Species and speciation in the *Hebeloma crustuliniforme* complex

Promotor: Dr. R.F. Hoekstra,
hoogleraar in de genetica, i.h.b. de populatie- en kwantitatieve genetica

Copromotor: Dr. Th.W. Kuyper, universitair hoofddocent, sectie bodemkunde en plantenvoeding

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Stellingen

1. Het *Hebeloma crustuliniforme*-complex vormt geen monofyletische groep.
   Dit proefschrift.

2. De twee verschillende kernen van sommige natuurlijke *Hebeloma*-dikaryons kunnen zeer verschillende ribosomale ITS-sequenties bevatten.
   Dit proefschrift.

3. Binnen het *Hebeloma crustuliniforme*-complex kan onderscheid gemaakt worden tussen een monofyletische groep intercompatibiliteitsgroepen die voornamelijk bij *Salicaceae* gevonden worden en een monofyletische groep intercompatibiliteitsgroepen die zelden bij *Salicaceae* gevonden worden.
   Dit proefschrift.

4. De meeste van de door verschillende auteurs onderscheiden morfologische soorten binnen het *Hebeloma crustuliniforme*-complex bestaan uit meerdere biologische soorten.
   Dit proefschrift.

5. De wens om morfologisch herkenbare, discrete eenheden te herkennen lijkt voor sommige taxonomen de vader van de gedachte te zijn.

6. Het oudste beroep ter wereld wordt in Europa met uitsterven bedreigd.
   Genesis 2: 19: En zoals de mens elk levend wezen noemen zou, zo zou het heten.
   B. Buyck. 1999. Taxonomists are an endangered species in Europe. Nature 401: 321.

7. In het artikel 'Human longevity at the cost of reproductive success' wordt gesuggereerd dat er een genetisch bepaalde trade-off is tussen levensduur en fertiliteit bij mensen. Uit het artikel blijkt echter niet dat er geen directe, niet-genetisch bepaalde trade-off is tussen levensduur en het aantal kinderen.
   R.G.J. Westendorp en T.B.L. Kirkwood. 1998. Human longevity at the cost of reproductive success. Nature 396: 743-746.

8. De door sommigen aan linksbenige voetballers toegeschreven extra kwaliteiten ten opzichte van rechtsbenige kunnen mogelijk worden verklaard doordat de -relatief zeldzame- linksbenige spelers een frequentieafhankelijk voordeel hebben ten opzichte van de -relatief talrijke- rechtsbenige.
   Johan Cruijff, 'Elf', februari 1984: 'Een linkspoot heb wat extra's, daar moet je van uit gaan'.

9. Resultaten van tests van medicijnen met een bijwerking kunnen onbetrouwbaar zijn doordat placebo's geen bijwerking hebben.
   Intermediair.

10. Rood-groenkleurenblindheid wordt in veel geneticaleerboeken gebruikt als een voorbeeld van een eigenschap bepaald door een recessief allel van één X-gekoppeld gen. In werkelijkheid is er echter sprake van twee X-gekoppelde genen waarvan er van beide een recessief allel is dat een vorm van rood-groenkleurenblindheid veroorzaakt. Dit betekent dat de werkelijke frequentie van kleurenblinde vrouwen nog lager is dan berekend uit de frequentie van kleurenblinde mannen op basis van het één-genmodel.
    G. Waaler. 1927. Zeit. Ind. Abst. Vererb. 45: 279.

11. Iedereen heeft verstand van evolutie. Vooral degenen die er geen verstand van hebben.
Vrij naar Rolf Hoekstra.

Stellingen behorende bij het proefschrift 'Species and speciation in the *Hebeloma crustuliniforme* complex'.
Duur Aanen, Wageningen, 17 november 1999.

2404

Duur Kornelis Aanen

Species and speciation in the *Hebeloma crustuliniforme* complex.

