M.Sc. Thesis

On the relationship of phylogeny, ecology and life span: The Sordariales

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Abstract

Certain naturally occurring strains of filamentous fungi cease mycelial extension when serially cultured. This observed behavior has been termed senescence. In *Podospora anserina* and certain species of *Neurospora* this phenomena has been extensively studied. It has been strongly correlated with mitochondrial degenerations, as well as with increased production of reactive oxygen species. Other factors such as extra-chromosomal genetic elements have also been related to the senescing phenotype. Surprisingly, in few (or non) other phylogenetically or ecologically related species gerontological research has been performed. Hence, senescence and relative life span has been poorly attributed to the ecology or to the phylogeny of the organism. The present study aims to correlate relative life span to phylogeny or to ecology and to examine if senescence is caused by similar causes, mainly, mitochondrial rearrangements and the consequences therein. In order to achieve this, different species from the order Sordariales were grown, and phylogenetic trees were constructed based on ITS regions and partial β-tubulin sequences. Parallel to this, experiments that measured relative life span were performed as well as detection and identification of extragenomic DNA sequences previously related to senescing phenotypes. The overall results suggest that ecology may be more strongly related to relative life span that phylogeny is, and that the presence of extrachromosomal DNA species -mainly senDNAα- is not strongly related with the onset of senescence.

1. Introduction

The Sordariales is an order of fungi within the class Sordariomycetes (also known as Pyrenomycetes). It gathers an economically important group, which contain species important for both biotechnological purposes such as paper digestion -in which several *Chaetomium* species are used, as well as species for fundamental research, presenting model organisms such as *Neurospora crassa* and *Podospora anserina.* Species within the order occur world wide living on a plethora of substrates such as dung, decaying wood, litter and/or soil. They form small fruiting bodies commonly known as ascomata that possess ascospores, which can greatly vary in morphology amongst genera, ranging from cylindrical hyaline to ellipsoidal brown ascospores, and presenting genera that exhibit different degrees of shapes within this range (Miller and Huhndorf, 2005). Generally, this difference in ascospore morphology has been used as an important morphological character to delimit genera. However, this traditional classification system renders most genera paraphyletic or polyphyletic when compared with multi-gene phylogenies, which additionally indicate that ascomal wall morphology is a better predictor of phylogenetic relationships than ascospore morphology is (Miller and Huhndorf, 2005).

At the gerontological research level, special attention has been given to certain species from the genus *Neurospora* and to *P. anserina,* both filamentous fungi classified within the order Sordariales. Senescing isolates within the genus *Neurospora*, typically contain intramitochondrial linear or circular plasmids and are phenotypically characterized -as is *P. anserina-* by rearrangements in the mitochondrial chromosome, increased production of reactive oxygen species (ROS), decreased growth rates and inability to resume growth at the hyphal tips. Nevertheless and in contrast to species of *Neurospora*, *P. anserina* exhibits senescence in the absence of extrachromosomal mitochondrial plasmids. Nonetheless, it is known that in *P. anserina* plasmids are senescence factors independent of intrinsic senescence (Diepeningen *et al.*, 2008), and that this plasmid-based senescence is similar to that observed in the genus *Neurospora.* Hence, it has been postulated that plasmid-based senescence can be viewed as a lethal disease that exerts its effect in the 'shadow of regular' fungal senescence (Maas *et al.*, 2007).

Besides these few species, little or no work has been done in other members from the Sordariales. Hence, it is of interest to understand if other members from this order show clear signs of senescence and thus, attempt to relate this trait to phylogeny. On the other hand, senescence might not be (strongly) related to phylogeny but rather to ecology, specifically to substrate availability and distribution, hence, niche being the key determinant for life span.

It is generally accepted that during the past, differential degrees of natural selection have occurred which have resulted in a variety of survival strategies. At the extremes of survival strategies, we find *r* and *K* adaptative strategies. Within the first, organisms typically exhibit short life spans, allocating a larger proportion of the available resources to reproduction. Organisms that are classified within this strategy are typical found in habitats characterized by plenty of “free energy”, in other words, in young, unstable ecosystems and/or in early stages of a succession. In the other extreme, *K*-selected organisms are found. These are organisms whose life span is relatively long, organisms that are more somatic, investing less energy in reproduction and yielding few but fit offspring. Within the *r-K* continuum, three different sub-strategies can be defined (Cooke and Rayner, 1984): the first, *C*-selection, has arouse as a combative strategy in which organisms maximize occupation and exploitation of resources in relatively undisturbed conditions. The second, *S*-selection, includes organisms that have evolved adaptations towards conditions of environmental stress, therefore, the incidence of competitors becomes partially excluded. Finally, *R*-selection has resulted in a so-called ruderal strategy, in which organisms exhibit a short life span coupled with a high reproductive potential which determines success in severely disturbed but nutrient rich conditions (Cooke and Rayner, 1984). At the plant-animal community level this ecological strategy scheme seems to fit and hence, slow growing trees that devote only a small fraction of their energy to reproduction are found in undisturbed mature habitats. In accordance with this, certain vertebrates that posses long life spans rendering few but very fit offspring are normally found in this mature ecosystems. In contrast, rapid growing shrubs and herbs with a high reproductive potential and relatively short life spans are generally present in disturbed ecosystems and/or first stages of successions, as well as their associate fauna However, the situation depicted above does not seem to apply to (some) filamentous fungi, for example, several species of *Neurospora* are found in fire-disturbed ecosystems were the availability of simple sugars is high. *Neurospora* typically grows and sporulates at high rates, resembling an *r*-selected strategy, however, its life span is not short. Moreover, it seems capable of indefinite growth (unless infected with a plasmid) as seen in laboratory experiments. On the other extreme lies *P. anserina*, a coprophagous fungus that colonizes herbivore dung in the middle-late stages of succession (Cooke and Rayner, 1984). This ascomycete grows and reproduces at slower rates than does *Neurospora*, however, its life span is shorter -life behavior that seems not to fit with any of the above mentioned strategies when these organisms are considered. Hence, this raises the questions of why *P. anserina* is ageing and why species of *Neurospora* are not. Although the answer is not simple and it is still a matter of scientific debate, it seems to rely on global metabolic efficiency, organization of the electron transport chain, repair mechanisms and particularly on the substrate were the organism has developed.

It has been repetitively shown that caloric restriction (CR) can substantially extend life span in *P. anserina* (Maas *et al.,* 2004) as well as in all other organisms were it has been tested (Bishop and Guarente, 2007). Hence, it can be concluded that substrate is a key factor delimiting relative life span. However, the importance of substrate does not only rely on the fact that it can prolong life span, for under CR most organisms are not able to complete their life cycle, they do not reproduce (Burks *et al.,* 2000). This observation may imply that reproduction and life span are opposite trades. In accordance with this view, it has been shown that from bacteria to human cells, cells become progressively less fit after each round of division (Jazwinski, 2006. Ackermann *et al.,* 2003. Takahashi *et al.,* 2007). Thus, it seems, that senescence is an autapomorphy of life.

Previously, the seemingly "immortality" of certain species of *Neurospora* has been discussed, however, it is noteworthy to mention that filamentous fungi posses a particular anatomy and organization. The fact that they contain hyphal fusions and perforated septa, offers the possibility to see the organism as a cytoplasmic continuum in which molecules and organelles can easily migrate. In addition, their multinuclear condition, may allow any deleterious mutation to be complemented by its functional gene/allele in the other nuclei (Maheshwari and Navaraj, 2007). These particular characteristics might contribute to prolonged life span. Yet, other factors have to be considered when senescence is brought to the test: the ecology of these organisms. As previously mentioned *P. anserina* lives exclusively on dung, where it appears at the middle-late stages of the succession. The first fungi that appear on this ephemeral substrate are those that can readily use simple sugars (e.g. *Mucor sp.*), followed by organisms that are capable of using more complex polymers (e.g. *P. anserina*). Just like the fungi that appear during the first stages of dung succession, *N. crassa* –living on fire-disturbed ecosystems- may have access to easily digestible carbon sources. In contrast, *P. anserina* appears when most of the simple sugars are exhausted from the media. Hence, it has evolved in these conditions and harbors an incredible enzymatic machinery (Espagne *et al.*, 2008), suggesting that it can digest several types of complex polymers including lignins, fact that is in accordance with its ecology. In accordance with the above mention statement, when *N. crassa* and *P. anserina* are compared, the latter posses the most complete enzymatic toolkit involved in lignin and other complex polymer degradation. Enzymes that are known to produce or use ROS during lignin degradation (Espagne *et al.*, 2008). Hence, we come to the same fundamental question, has the adaptation to different substrates and thus, the evolution of particular sets of enzymes –with overall metabolic efficiency consequences- (e.g. with ROS producing/using effects) an influence over life span? In order to make a first intend in answering these questions, phylogenetically related species living on diverse substrates must be tested for senescence. Thus, making an attempt to relate this trade to phylogeny or rather to ecology. Precisely this approach was the one that was taken in order to answer several questions during this research experience.

