biology of alkaliphilic fungi (2011)

Writing of project proposal (3 ECTS)

- On the biology and evolution of fungi from soda soils (2012)

Post-graduate courses (4 ECTS)

- Mycology & phycology; MSU, Moscow, Russia (2008, 2009)
- Introduction to R; PE&RC, Wageningen, the Netherlands (2013)

Laboratory training and working visits (4.5 ECTS)

- Enzyme assays; CBS-KNAW, Utrecht, the Netherlands (2014)

Invited review of (unpublished) journal manuscript (2 ECTS)

- Fungal Diversity: extremophilic fungi (2015)
- PLoS ONE: diversity of filamentous fungi from mangrove sediments in Brazil (2015)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics; Wageningen, the Netherlands (2013)

Competence strengthening / skills courses (5.2 ECTS)

- Scientific writing; Wageningen in'to Languages, the Netherlands (2014)
- Philosophy; MSU, Moscow, Russia (2008-2009)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- PE&RC PhD Day extremophiles; Wageningen, the Netherlands (2012)
- PE&RC Weekend for last year PhD students; Doorwerth, the Netherlands (2013)
- PE&RC PhD Day on biomimicry at Naturalis; Leiden, the Netherlands (2013)

Discussion groups / local seminars / other scientific meetings (8.3 ECTS)

- Wageningen evolution and ecology seminars; Wageningen, the Netherlands (2009, 2011-2015)
- KNVM Mycology symposium at CBS-KNAW; oral presentations; Utrecht, the Netherlands

(2011, 2013-2014)

- CBS-KNAW, Sybren de Hoog group; oral presentation; Utrecht, the Netherlands (2012)
- Experimental evolution discussion group; Wageningen, the Netherlands (2013-2014)
- Illumina HiSeq workshop; Wageningen, the Netherlands (2013)
- NIOO-KNAW; oral presentation; Wageningen, the Netherlands (2014)

International symposia, workshops and conferences (5.7 ECTS)

- ECFG11 Conference; poster presentation; Marburg, Germany (2012)
- Population genomics meeting at John Taylor lab; oral presentation; Berkeley, CA, USA (2013)
- Fungal Genetic Conference; poster presentation; Asilomar, CA, USA (2013)

Lecturing / supervision of practical's / tutorials (10 ECTS)

- Aspects of mycology teaching for students; MSU (2008-2009)
- Mycology & phycology practical course; MSU (2009)
- Molecular & evolutionary ecology; WUR (2010-2011, 2013-2014)

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