Proefschrift

ter verkrijging van de graad van doctor

op gezag van de rector magnificus

van Wageningen Universiteit,

Dr. C.M. Karssen,

in het openbaar te verdedigen op woensdag 17 november 1999

om half twee 's middags in de Aula.

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[illegible]
LANDBOUWUNIVERSITEIT
WAGENINGEN

aan mijn ouders,

aan Birgith

Contents

1

Introduction

In the next paragraphs the scope of this thesis will be presented. Different aspects of 'Species and speciation in the *Hebeloma crustuliniforme* complex' will be introduced. First the genus *Hebeloma* will be presented. Traditional views on species delimitation and infrageneric subdivisions will be discussed. Species of the genus *Hebeloma* form ectomycorrhizas, and this symbiosis will be the subject of a separate paragraph. Since one of central themes of this thesis is the way to delimit species, a paragraph will be devoted to species concepts. The most important way to delimit species in this thesis is by testing sexual intercompatibility (the 'biological species concept'). Agaricales life cycles and mating systems and the way to use the biological species concept for Agaricales will be discussed. An important part of this thesis is formed by reconstructing phylogenies and some aspects of the data and methods used to reconstruct phylogenies will be discussed. Speciation will be the subject of the next paragraph. The definition of speciation used in this thesis is given and theories on speciation will be discussed. In a final paragraph the aim and outline of this thesis will be presented.

Hebeloma, the genus, infrageneric divisions and species delimitation

In 1821 Fries described *Hebeloma* as a tribus of *Agaricus*, and 50 years later Kummer elevated *Hebeloma* to generic rank. The genus *Hebeloma* is characterized by a rather uniform external appearance. The pileus colour is pinkish-cinnamon to ochraceous cinnamon fading all the way to white near the margin (a few species are predominantly white), and tending to become deeper and richer reddish brown or fuscous in the center. Pileus size ranges from a few milimeters to more than 30 centimeters! The lamellae are dull brownish with whitish edges. Many species have a characteristic odour, ranging from raphanoid to very sweet. The stipe is rather fleshy to fibrous-fleshy, solid, hollow or stuffed. The colour of the stipe is often pure white, sometimes brownish for older specimens, but never lilac or violet. A cortina is present in some species but lacks in many

species. In at least one species a distinct veil is present which assumes the shape of an annulus in mature specimens. A pseudorhiza can be present in some species. All hyphae bear clamp connections. Spores are yellow-brown and usually warty rough. The shape of the spores is usually fusoid to boat-shaped, more rarely ellipsoid-oblong or ellipsoid. Basidia are tetrasporous, but bisporous forms are not rare. No pleurocystidia are present. Cheilocystidia are hyaline, versiform, always distinct and crowded.

The taxonomy of *Hebeloma* is very controversial and has led Favre (1960) to state: "Il n'est pas de genre où la taxonomie des espèces soit plus embrouillée. C'est un véritable chaos." The genus must be considered as one of the less investigated agaric genera. Species delimitations and relationships between species traditionally have been based on morphological characters (Bruchet 1970, Boekhout 1982, Singer 1986, Vesterholt 1989). However, species identification is difficult and species concepts and infrageneric classifications are controversial (Table 1-I). Relatively few morphological characters have been used in existing taxonomic treatments. A submembranaceous ring, unique to *H. radicosum* (Bull.: Fr.) Rick., has been used to delimit subgenus *Myxocybe*, and the presence of a reddish spore print uniquely characterizes *H. sarcophyllum* (Peck) Sacc., which has been assigned to section or subgenus *Porphyrospora* (Bruchet 1970; Singer 1986). Of the remaining species, those with a velum partiale have been placed in section *Indusiata* (Vesterholt 1989), and those without a velum partiale in section *Hebeloma* (formerly called sect. *Denudata*, see Kuyper and Vesterholt 1990). The latter group is fairly heterogeneous and various attempts have been made to arrive at more homogeneous groupings. Vesterholt (1989) proposed that the species with a rooting stipe of section *Hebeloma* should be transferred to *Myxocybe*. Boekhout (1982) recognized two groups within the group without a velum partiale, i) species with strongly ornamented, usually dextrinoid spores with a loosening perispore, cylindrical cheilocystidia, and the potential of having a rooting stipe (section *Anthracophila*), and ii) species with less ornamented spores without a loosening perispore, the presence of weeping lamellae, and a non rooting stipe (section *Denudata*; section *Denudata* subsect. A in Bruchet (1970)). This latter group is called the *H. crustuliniforme* complex, the subject of this thesis. Although the *H. crustuliniforme* complex is generally considered a homogeneous grouping (Bruchet 1970), Boekhout (1982) noted that two separate subgroups within that complex could