Now, let us consider senescence from a (non linear) thermodynamic point of view. Ilya Prigogine with his work on dissipative structures, complex systems, and irreversibility, realized that in far-from-equilibrium conditions various types of self-organization processes may occur and that these far-from-equilibrium systems have the possibility to evolve, to generate new molecular orders spontaneously (Prigogine and Stengers, 1984). Cells are well organized entities, they present a remarkable organization with a highly non uniform distribution of ions and molecules were specific and coherently controlled chemical transformations take place. The presence of autocatalysis (e.g. autocatalytic RNAs) auto-inhibition (e.g. ADP/ATP oscillations in glycolysis), and cross-catalysis (e.g. self replicating peptides), between others, provides the basic regulatory mechanisms that guaranty the coherence of metabolic functions within a living cell. However, this far-from-equilibrium conditions have to be maintained in order to remain as a living entity. For instance, it has been shown that if calcium ion concentrations are not maintained within certain limits, plant cells suffer from programmed cell death (Geydan and Spinel, 2007).

The origin of life goes beyond the scope of this writing, however, for practical reasons we will consider the origin of life as an spontaneous process were self-organizing molecules arose and evolved in conditions far-from-equilibrium. When the system arose, it necessarily had to interact with the outside world, thus, becoming a dissipative structure, a system ruled by the laws of physics, by laws that establish that all physical systems tend to states of maximum entropy, to states of equilibrium. Yet, on systems far-from-equilibrium the situation is not that simple, for thermodynamic forces that generate fluctuations not simply "disturb" the system momentarily, but may be amplified invading the entire system, obliging it to evolve towards new states that may be qualitatively different from the stationary states corresponding to minimum entropy production (Prigogine and Stengers, 1984). Once this states have been reached they may be registered in time as information coded in the form of ribonucleic acids (be RNA or DNA), as a memory. In other words, biological systems have a past, they are the result of evolution, they have been selected based on their states that differ from those of maximum entropy, on their capacity to replicate.

Now let us consider Boltzmann observations: through probability he realized that irreversible increase in entropy could be seen as the expression of a growing molecular disorder, of a gradual forgetting of any initial dissymmetry. As mentioned before cells are physical systems built up of molecules, however, these molecules are also subject to states of maximum entropy, characteristic that can easily be observed in any cell: its components continuously have to be repaired, synthesized. However, there seems to be a point in which certain components can no longer be repaired or synthesized, a point were the initial dissymmetry is broken, it is forgotten. In order to exemplify this consider the mitochondrial rearrangements that *P. anserina* (and other organisms) suffer when it senesce, isn't this forgetting the initial conditions? The impossibility to correctly interpret information in form of DNA? And if metabolic efficiency is hampered and hence disorder within the cell arises (e.g. due to elevated production of ROS) wouldn't this be considered as an increase in entropy? Increases that accumulate until the system reaches a state of maximum entropy, of equilibrium. Hence, senescence itself is the gradual process of forgetting the initial conditions, steps towards chaos. Hence, it does not evolve, it is simply the fall after the rise, the system dissipating, gaining chaos once again. Instead, what evolves is the global metabolic efficiency, organization of the ETC, repair and cell signaling mechanisms, intramolecular organization, the *phenotype*; all this with a dependency on the substrate (and the environment in general) were the organism has developed, intermixed with the plasticity of the previously registered memory (what has been learnt, registered in time), bringing as a consequence differential life spans. The fact that certain genetic programs such as programmed cell death and apoptosis like mechanisms have evolved, only exemplifies the fact that evolution not only relies on the organism itself but on others akin to it and hence, populations evolve as a whole, rendering -in this case- apoptosis like mechanisms that perhaps do not necessarily benefit the individual organism itself, but the population, that that is kept in time -the genes. By this means conditions far-from-equilibrium persist and further expand, generating what we define as a *population.* In doing so (and following similar principles), new levels of complexity arise –emerging properties- that can only be maintained when systems (now a population) generate new states of order where entropy production is minimized. In order to exemplify this, let us consider an age-structured population of yeasts, just as it naturally occurs. When mathematical models are applied to experimental data on the oscillating age-structure of a non-equilibrium populations of yeast (where asymmetry of cell division and control of the cell cycle are the parameters considered) the model predicts both the transient behavior and the equilibrium structure of such populations. Moreover, the model shows that, (1) the asymmetry in cell division can explain the "excess" of newly formed daughter cells in the population as compared to the frequency of older cells, and (2) the damped oscillations in the frequencies of cells of different ages as demographic equilibrium is approached (in words of Adams *et al.*, 1981). Hence, only with a continual "removal" of (old) cells that have presumably accumulated damaged components -that have increased their molecular disorder-, can minimum entropy production levels be maintained within the emerging property-the population. Thus, we can see that senescence has not evolved *per se*, what has evolved is a new (more complex) macroscopic system that requires certain (microscopic) conditions in order to remain in time.

The following research aims to relate differential life span to phylogeny or to ecology, and to establish if senescence renders a particular phenotype within the Sordariales.

2. Methods

2.1. Taxon sampling

All taxa used in this study were obtained form the Centraalbureau voor Schimmelcultures (CBS) and are listed in Table 1 along with their origin and source of collection. Multiple representatives were included from eight genera within the Sordariales and two species –not belonging to the Sordariales- were used as outgroups. Hence, *Phomopsis subordinaria* represented the Diaportales, while *Coniochaeta savoryi* represented the Coniochaetales.

2.2 Culture conditions and life span analysis

Isolates were grown on *Podospora anserina* synthetic medium (PASM). The PASM used in these experiments is based on the minimal media described by Diepeningen *et al.,* 2008 and contained *per* liter 0.75 g KH_2PO_4, 0.5 g $MgSO_4$, 1 g urea, and 20 g agar. Both biotin and thiamin were added to a final concentration of 100 µg/mL and 1 mL trace element solution *per* liter was added. All incubations were done at 25°C in the dark, unless stated otherwise. At least triplicates of each fungal isolate were grown for the life span experiments.

Lifespan was measured in time (days) and length (cm) of continuous growth, using big Petri dishes (14 cm diameter) and/or 30 cm long glass race tubes with an inner diameter of 1 cm filled with 25 mL of medium. Growth was marked Mondays, Wednesdays and Fridays, however, one marking was normalized to 2.33 days.

Inoculation material was obtained from the CBS as living cultures (mycelia) or as frozen grounded mycelial powder, and was from that material that life span analysis were performed. Explants from full race tubes or from the rim of plates were transferred to fresh tubes/plates with medium for continuous growth. For each isolate, growth experiments were performed for at least six weeks or until three continuous race tubes were filled with mycelia. A senescing phenotype was attributed to the isolate when growth rate decreased substantially and/or when growth rate came to a halt.

Table 1. Taxa used during this study

Taxon	CBS No.	Origin	Source	ITS 1-4	β-tubulin
Podospora anserina†	124.78	India	goat dung	+®	+®
Podospora	253.71	Central	cobus dung	+®	+®

anserina†		African Republic			
Podospora anserina†	333.63	Argentina	cow dung	+®	+®
Podospora anserina†	415.72	Pakistan	soil	+®	-
Podospora anserina†	451.62	Argentina	cow dung	+®	+®
Podospora anserina†	455.64	Switzerland	cow dung	+®	+®
Apiosordaria verruculosa	438.67	UK	alluvial soil	+¢	+¢
Apiosordaria backusii	106.77	Japan	sandy soil	+§	+®
Apiosordaria otanii	317.91	S. Udagawa	forest soil	+§	+§
Cercophora grandiuscula †	120013	Australia	wombat dung	+§	+§
Cercophora samala	307.81	Japan	dung of cow	-	+®
Chaetomium thermophilum	143.50	UK	decaying wheat straw	+®	+§
Chaetomium pachypodioides †	164.52	USA	vegetable detritus	+¢	-
Chaetomium longicolleum	119.57	Madagascar	soil, under *Theobroma cacao*	-	+§
Lasiosphaeris hispida	955.72	Germany	decaying wood	+*	+¢
Lasiosphaeria ovina	725.69	Canada, Ontario;	dead wood	+®	+®
Neurospora pannonica	270.91	Hungary; Hortobagyi	open soil in Puszta	+®	+®
Neurospora africana	571.69	Nigeria; Eastern	soil	+®	+®
Neurospora tetrasperma	377.74	Kuwait	desert soil	+®	+®
Podospora vesticola	244.28	Netherlands	rhizomorph in peat-boat	+¢	+¢
Podospora setosa	435.50	Germany; Tamsel	dung of dog	+®	+§
Podospora longicollis	368.52	Panama; Canal Zone	deteriorating material	+§	+§
Podospora austroamericana	724.68	India; Allahabad	*Carica papaya*, flower	+®	+®
Podospora	247.71	South Africa	dung of	+§	+§

ampullacea			*Hippotragus* sp.		
Podospora inflatula	258.78	Venezuela; Edo Monagas	dung of burro	+®	+§
Podospora fibrinocaudata	315.91	USA, California; San Mateo Co	dung of dusky footed wood rat	+§	+®
Podospora prethopodalis	121128	Australia	marsupial dung	+*	+§
Podospora minicauda	227.87	Egypt; Western Desert	soil	+§	+§
Zopfiella longicaudata †	971.73	Tanzania	dung of elephant	+¢	-
Zopfiella marina	155.77	Taiwan; Chinese Sea	marine mud	+§	+§
Coniochaeta savoryi♣	415.73	Netherlands; Drente	sandy soil	+§	+®
Phomopsis subordinaria♣	104.84	Netherlands; Papendal near Arnhem	*Plantago lanceolata*, stem of diseased plant	+§	+§

+ Indicates gene sequenced for taxon.