TABLE 1-I. Summary of most important infrageneric classifications in *Hebeloma*

Species	Bruchet (1970)	Singer (1986)	Boekhout (1982)	Vesterholt (1989)
	genus *Hebeloma*	= genus *Hebeloma*	= genus *Hebeloma*	= genus *Hebeloma*
	subgenus *Hebeloma*	subgenus *Hebeloma*	subgenus *Hebeloma*	
H. mesophaeum, H. collariatum	sect *Hebeloma*	= sect *Hebeloma*	= sect *Hebeloma*	= sect *Indusiata*
	sect *Denudata*	= sect *Denudata*		
	subsect A		= sect *Denudata*	
H. pusillum, H. lutense, H. helodes	stirps *pusillum*			
H. alpinum	stirps *alpinum*		= subsect *Denudata*	
H. crustuliniforme	stirps *crustuliniforme*			= sect *Hebeloma*
H. velutipes, H. incarnatulum, H. bulbiferum			= subsect *Attenuatocystis*	
			= sect *Anthracophila*	
H. sacchariolens , H. tomentosum	subsect B		subsect *Sacchariolentia*	
H. sinapizans, H. truncatum	subsect C		subsect *Lagenicystis*	
H. anthracophilum,			subsect *Anthracophila*	
H. danicum, H. circinans , H. birrus				
H. cylindrosporum, H. edurum,				
H. sarcophyllum	sect *Porphyrospora*	= subgenus *Porphyrospora*	= sect *Porphyrospora*	= sect *Myxocybe*
H. radicosum	subgenus *Myxocybe*	= subgenus *Myxocybe*	= subgenus *Myxocybe*	

be recognized, exemplified by *H. velutipes* Bruchet (subsection *Attenuatocystis*; dextrinoid spores; slenderly clavate cheilocystidia) and *H. crustuliniforme* (Bull.) Quél. (subsection *Denudata*; non-dextrinoid spores; capitate cheilocystidia). Within the *H. crustuliniforme* complex, one of the best known names is *H. crustuliniforme*, a name regularly encountered in the mycorrhizal literature. However, it is likely that this name is used for a species complex of taxa with relatively pale (but sometimes more brown-tinged), veilless carpophores with weeping lamellae. This complex also includes species such as *H. alpinum* (J. Favre) Bruchet, *H. fragilipes* Romagn., *H. helodes* J. Favre, *H. leucosarx* P.D. Orton, *H. longicaudum* (Pers.: Fr.) Kumm., *H. lutense* Romagn., *H. pusillum* J. Lange, and *H. velutipes* Bruchet (Bruchet 1970, Vesterholt 1993, 1995).