+* Indicates gene was not sequenced for taxon and data was taken from Genebank.

- Indicates gene could not be sequenced for taxon and sequence was not present in Genebank

♣ Indicates the species used as outgroups.

§Indicates newly sequenced taxa.

¢ Indicates taxa whose taxomical identity remains controversial.

® Indicates taxa that show high and correct sequence similarity against NCBI database when Blasted.

† Indicates isolates that exhibit senescence.

2.3 *DNA extraction, amplification and sequencing*

Mycelial cultures were grown on PASM with 2% glucose covered with cellophane for 2–3 days. Harvested mycelium was frozen with liquid nitrogen, ground in a bead beater two times and after, 50ml of Chelex X-100 and 5µl of proteinase K (20mg/ml) was added to each sample and incubated for at least one hour. Afterward, samples were incubated at 95°C for at least 15 min and centrifuged for 1 min at 13000 rpm. The relative quantity of total genomic DNA was observed on a 1% TAE agarose gel stained with Gel Red and 1:10 dilutions were made for each extracted DNA sample. ITS and β-tubulin gene fragments were PCR-amplified on a Bio-Rad I-Cycler using the primers developed by Glass and Donaldson, 1995, while intron alpha fragments were PCR-amplified using the primers developed by Cummings *et al.*, 1985 and listed on Table 2.

PCRs were performed in 25µl volumes containing 5µl 5x green GoTaq buffer, 3 µl of mM $MgCl_2$, 1µl of 10mM dNTP, 0,5µl of each primer, 0,12µl of GoTaq enzime, 12.88µl of MQ water and 2µl of DNA extract.

ITS was amplified using the following thermocycling parameters: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min followed by a final extension step of 72°C for 10 min. Parameters for amplifying β-tubulin partial sequences were identical except that annealing was conducted at 58°C for Bt1 and at 59°C for Bt2. Intron alpha was amplified using the following thermocycling parameters: denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 4 min followed by a final extension step of 72°C for 10 min. After verification on an Gel Red stained TAE 1% agarose gel, all PCR products were gel-purified using a Gene elute PCR Clean-up Kit (Sigma-Aldrich) and dsDNA concentrations were measured using a Bio-Rad Smart spectrophotometer 3000. Adequate amounts of the PCR-products were sent to Eurofins MWG Operon sequencing service for single strand sequencing.

2.4 Blast search

After successful sequencing, each amplified fragment was subjected to a BLAST search to verify its identity. The program selection was optimized towards highly similar sequences (megablast), with an expected threshold of 10 and a word size of 28, the gap cost were considered linear and match/mismatch scores were set to 1,-2.

The Blast search was done by blasting the complete amplified gene fragment of the ITS, Bt1, Bt2 and Bt1 + Bt2 combined. A sequence was considered as “newly sequenced” when two criteria were met: the amplified gene fragment showed no considerable homology (less than 95% similarity) to any sequence in the database, and/or when the scientific name given by the CBS to the isolate -from which the amplified fragment came from- was not found within the NCBI database. In order to consider a positive hit, sequence homology with sequences previously entered in the database had to be of at least 98% similarity with E-values very close to or 0.0 plus the taxonomic names had to overlap.

2.5 Sequence alignments and phylogenetic reconstructions

For each fungal strain, sequences obtained from single strands were assembled and aligned using the Lasergene 8.0 package, optimized by eye and manually corrected when necessary. For both β-tubulin sequence fragments, noise (first 39 bp of the sequence) and introns were manually removed, and from the resulting fragments, phylogenetic trees were built. For ITS, only noise was removed from the sequence fragments and from this, distance trees were built. For all phylogenetic reconstructions the

Clustal W method was used and all trees were generated with a bootstrap of 1000 and a seed of 111.

Table 2. Primers used in this study for PCR amplification and sequencing.

Target gene	Primer	Primer DNA sequence	Position
β-tubulin	Bt1a	5' TTCCCCCGTCTCCACTTCTTCATG 3'	1298-1321
	Bt1b	5'GACGAGATCGTTCATGTTGAACTC 3'	1812-1835
	Bt2a	5' GGTAACCAAATCGGTGCTGCTTTC 3'	352-375
	Bt2b	5' ACCCTCAGTGTAGTGACCCTTGGC 3'	824-847
ITS	ITS1	5' TCCGTAGGTGAACCTGCGG 3'	-
	ITS4	5' TCCTCCGCTTATTGATATGC 3'	-
Intron α	F3	5' AGAATGGAGTTGAGCCGGCC 3'	38640
	R3	5'ATCATCAGT AAGGCATGCGC 3'	38971
	Inseq R	5'ATCCTCTCTCCGCGGTAGTA 3'	41238

3. Results and Discussion

3.1 Growth and Life span analysis

The experiment began with 37 different fungal isolates; 8 were *P. anserinas* (also labeled as *P. pauciseta*) already present in the genetics laboratory which were chosen on dependence of their adeand 29 were newly ordered strains from the CBS. The *P. anserinas* were chosen based on the fact that they were tagged with specimen country of origin and substrate where it was collected. Regarding the *P. anseinas*, one of them (isolate 112042) was infected with a bacteria that could not be removed by using streptomycin, hence, this strain was not used for further research. Strain 415.72 was infected with a parasitic fungi identified as *Piptocephalis sp.* nevertheless, for the DNA isolation experiments strain this could be grown without the *Piptocephalis sp.* Finally, strain 433.50 did not grow at all, presumably the mycelia stored at -80C is old tissue that could not resume growth anymore and hence, can be considered senescent. Regarding the newly ordered CBS strains, three of them (215.60-*Cercophora ambigua*, 114597-*Lasiosphaeris hirsuta* and 725.69-*Lasisphaeria ovina*) did not grow on PASM 2% glucose or in dung media, hence, they first two were not used for further research. However, the third specie grew enough to gather mycelia only for molecular analysis. When strain 671.82- *Zopfiella ovina* was grown for the first time, it was noticed that three different fungi were growing with it, one of them was identified as *Penicillium sp.* and the other remained unidentified, hence, this strain was not used for further analysis, for *Penicillium sp* remained persistent in subsequent cultures. Finally, strain 247.71- *P. ampullacea* was sent with a bacterial contamination (informed by the CBS), this bacteria was very persistent and could not be removed by using streptomycin. However, when high doses of tetracycline were used –in some cases- the bacteria was removed and the fungal isolate could be grown alone. Hence, for this isolate molecular analysis could be preformed but the growth experiments are still on-going.

30 taxa were analyzed for life span analysis. Most fungal isolates did not show clear signs of senescence during the time the experiments lasted, consequently, their growth rates can be considered linear (Annex A). All *P. anserinas* tested showed clear signs of senescence and exhibited growth rate patterns that resembled a mirror inverted Z (Annex A). The growth rate of these strains was linear until they reached the so called 'presenescent' phase (Gredilla *et al.*, 2006), and from that moment on, the growth rate decreased, the peripheral hyphae became undulate and thin, pigmentation of the mycelium increased, and finally the culture stopped growing. Other species representative of the "genus" *Podospora* did not show clear signs of aging during the time the experiment lasted. However, growth rates amongst the genus varied in a great extent, presenting growth rates of less than half a cm per marking (2.33 days) in *P. minicauda* to more than 5 cm per marking in *P. vesticola.*

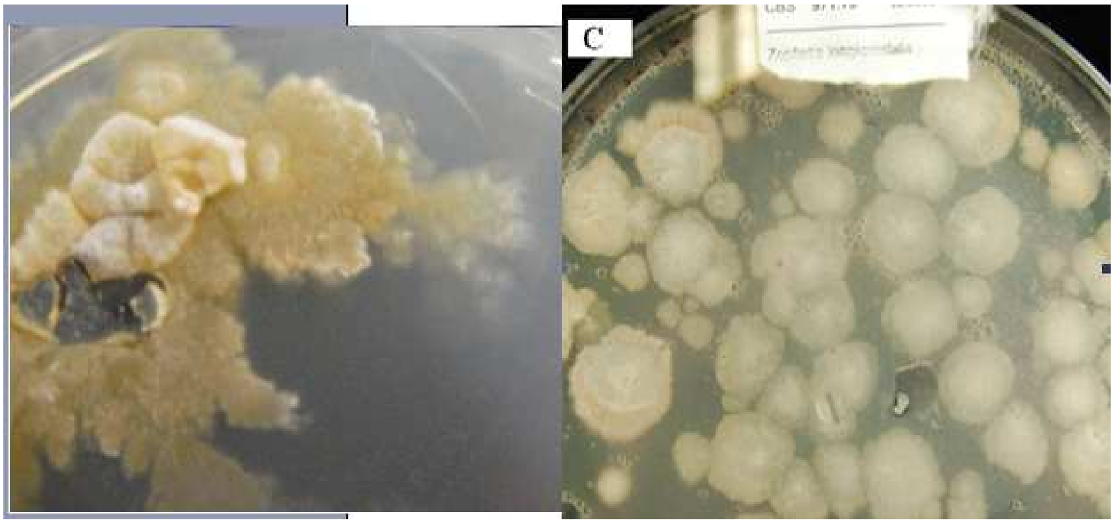
A part from the aging *P. anserinas*, three other species aged as seen in the growth experiments, for their growth rates decreased substantially and/or came to a halt (Annex A). *Chaetomium pachypoides (164.52)* -a member of the Chaetomiaceae family which was isolated from vegetable detritus-showed decreased growth rates and in some cases mycelia growth came to a halt. Interestingly, when the isolate was grown on a Petri dish, expansion of the mycelia did not occurred uniformly along the dish, rather, mycelia showed clear sectors of reduced growth or even growth halt, while other sectors continuously grew until eventually the also came to a halt (Fig. 1A). This growth behavior may imply that not all cells posses the same fitness, hence, some continuing growth whilst others stopping. If this is true and not all cells exhibit the same fitness, is this due to accumulation/translocation of damaged components? Increased production of ROS? Or is it a signaling mechanism within the mycelia-as a whole-that allow some cells to continue growth while others perform other actions, just like the observed signaling mechanisms in higher plants? Parallel to this, it was observed that the mycelia became darker and "harder", sometimes even growing a few µm vertically, all this just before its growth rates came to a halt (Fig 1 A).