Ectomycorrhiza

Species of *Hebeloma* form ectomycorrhiza (Hacskaylo and Bruchet, 1972) but a few species simultaneously decompose animal wastes (Sagara, 1995). Many species are pioneers, and some species are among the first ectomycorrhizal species to appear during succession (Gryta *et al.*, 1997). The term mycorrhiza refers to an intimate association between a root or other underground organ of a plant and a fungus (Deacon, 1997). Often these associations are mutualistic, because the fungus gains most or all of its carbon from the plant, while the plant obtains mineral nutrients from soil via the fungal hyphae. There are several types of mycorrhiza, the most important of which are arbuscular mycorrhiza and ectomycorrhiza. Arbuscular mycorrhizas are the most common type of mycorrhiza and they are found worldwide on most crop plants, wild herbaceous plants and trees, as well as on pteridophytes and bryophytes. The fungi are members of the Zygomycota. They have a very ancient origin possibly as old as the earliest land plants and could have been a major factor or even the prerequisite in the colonization of land (Smith and Read, 1997).

Ectomycorrhizas are the second most common type of mycorrhizas. In this type of mycorrhiza the fungus forms a structure called the mantle that encloses the rootlet. Hyphae or rhizomorphs radiate outward from the mantle into the substrate. Hyphae also penetrate inwards between the cells of the root to form a complex intercellular system, which appears as a network of hyphae in section, called the Hartig net (Smith and Read, 1997). Ectomycorrhizae are found on coniferous and broad-leaved trees in temperate and boreal

environments. However, most tropical trees and some temperate trees have arbuscular mycorrhiza and some can have both ecto- and arbuscular mycorrhizas (*e.g.* members of the *Salicaceae*). The fungi involved are principally Basidiomycota, but ectomycorrhizas also are formed by some Ascomycota (for example the truffle fungi). There is a wide range in levels of host specificity (defined as the number of tree genera with which a particular fungus associates) in ectomycorrhizal fungi (Molina *et al.*, 1992).

Hebeloma species are generally considered to be 'generalists', *i.e.* with wide host ranges (Molina *et al.*, 1992, Smith and Read, 1997). However, claims on (lack of) host specificity for *Hebeloma* species are difficult to judge, since the delimitation of species is problematical.

Systematics, species and species concepts

For centuries, naturalists have tried to describe diversity in the biological world; this endeavor is known as systematics. The species is generally considered as the basal taxonomic category. Whereas other taxonomic categories have been considered to be defined rather arbitrarily, the species has been thought by many to be real and nonarbitrary and to play a critical role in evolution (Futuyma, 1986). However, different species concepts have been applied and have often led to different species circumscriptions. For fungi, traditionally species were defined typologically by their morphological differences. Characters of individual organisms were compared and similar individuals were designated as a species. Inherent in this construction was an assumed genetic hiatus between dissimilar organisms. Decisions about similarity and dissimilarity of characters, however, were left to the taxonomist. This lack of an objective criterion for the recognition of species has been seen as a big disadvantage of the **morphological species concept** (Kuyper, 1988). There are other reasons why the use of morphological characters to determine species boundaries in fungi has proven inadequate (Petersen and Hughes, 1999). The mushroom fruitbody is relatively simple, so a limited number of characters is available for separating species. Moreover, the infraspecific plasticity and variation for many characters is unknown. Convergent evolution seems to be rather frequent and as a consequence, fungi of divergent evolutionary lineages may share morphological characters. It has been found, for example,

that gilled mushrooms do not form a monophyletic group: gills have evolved at least six times, from morphologically diverse ancestors (Hibbett *et al.*, 1997).

Several species concepts exist that stress evolutionary independence as a criterion for the recognition of species. These concepts have in common that they are applicable to both living and extinct groups, and to sexual and asexual organisms. Under the **evolutionary species concept** of Simpson (1951) a species is 'a lineage (ancestral-descendant sequence of populations) evolving separately from others and with its own unitary evolutionary role and tendencies'. This concept is rather vague operationally in what is meant by 'unitary evolutionary role and tendencies'. Cracraft (1983) has proposed the **phylogenetic species concept**, based on monophyly: 'a species is a monophyletic group composed of the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent'. Although there is now an objective criterion, *viz.* monophyly, a serious problem is how monophyly is to be recognized.

A theoretically more objective species concept is the **biological species concept** (Mayr, 1942), defined as follows: Species are groups of actually or potentially interbreeding populations, which are reproductively isolated from other such groups. According to this view a species is a group of organisms with a common gene pool that is reproductively isolated from other such groups. An argument for the importance of reproductive isolation is that groups that are reproductively isolated for long periods of time are evolutionarily independent, making them effectively separate entities (Mishler and Donoghue, 1982). Breeding discontinuities are thought to be relatively clear-cut and therefore little arbitrary. However, it appears that this is not always the case and variation to interbreed exists in many different degrees (Mishler and Donoghue, 1982). Other difficulties exist in applying the biological species concept. The biological species concept can never be tested in fossil forms and it is irrelevant to asexual populations. Moreover, in some groups of organisms such as plants, the potential of populations, which represent distinct evolutionary units, to hybridise has been maintained (Templeton, 1989). Since it is not the ability to (still) interbreed that determines the status of those distinct evolutionary units, but rather the actual gene flow that occurs *within* those units, other species concepts have been proposed that focus on cohesion within rather than on isolation between populations (**recognition species concept**, Paterson 1985; **cohesion species concept**, Templeton, 1989). However,

the application of these species concepts has some of the same problems as the application of the biological species concept.

Intuitively, the notion that species really exist, independent of our ability to recognize them, is very attractive. A species concept that tests the ability of the organisms to recognize themselves as conspecific (instead of our ability to consider them conspecific) has therefore major advantages. For that reason the basal species concept applied in this thesis is the biological species concept. Most students of speciation recognize the importance of reproductive isolation in the process of speciation, whichever species concept is adopted. Therefore, the study of speciation can best be referred to as the origin of reproductive isolation.

I will first consider life cycles and mating systems before I will discuss the application of this species concept for Agaricales.

Agaricales life cycles and mating systems

Most Agaricales are sexual, only very few are strictly asexual. Within the sexual species, the majority is outcrossing. The predominant life cycle in these outcrossing Agaricales is the haploid-dikaryotic life cycle (Fig. 1-1; Raper, 1966). This life cycle has also been found in species of *Hebeloma* studied so far (Lamoure, 1989; Meyselle *et al.*, 1991). In this life cycle two different vegetative states can be distinguished, the monokaryon and the dikaryon. The monokaryon is the primary mycelium, which develops on germination of a single spore. A dikaryon is derived from the interaction between two monokaryons. This interaction involves the fusion of hyphal cells, without fusion of nuclei. Nuclei generally migrate bidirectionally throughout both mycelia, whereas mitochondria do not migrate. This results in dikaryons having binucleate cells with one nucleus from each monokaryon, that are mosaic for mitochondria from the two monokaryons. Heteroplasmy only occurs in the region where the hyphae of the two monokaryons meet. Recombination between different populations of mitochondrial genomes in a natural population of *Armillaria gallica* has been shown to occur (Saville *et al.*, 1998). In most species, nuclear division is regulated by a special kind of cell division resulting in clamp connections or clamps (Fig. 1-2). The dikaryon can produce fruitbodies (basidiomata) bearing basidia. In

these basidia nuclear fusion occurs, followed by a meiosis and the production of basidiospores.