Cercophora grandiuscula (120013) -a member of the Lasiosphaeriaceae - the same family that harbors *P. anserina* -and isolated from wombat dung, also exhibited signs of senescence, for its growth rate decreased substantially and/or came to a halt (Annex A). *C. grandiuscula* old mycelia looks thinner and presents "airy mycelia" when compared to the younger one, that looks more dense and compact (Fig 1 B). Old mycelia has a shorter life span when explanted onto new plates for continuous growth and does not produce the white little cotton like dots that interestingly, have a longer life span that grey mycelia of their surrounding areas (Fig 1 B)(data not shown, on going observations).

Zopfiella longicaudata (971.73) -a member of the family Chaetomiaceae and isolated from elephant dung also revealed signs of senescence. During the first growth experiments, *Z. longicaudata* displayed a linear growth rate during the first two and a half weeks. At this point, its growth rate decreased but kept continuous growth (Annex A). However, when explants were taken from the rim of growing (old) mycelia and were transferred to new plates for continuous growth, the growth rate of the explants decreased substantially and eventually came to a halt (Annex A). An additional senescing phenotype can be attributed to the isolate: young mycelia grows whitish and expands uniformly throughout the agar, while old mycelia attains a darker beige pigmentation expanding unevenly throughout the agar showing sectors of growth and sectors of growth halt (Fig 1C). Noteworthy of mentioning, is the fact that the mycelia that is capable of growing further is characterized by thinner mycelia and in some cases even individual hyphae can be observed –phenomena that does not occur in young tissue.

Phomopsis subordinaria (104.84) is an interesting isolate which was found on a diseased stem of *Plantago lanceolata.* During the first growth experiments -and in some replicas- it exhibited decreased growth rates and in some plates even growth came to a halt (Annex A). However, when explants were transferred onto new plates its growth rate increased substantially, was maintained, and halts in growth rate were not experienced again. No obvious differences in mycelia morphology were observed throughout the growth experiments. Hence, a possible explanation for the observed growth patterns is that the isolate was suffering –during the first experiments- an adaptation towards the growth media and thus, the observed behavior was experienced. Hence, during the second part of the experiments the isolate may had already adapted to the "new environment" exhibiting a linear growth rate. Interestingly, *Ph. subordinaria*, has previously been reported as specie that senesce when grown on potato dextrose agar (personal observations E. Linders). However, the present results are not in accordance with the previous observation. This generates questions such as: are certain species senescing only on certain substrates? Hence, substrate being the key factor delimiting life span? Has selection for longevity taken place by successive rounds of culturing under certain laboratory conditions? If any or both are true, what are those cellular components that under selection?

Fig. 1. Illustrating phenotypic differences between old (left) and young (right) mycelia of *Chaetomium pachypoides (A), Cercophora grandiuscula (B) and Zopfiella longicaudata (C).* Notice the uneven mycelial expansion, coloration and texture of the old material when compared to the younger one.



3.2 Amplification, sequencing and analysis of target DNA

Full data sets contained 29 and 30 taxa for the β-tubulin and ITS genes respectively. While reduced data sets sampled 27 taxa for each of the two genes and will be subsequently used in combined analysis.

At an annealing of 55°C, the ITS1-ITS4 primer set was successful in amplifying fragments from all isolates tested for the exception of four of them (Table 1), which could not be amplified even if the PCR conditions were extensively varied. The ITS amplification product varied in size from approximately 552bp in *C. termophilum* to 429 bp in *A. verruculosa*, and as expected, contained the two internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S rRNA gene. Most variation within the amplified product was observed within the two ITSs.

The Bt1 and Bt2 primer sets were successful in amplifying fragments from 29 isolates when the annealing was conducted at 58°C and 59°C respectively (Table 1). The Bt1 amplification product varied in size from approximately 503 bp in *P. anserina* CBS 124.28 to 413 bp in *P. ampullacea.* In most isolates, one intron was detected and it exhibited high sequence homology with intron IVS6 of *Neurospora crassa's* β-tubulin gene (Orbach *et al.,* 1986) (Annex B1). The coding product of the Bt1 amplified product *vari*ed in size from 439-bp in 124.78 *P.* anserina to 377-bp in *Cercophora grandiuscula.*

The Bt2 product varied in size from approximately 588 bp in *P. ampullaceal* to 292 in *A. verruculosa.* Three introns were observed within the Bt2 amplified fragment: IVS3, IVS4 and IVS 5 in accordance to *N. crassa's* β-tubulin gene (Orbach *et al.,* 1986). IVS5 seemed to be the most 'degenerated', possessing little sequence homology in its 5' and 3' splicing sites as well as in its internal conserved sequence (Annex B2-4).

The coding region of the Bt2 amplified product varied from 227 bp in *P. inflatula* to 300bp in *A. backusii.* Special is the case of *P. ampullacea*, whose Bt2 sequence length (after removal of known introns) was found to be unusually long (427 bp), however, when all sequences are aligned it can be observed that this unusual length might be due to the acquisition/insertion of a sequence at the 3'end of the coding region that resembles IVS type introns, for its 5', 3' and bp length shows similarity towards this type of genetic elements. In no other isolate similar sequences were found. Consequently, the Bt1 and (specially) Bt2 size polymorphisms observed in the amplified products reflects variability in the number of introns as well as in intron and exon length within the β-tubulin gene.

3.4 Analysis of introns from the β-tubulin amplified fragments

Orbach *et al.*, 1986 characterized the β-tubulin gene from a benomyl-resistant mutant of *Neurospora crassa* and found out that the gene is coded by a nucleotide sequence of more less 1925-bp. However, the protein consists of only 447 amino acid residues. Hence, six introns were found to interrupt the gene and were designated IVS1 to IVS6. The six introns of the *N. crassa* β-tubulin gene contain conserved 5'and 3' splicing sites along with a conserved internal sequence (Orbach *et al.*, 1986). Other studies on fungal β-tubulin genes have shown that there is a considerable conservation of intron position within the β-tubulin gene (Glass and Donaldson, 1995).

The primers used in the present study successfully amplified β-tubulin gene fragments that were -in most cases- interrupted by one or more introns (Annex B). Additionally, examination of the β-tubulin genes from all isolates, revealed that there is a considerable conservation of intron position, which is in accordance with observations from previous studies.

The Bt1 primer set amplified one intron designated IVS6 and was contained in 24 out of 29 isolates tested (Annex B). This intron contained relatively conserved 5' and 3' splicing sites as well as a conserved internal sequence localized in proximity to the 3'end. IVS6 varied in length from 67-bp in *C. samala* to 52-bp in *A. otanii.*

The Bt2 primer set amplified three introns designated IVS3 to IVS5. 26 out of 29 isolates tested were found to posses all three introns. Both *A. verruculosa* and *P. inflatula* lacked IVS4 and IVS3, whilst *L. ovina* lacked only IVS3. Interestingly, non of the isolates lacked IVS5, intron that contains the least conserved 5' splicing site as well as the least conserved internal conserved sequence (Annex B). IVS5 varied in length from 97-bp in *L. ovina* to 52-bp in *A. verruculosa.* IVS4 varied in length from 82-bp in *L. hispida* to 53-bp in *P. prethopodalis*. The position of the Bt2a primer is just before the 5'end of IVS3 (Table 2). Hence, its length could not be elucidated due to the removal of the first 39-bp (noise) from isolates, however, it could be observed that it contained a relatively conserved internal sequence as well as a 3'end (Annex B).

Studies in *Schizosaccharomyces pombe* and *Aspergillius nidulans* have revealed that most introns occur at the same positions as they do in *N. crassa* β-tubulin gene (in Orbach *et al.*, 1986). In contrast, the *Saccaromyces cerevisae* β-tubulin gene was found to lack introns within the β-tubulin gene (Neff *et al.*, 1983). The above mentioned observations plus the results from this study beg for whether introns have been gained or lost during evolution. As previously mentioned IVS5 was the intron that exhibited less sequence homology regarding splicing sites when compared to other introns. Hence, is IVS5 could be the oldest intron and has suffered mutations over time thus, rendering it the least conserved; alternatively, IVS5 could be the most recently gained intron and thus, evolutionary time has not been enough to render it as conserved as the other introns are. But if any of this is true, it implies that selection also occurs in non-coding sites of the genome and that sequences with conserved splicing sites (introns) are non-randomly inserted into the genome at specific positions. Alternatively, relatively random sequences are inserted into conserved regions and during evolution suffer similar selective pressures (in most fungi) which renders them with conserved splicing sites.