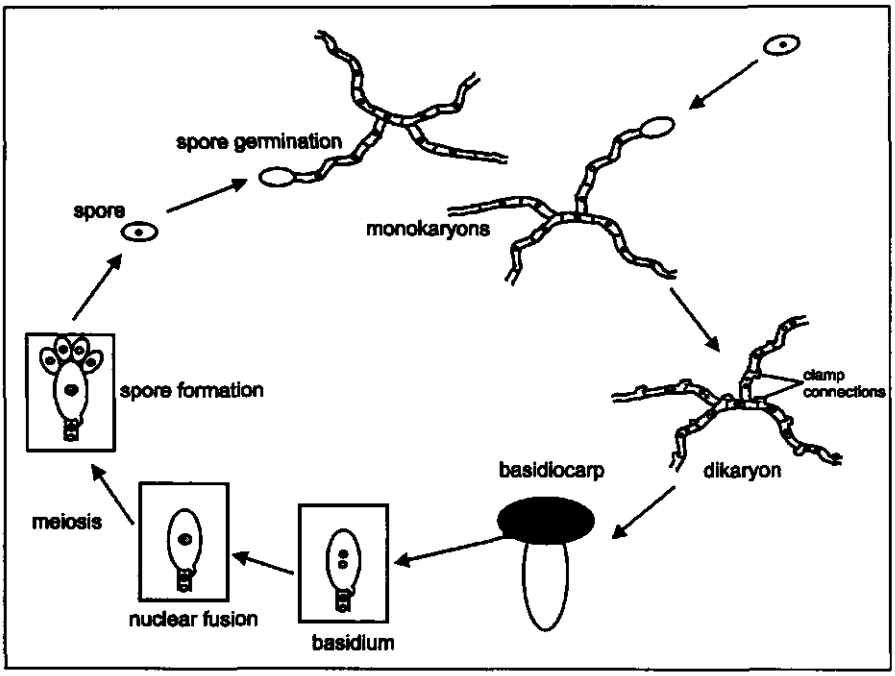

Fig. 1-1. The haploid-dikaryotic life cycle of Basidiomycetes.

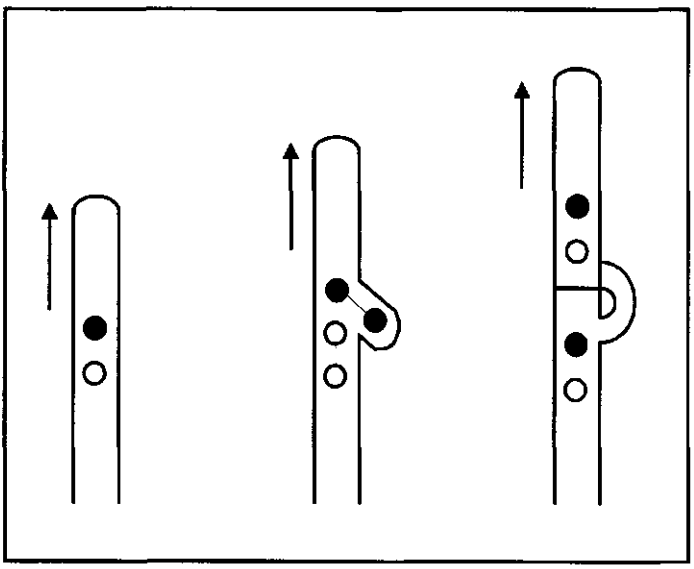

Fig. 1-2. Cellular and nuclear division in a dikaryotic cell of a Basidiomycete. A small branch cell receives the posterior daughter of the leading nucleus, septa form and the hook cell then fuses with the penultimate cell, leaving a clamp connection (right).

The mating system is usually such that inbreeding is suppressed and outbreeding promoted. Most species have a diaphoromictic mating sytem. In this system, mating is regulated by mating type factors. In unifactorial or bipolar species, one factor determines the mating type (A factor), in bifactorial or tetrapolar species, two unlinked factors determine the mating type (the A and B factor). For a successful mating to occur, all factors that determine the mating types must be different. That implies that the descendants of a single dikaryon of a unifactorial species can fuse with half of their siblings, and those of a bifactorial species with only a quarter. However, many mating types occur in a species (in tetrapolar species the number of A factors ranges from 5 to 339 and of B factors from 5 to 240; Murphy and Miller, 1997) which makes for a highly efficient outbreeding system. Some 90 % of higher fungi (Aphyllophorales, Agaricales and Gasteromycetes) are diaphoromictic, and 39 % of these are unifactorial and 61 % bifactorial (Whitehouse, 1949).