3.5 *Blast search and homology with databases*

The following procedure leaned on three central but implicit assumptions:

as literally stated by Nilsson *et al.*, 2006:

1. that the reference database features a satisfactory taxonomic sampling of sequences
2. that the sequences in the reference database are correctly

identified and annotated
3. that the process of translating the comparison into species names is standardized, universally adopted, and not easily misunderstood.

Both ITS and β-tubulin amplified fragments were subject to a Blast search in order to verify their identity. The ITS data set consisted of 10 newly sequenced taxa, 14 sequences that exhibit high homology (at least 98% similarity and E-values very close to 0.0) to sequences present in the NCBI database, and finally 4 sequences that -to date- should be taken with precaution for their taxonomic identity remains controversial (Table 1). The combined Bt1+Bt2 sets rendered 12 newly sequenced taxa, 14 sequences that exhibit high homology to sequences present in the NCBI database, and finally 3 sequences that -to date- should be taken with caution for their taxonomic identity remains controversial (Table 1). Taking together these results, a total of 5 isolates lack reliable taxonomic identity and would be further discussed:

From *Z. longicaudata* CBS No 971.73 only ITS could be sequenced. The resulting sequenced gene presented only 91% sequence homology to the *Z, longicaudata* strain IFO 30296 found in the NCBI database. The best hit that CBS 971.73 ITS region had, was to *Zopfiella latipes* and to *Apodus oryzae*, both with a sequence similarity of 97%. Important to mention is the fact that *A. oryzae* is a member of the Lasiosphaeriaceae family, while *Z. latipes* and *Z. longicaudata* are both members of the Chaetomiaceae family. Hence as a priority, the β-tubulin gene fragment of *Z. longicaudata* CBS 971.73 should be sequenced in order to clarify its taxonomic identity.

Lasiosphaeria hispida CBS 955.72 is another isolate whose taxonomic identity remains controversial. Only the β-tubulin gene fragment could be successfully sequenced, and when blasted, no hits with sequence similarity above 90% could be observed. Nevertheless, when its β-tubulin gene fragment was compared with that of the *Lasiosphaeria hispida* strain SMH3336 present in the database, only 84% similarity was found between the two sequences, which is to little homology to consider them as the same specie. Hence, the ITS region should be sequenced in order to clarify the taxonomic identity of the isolate.

CBS 164.52 *Chaetomium pachypodioides*, also presents taxonomic constrains. First of all, only its ITS region could be sequenced, however, this region hits *N. crassa* ITS region with 99% similarity. Second, the name *C. pachypodioides* does not exist within the database, so a two sequence blast (or a pairwise alignment) could not be performed. The similarity with *N. crassa* is intriguing due to the fact that contamination-although not completely ruled out- is very improbable (all cultures are checked) and also because *N. crassa* and *C. pachypodioides* currently belong to different families. Again, the sequencing of other genes might help clarify this conundrum.

Apiosordaria verruculosa CBS 438.67 is the fourth isolate that presents taxonomic uncertainties. Both ITS and β-tubulin gene fragments were successfully sequenced from this isolate. When both sequences are blasted against the database, both sequences hit with 99% similarity *Phaeosphaeria nodorum* a member of the order Pleosporales. Furthermore, when the β-tubulin gene fragment is blasted against *Apiosordaria verruculosa* strain F-152365 presented in the NCBI database, only 94% sequence homology can be found. ITS has not been sequenced for *Apiosordaria verruculosa* strain F-152365. The above mentioned facts are quite intriguing, for different orders should be quite divergent in conserved gene sequences such as ITS and β-tubulin. Hence, although CBS 438.67 is named *Apiosordaria verruculosa* it is very probable that this is a mistake and that the correct name for this isolate should be *Phaeosphaeria nodorum.*

Finally, CBS No. 244.28 named as *Podospora vesticola* has a similar story as the previous isolate. Both genes were successfully sequenced, however ITS hits with 99% similarity *Neurospora* terricola, *Sordaria tomento* and *Gelasinospora tetrasperma.* However, the β-tubulin gene fragment of CBS 244.28 only hits –with 100% similarity- *Gelasinospora tetrasperma* and non of the other species that were hit with the ITS blast search. Additionally, when CBS 244.28 ITS is blasted against the ITS from *Podospora vesticola* under accession AY515365, only 88% similarity can be found between both sequences. Hence, all data points towards a mistake in labeling CBS 244.28 as *Podospora vesticola,* a more accurate name would be *Gelasinospora tetrasperma.*

3.6 *Phylogenetic reconstructions*

The ITS phylogenetic tree consists of 30 taxa that were aligned using the Clustal W method. This method was chosen for it has be shown to improve the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice (Thompson *et al.,* 1994). The sequences used for building the tree consist of ITS1, 5.8S rDNA and ITS2. In the resulting tree, three well supported clades-all showing 100 support- can be observed (Fig 2a). The first clade termed "A" consists of nine isolates: 435.30-*P. setosa*, 724.68-*P. austroamericana*, 106.77-*A. backusii* and the six *P. anserinas* from our laboratory –which additionally cluster into a smaller subclade with 100 support and termed "*P. anserina* subclade". Interestingly, non of the other species of from the genus *Apiosordaria* (CBS 438.67 and 317.91) clustered together, which exhibits the paraphyletic nature of the genus *Apiosordaria.* A second well supported clade termed "B", consists of all the Neurosporas used within this study (table 1) plus 244.28-*P vesticola* and the ageing 164.52- *Chaetomium pachypodioides* (Fig 2a). However, special care should be taken with 164.52 due to its taxonomic uncertainty. The third well-supported clade consists of three isolates: 247.71-*P. ampullacea*, the ageing 971.73-*Zopfiella longicaudata* and *Z. marina.* One other pair of isolates clusters together with a good support of 99.4 and this pair of isolates has temporarily been termed * . This pair is built with 227.87-*P. minicaudata* and 317.91-*A. otanii.* Although this phylogenetic tree still needs refinement, it can already be concluded –based on the well supported clades- that the genus *Podospora* is not a monophyletic genera due to its differential distribution within the different clades (Fig 2).

The β- tubulin tree obtained from the Bt1+Bt2 set of primers consists of 29 taxa that were align using the Clustal W method and corrected manually. The resulting phylogenetic tree rendered 3 well supported clades (Fig 2b), of which two of them (termed *P. anserina* and "B" in homology with those of the ITS tree) show certain degree of homology with those observed from the ITS phylogenetic tree. The first clade, the *P. anserina* clade comprises all the *P. anserina* isolates plus CBS 307.81 *Cercophora samala* (whose ITS region could unfortunately not be amplified). *C. samala* is an interesting strain isolated from cow dung. During the growth experiments only one (out of seven) of the replicas showed halt in its growth rate, so this isolate was considered as a non ageing one. However, recent observations of the isolate, when kept under continuous growth, have revealed that certain sectors from the rim have stopped growing, a pattern that resembles that observed in the senescing 971.73-*Zopfiella longicaudata.* Further experiments will hopefully confirm this just observed behavior. The second well supported clade is that that clusters all the species of *Neurospora* plus what is called *P. vesticola* but presumably is *Gelasinospora tetrasperma.* Hence, this clade was termed "B" in accordance with the named assigned in the ITS tree. Finally, a pair of isolates with an perfect support can be observed in the β- tubulin tree (Fig 2b), this pair is formed by 104.84 *Ph. subordinaria* and 106.77 *A. backusii* and has been termed "Y" because it is not known why they cluster together being both members of different taxonomical orders. In addition, they do not cluster together in the ITS tree.

The overall results suggest that the morphological character(s) –which has generally been ascorpore morphology- is not an adequate morphological character to delimit genera within the Sordariales. As can be seen in Fig 2, most genera are clustering with other genera apart from their own. As a result, most genera studied are rendered para or polyphylectic. This observation suggests that similar ascospore morphologies have evolved independently and perhaps several times within the Sordariales. The above mentioned observations are in accordance with previous phylogenetic studies that reported similar observations and additionally indicate that ascomal wall morphology is a better predictor of phylogenetic relationships than ascospore morphology (Miller and Huhndorf, 2005).

The combined information obtained from the Blast search and from the phylogenetic trees indicates that -at the phylogenetic level- the *P. anserinas* tested are indeed *P. anserina* and not other species. However, in order to confirm this, the biological concept of specie should be put the test and hence, all the strains should be crossed with each other, and their ability to produce sexual offspring will determine their nature.

Although both phylogenetic trees must be further analyze and refined, and posses certain degree of incongruence, it is tempting to speculate (as seen from the well supported clades) that life span is not strongly related to phylogeny. Further analysis will hopefully prove this hypothesis.

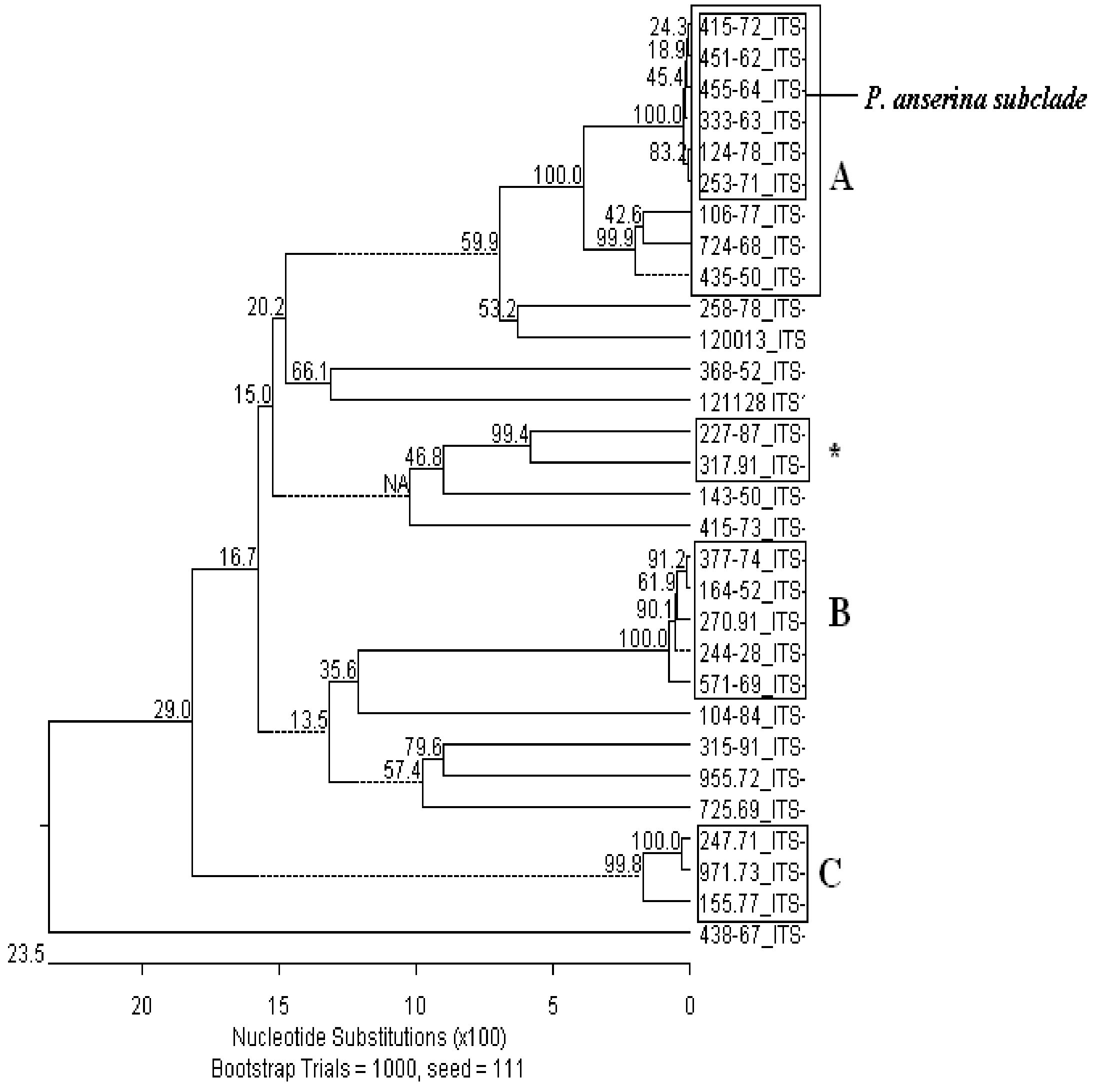


Figure 2a. Phylogenetic tree based on ITS regions. Three clades can be observed termed A, B and C respectively. Notice that within clade A one sub-clade exists termed *P. anserina* sub-clade.

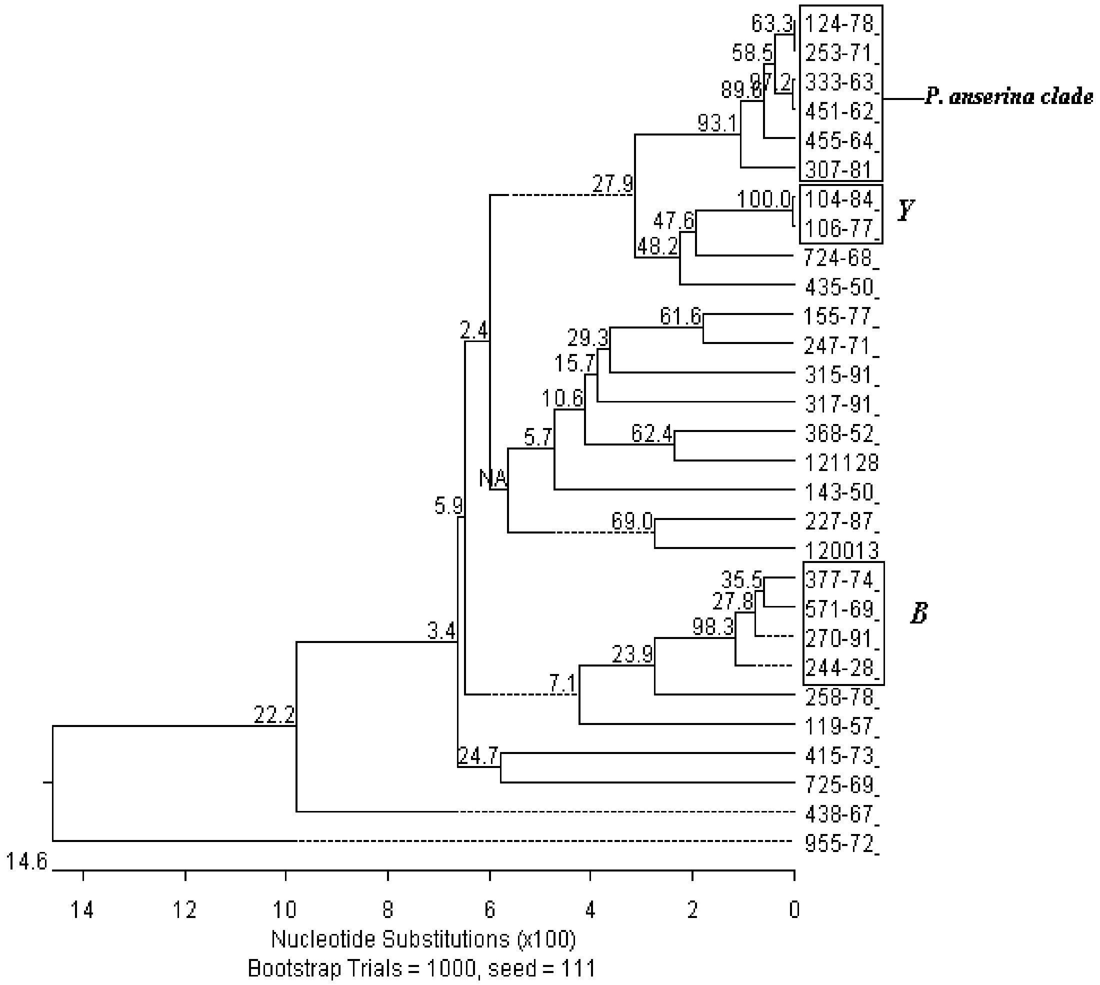


Figure 2b. Phylogenetic tree based on β-tubulin gene fragments. Three clades can be observed termed *P. anserina*, B and Y.

3.7. *Intron alpha*

Molecular analysis of mitochondrial DNA in senescent wild type *P. anserina* isolates has revealed that this state is always correlated with the presence and accumulation of circular DNA molecules termed senDNAs, of which senDNAα seems to be the most prominent, for it has been found in all senescent cultures (Begel *et al.*,1999). It correspond to the first intron of the COX1 gene, which interestingly encodes for the first subunit of cytochrome *c* oxidase (Cummings *et al.*, 1985) -pathway strongly correlated with mitochondrial free radical generation and lifespan control (Gredilla *et al.*, 2006).

In order to test if the presence of senDNAα is correlated with senescence, PCR-amplifications of this genetic element was performed in all studied isolates. Intron alpha was confirmed (sequenced and blasted) to be present in six isolates of which four of them correspond to different strains of *P. anserina.* Result that is accordance with previous observations that show that intron alpha is found in all senescing cultures of *P. anserina* (Begel *et al.*,1999). Interestingly, the two other isolates in which intron alpha was confirmed to be present (*N. pannonica* and *Lasiosphaeris hispida)* are isolates that did not show clear signs of senescence during the experiments. Additionally, when an intron alpha PCR-gel electrophoresis was photographed, several other isolates showed amplification of fragments that show similar sizes as intron alpha does (Fig. 3). Unfortunately, this fragments could not be successfully sequenced. Nonetheless, it is noteworthy to mention that non of the isolates that expressed this ‘intron alpha-like amplified fragment’ (for the exception of all *P. anserinas*) were senescing. Moreover, non of the senescing isolates showed a positive amplification product when an intron alpha PCR program was run (Fig 3). A part from this ‘intron alpha-like amplified fragment’ other bigger and smaller fragments were amplified. However, the exact nature of this fragments remains to be elucidated.

Although further research should be done regarding this aspect, it can be concluded that the presence or absence of intron alpha does not seem to be related with the onset of senescence within the Sordariales.

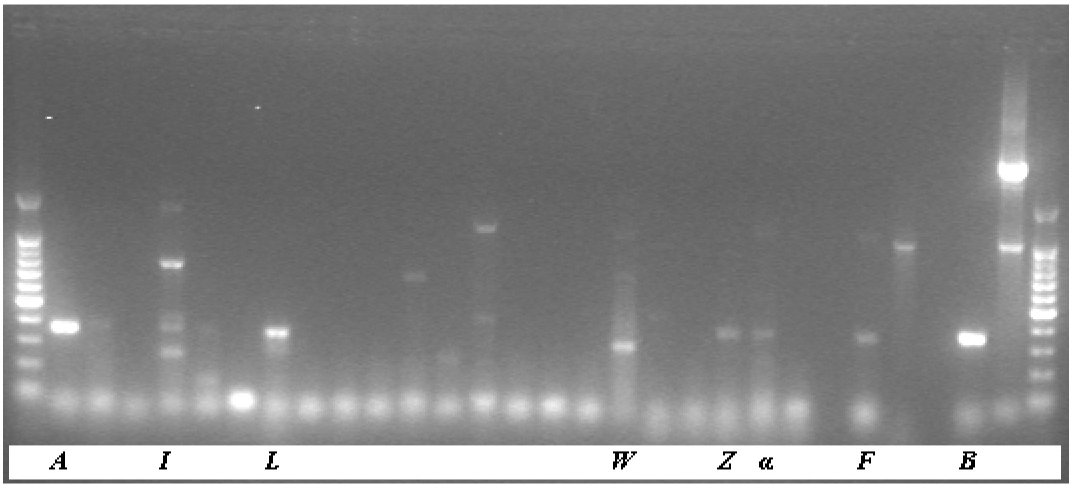


Figure 3. PCR-gel electrophoresis of intron alpha which yields products of approximately 370-bp. A, B and F show intron alpha in three *P. anserina* isolates; L represents *Apiosordaria verruculosa* an non-senescing isolate; W, Z and α represent the non-senescing *Podospora prethopodalis*, *P. vesticola* and *P. longicollis* respectively. In all other isolates intron alpha was not detected.

4. Conclusions

This study contributes to the understanding of the relationship that phylogeny and ecology have over differential life spans in ascomycetes. The fixation of a key variable –the substrate- allows the researcher to examine how different life histories render specific phenotypes on the dependence of the fixed variable. Additionally, it permits further research over the phenotypes of interest, which again, may help correlate specific intramolecular organizations to such phenotype -the senescing phenotype. Hence, it opens the possibility to draw preliminary conclusions with respect to genotype responses towards specific substrates. The fact that a specific genotype is the reflection of the life history of the organism, permits the resulting phenotypes to be correlated to phylogeny or rather to ecology when a phylogenetic reconstruction of a group of organisms is carried out. By these means, and as a preliminary conclusion, it could be observed that -within the Sordariales- the ecology of the organism, specifically the substrate where it develops, influences the relative life span in a greater extent than the phylogeny does. In addition to this, there seems to be no strong correlation between the presence of extrachromosomal DNA species -mainly senDNAα- and the onset of senescence. However, in order to validate the above mentioned additional information regarding responses towards different substrates should be collected, bioinformatic analysis should be refined, and DNA sequences of interest should be confirmed.

5. Acknowledgments

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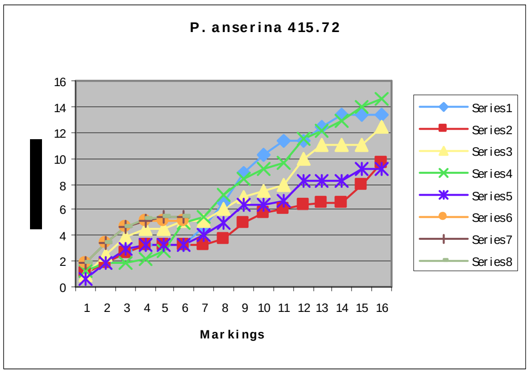
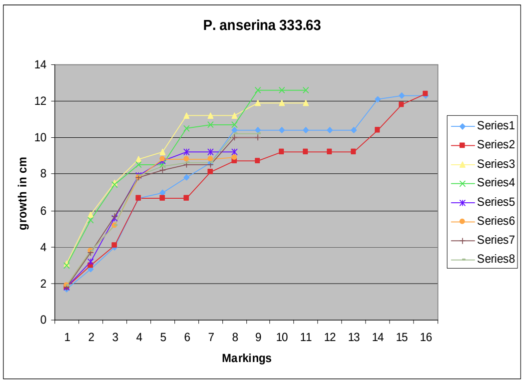
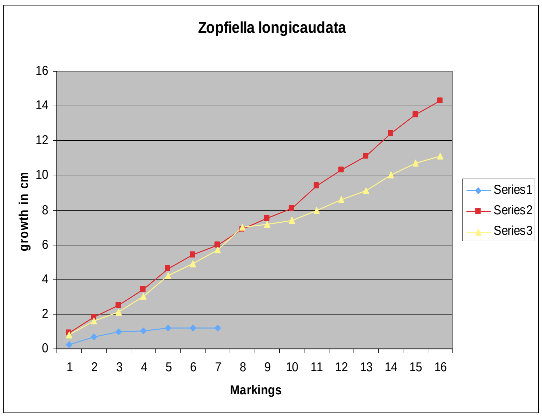
Annexes.

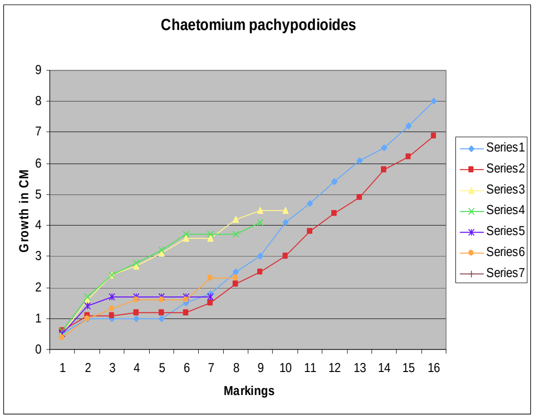
A.

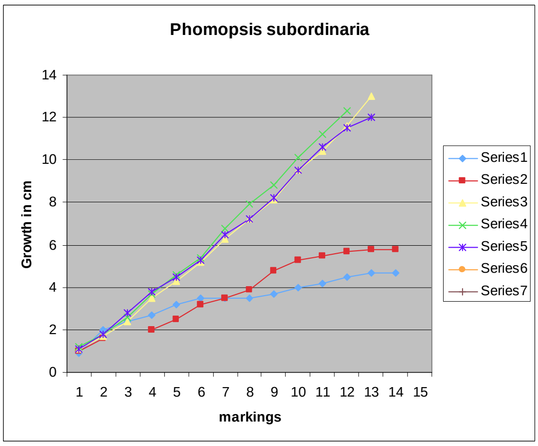
Showing growth rate patterns of ageing isolates when compared to non ageing isolates. Notice the linear growth rate of the non-ageing isolates (last graphs) when compared to other isolates that show senescence.

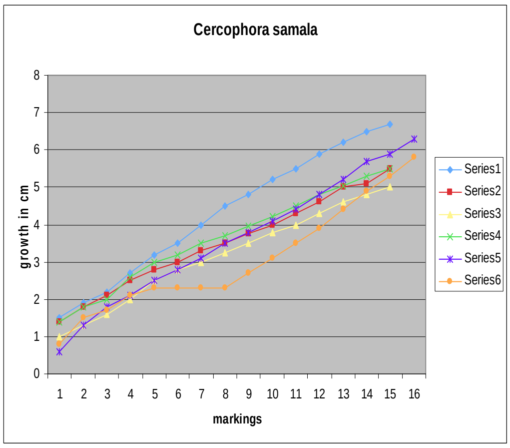


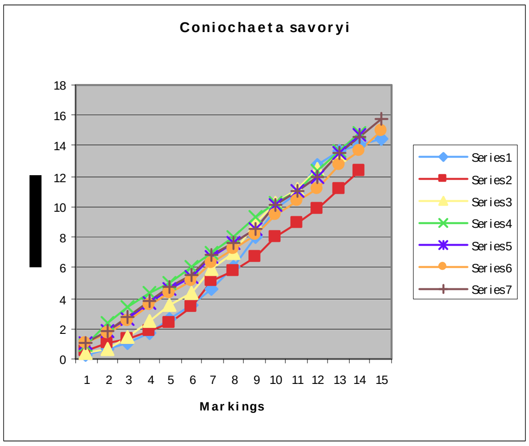


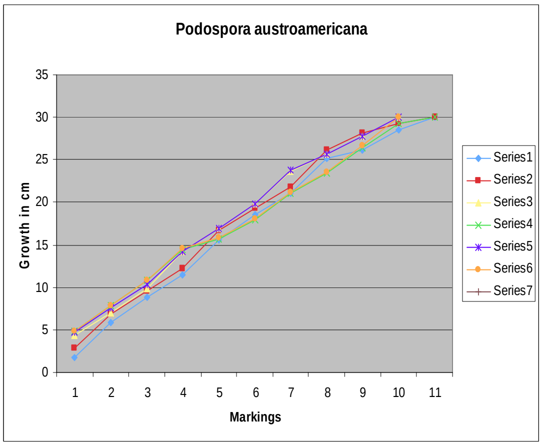












B.

B.1. Comparison of the length, 5'and 3'splicing sites and Internal conserved sequence of the β-tubulin IVS6 intron.

CBS No.	IVS	5'	ICS	3'	length bp
		CTT before			
104.84	6	GTAAGT	GCTGACA	TAG	62
106.77	6	GTAAGT	ACTGATT	CAG	62
119.57	6	GTAAGT	ACTAATA	CAG	56
120013	6	GTAAGT	GCTAACT	CAG	60
121128	6	#	#	#	
124.78	6	GTAAGT	ACTGACT	TAG	64
143.5	6	GTGAGT	GCTAACA	CAG	62
155.77	6	#	#	#	
227.87	6	GTAAGT	GCTAACA	CAG	52
244.28	6	GTAAGT	ACTAACA	TAG	59
247.71	6	#	#	#	
253.71	6	GTAAGT	ACTGACT	TAG	64
258.78	6	GTAAGT	ACTAACC	CAG	63
270.91	6	GTAAGT	ACTAACA	AAG	59
307.81	6	GTAAGT	ACTGACT	TAG	67
315.91	6	GTAAGT	ACTAACC	CAG	55
317.91	6	GTAAGT	GCTAACA	CAG	52
333.63	6	GTAAGT	ACTGACT	TAG	64
368.52	6	#	#	#	
377.74	6	GTAAGT	ACTAACG	CAG	57
415.73	6	GTGAGT	GCTAACA	TAG	56
435.5	6	GTAAGT	ACTGACT	CAG	64
438.67	6	#	#	#	
451.62	6	GTAAGT	ACTGACT	TAG	64
455.64	6	GTAAGT	ACTGACT	TAG	64
571.69	6	GTGAGT	ACTGACA	CAG	57
724.68	6	GTAAGT	ACTAACG	CAG	60
725.69	6	GTGAGT	GCTGACC	TAG	54
955.72	6	GTAAGA	GCTAACA	CAG	57
		5'consensus	ICS	3'consensus	

			consensus		
		GT A/G A GT	G/A CT G/A AC A/T	PyAG	

# indicates that the fungal isolate lacks the intron
ICS= Internal Conserved sequence.
At the bottom of the table the consensus sequences for the 5’, 3’ and ICS have been established. Slashes between bases denote that in that position any of the bases showed can be found.
CTT before= indicates that CTT is always present in the coding region before the intron’s 5’splicing site.

B.2. Comparison of the length, 5’and 3’splicing sites and Internal conserved sequence of the β-tubulin IVS5 intron.

CBS No.	**IVS**	**5'**	**ICS**	**3'**	**length bp**
		GAG before			
104.84	5	GTGAGT	ACTGACG	CAG	95
106.77	5	GTAAAT	GCTGACA	CAG	69
119.57	5	GTTCGT	GCTGACA	CAG	71
120013	5	GTGCGA	GCTGACT	CAG	72
121128	5	GTCAGT	ACTGACA	CAG	80
124.78	5	GTACAT	GCTAACA	CAG	71
143.50	5	GTGAGT	GCTCAAG	CAG	66
155.77	5	GTCAGT	ACTGATG	CAG	68
227.87	5	GTGCGT	GCTAACG	CAG	69
244.28	5	GTGAGC	GCTCGCT	CAG	70
247.71	5	GTCAGT	GCTGATG	CAG	61
253.71	5	GTACAT	GCTAACA	CAG	71
258.78	5	GTGAGT	GCTAATC	CAG	71
270.91	5	GTGAGC	GCTAATT	CAG	70
307.81	5	GTAAAT	GCTAACA	CAG	72
315.91	5	GTCAGT	ACTAACA	CAG	85
317.91	5	GTAGGA	GCTAATC	CAG	62
333.63	5	GTACAT	GCTAACA	CAG	71
368.52	5	GTCAGT	?	CAG	71
377.74	5	GTGAGC	ACTAGCT	AAG	73
415.73	5	GTAAGC	GCTAATT	TAG	66
435.50	5	GTAAAT	GCTAACC	CAG	71
438.67	5	GTGCGG	ACTGACA	CAG	52
451.62	5	GTACAT	GCTAACA	CAG	71
455.64	5	GTACAT	GCTAACA	CAG	71
571.69	5	GTGAGC	GCTAACT	CAG	70
724.68	5	GTAAGC	GCTAACA	CAG	70
725.69	5	GTTAGT	ACTGACT	TAG	97
955.72	5	GTATGG	GCTCACA	AAG	69
		5’consensus	ICS consensus	3’consensus	
		GT-A/C G/A -	A/T CT - A - -	PyAG	

# indicates that the fungal isolate lacks the intron
ICS= Internal Conserved sequence.

?= indicates that no conserved ICS was observed.
GAG before= indicates that GAG is always present in the coding region before the intron’s 5’splicing site.
At the bottom of the table the consensus sequences for the 5’, 3’ and ICS have been established. Slashes between bases denote that in that position any of the bases showed can be found. Dashes indicate that any base pair can be found at that position.

B.3. Comparison of the length, 5’and 3’splicing sites and Internal conserved sequence of the β-tubulin IVS4 intron.

CBS No.	**IVS**	**5'**	**ICS**	**3'**	**length bp**
		GT before*			
104.84	4	GTATGC	ACTGATG	CAG	70
106.77	4	ATATGT	ACTCACC	TAG	56
119.57	4	GTACGT	GCTCACC	CAG	61
120013	4	GTACGT	GCTCACC	TAG	56
121128	4	GTACGT	GCTCACC	TAG	53
124.78	4	GTATGT	ACTCACC	TAG	56
143.50	4	GTATGT	GCTCACC	CAG	59
155.77	4	GTACGT	ACTCACC	TAG	65
227.87	4	GTACGT	GCTCACC	TAG	55
244.28	4	GTACGT	GCTCACC	CAG	65
247.71	4	GTACGT	ACTCACC	TAG	65
253.71	4	GTATGT	ACTCACC	TAG	56
258.78		#	#	#	
270.91	4	GTACGT	GCTCACC	CAG	65
307.81	4	GTATGT	ACTCACC	TAG	56
315.91	4	GTATGT	GCTCACC	TAG	62
317.91	4	GTATGT	GCTCACG	CAG	58
333.63	4	GTATGT	ACTCACC	TAG	56
368.52	4	GTACGT	GCTCACC	TAG	55
377.74	4	GTACGT	GCTCACC	CAG	65
415.73	4	GTATGT	GCTCACC	TAG	54
435.50	4	GTATGT	ACTCACC	TAG	56
438.67		#	#	#	
451.62	4	GTATGT	ACTCACC	TAG	56
455.64	4	GTATGT	ACTCACC	TAG	56
571.69	4	GTACGT	GCTCACC	CAG	65
724.68	4	GTATGT	ACTCACC	TAG	56
725.69	4	GTACGT	GCTCACC	TAG	61
955.72	4	GTACGT	GCTGACC	TAG	82
		*725.91 has AT before			
		5’consensus	ICS consensus	3’consensus	
		GTA T/C GT	G/A CT C ACC	PyAG	

# indicates that the fungal isolate lacks the intron
ICS= Internal Conserved sequence.
GT before= indicates that GT is always present in the coding region before the intron’s 5’splicing site (for the exception of isolate 725.91).

At the bottom of the table the consensus sequences for the 5’, 3’ and ICS have been established. Slashes between bases denote that in that position any of the bases showed can be found.

B.4. Comparison of the 3’splicing sites and Internal conserved sequence of the β-tubulin IVS3 intron.

CBS No.	IVS	5'	ICS	3'
		Not amplified		
104.84	3		ACTGACC	TAG
106.77	3		ACTGACT	CAG
119.57	3		GCTGACT	CAG
120013	3		ACTGACA	CAG
121128	3		ACTGACC	CAG
124.78	3		ACTGACC	CAG
143.50	3		GCTAACT	CAG
155.77	3		ACTAACC	CAG
227.87	3	Not amplified	GCTGACT	CAG
244.28	3		GCTGACA	CAG
247.71	3		GCTAACA	CAG
253.71	3		ACTGACA	CAG
258.78	3		#	#
270.91	3		GCTGACA	CAG
307.81	3		ACTGACA	CAG
315.91	3		ATTGACT	TAG
317.91	3		ACTAACC	TAG
333.63	3		ACTGACA	CAG
368.52	3	Not amplified	GCTGACA	CAG
377.74	3		GCTGACA	CAG
415.73	3		CCTAACA	CAG
435.50	3		ACTGACA	CAG
438.67	3		#	#
451.62	3		ACTGACA	CAG
455.64	3		ACTGACA	CAG
571.69	3		GCTGATA	CAG
724.68	3		ACTGACA	CAG
725.69	3		#	#
955.72	3	Not amplified	ACTTATT	TAG
			ICS consensus	3’consensus
		Not amplified	A/G CT G/A AC -	PyAG

# indicates that the fungal isolate lacks the intron
ICS= Internal Conserved sequence.
The 5’ splicing site could not be defined due to amount of noise present in the sequence. At the bottom of the table the consensus sequences for the 5’, 3’ and ICS have been established. Slashes between bases denote that in that position any of the bases showed can be found. Dashes indicate that any base pair can be found at that position.