Some species have mechanisms that promote inbreeding. Some of these species are strictly homothallic (haploid, selfing). Others have -probably- secondary mechanisms that promote inbreeding and are called secondarily homothallic. They are probably derived from heterothallics. For these species more than one nucleus is put into one spore, sometimes randomly (random amphithallism, Petersen, 1995), with the additional possibility of outcrossing, sometimes directed (directed amphithallism, Petersen, 1995), without the possibility of outcrossing. The species of the genus *Hebeloma* studied so far are bifactorial heterothallic (Lamoure, 1989; Meyselle *et al.*, 1991). Spores are binucleate with identical nuclei, because of an extra mitosis in the spores (Kühner, 1980).

Sexual incompatibility and the biological species concept

In order to apply the biological species concept, one has to test wether progeny is formed, but also if this progeny is viable and fertile. In sexual reproduction, three stages are recognizable: plasmogamy, karyogamy and meiosis. In Agaricales, plasmogamy occurs long before karyogamy and meiosis. Relatively few species of Agaricales sporulate readily in culture (Boidin, 1986). Therefore, usually only plasmogamy is tested between monokaryons when the biological species concept is applied. Plasmogamy is derived from observations of the nuclear state of the mated thalli and the presence or absence of clamps. Theoretically,

pairings between intercompatible populations may not result in viable spores and could thus be considered as infertile. Therefore, crosses resulting in stable dikaryons are best referred to as indicating **intercompatibility** between the mated strains rather than indicating **interfertility** (Boidin, 1986). Strictly speaking, the term **InterCompatibility Group** (ICG) would therefore be more correct than InterSterility Group or **biological species** (Boidin, 1986), although in this thesis the terms InterCompatibility Group and biological species are, following practice among students of Agaricales, used interchangeably.

For ectomycorrhizal fungi, the use of the biological species concept has been restricted to a few genera (*Laccaria*, Mueller and Gardes 1991; Fries and Mueller 1984; *Suillus*, Fries and Neumann 1990; *Paxillus*, Fries 1985). The reason for this limited impact of the biological species concept on ectomycorrhizal fungi is that in vitro spore germination is problematic for most ectomycorrhizal genera. In vitro germination of spores of ectomycorrhizal species has been stimulated in different ways, for example by co-inoculation of certain micro-organisms, such as *Rhodotorula*, growth with living tree roots or dikaryotic mycelium of the same fungal species (see the different studies of Fries and coworkers). Germination of *Hebeloma* spores had previously been shown to be stimulated by living plant roots (Fries and Birraux, 1980).

A fundamental problem with the biological species concept is that intercompatibility relationships need not be transitive, *e.g.* two interincompatible populations are both intercompatible with a third population. Do we then have to recognize one, two or three biological species? I return to this question later.

Reconstruction of phylogenetic history

Evolutionary biology is founded on the fact that organisms share a common history and have subsequently diverged through time. The evolutionary history of a group of organisms is called its phylogeny. Since the history of a group can never be known with absolute certainty, we have to estimate it from data. A phylogenetic tree is a graphical summary of this estimate for a group of organisms.

Traditionally, morphological characters were used to reconstruct phylogenies. For fungi, the scarcity of morphological characters because of their relatively simple morphology, and the lack of a fossil record have impeded this reconstruction. With the

development of molecular techniques, many new characters have become available. Nucleic acid sequence data are the most important kind of data used nowadays.

Phylogenies based on a gene sequence are called gene trees. Traditionally, these gene trees were used to infer phylogenetic relationships between organisms representing species. However, processes like hybridisation and lineage sorting can cause incongruence between gene trees and organismal trees. Therefore caution must be taken to translate single gene trees to organismal trees. It is often argued that congruence among different gene trees provides some of the strongest evidence that a particular estimate for an organismal tree is accurate (Hillis et al., 1996). A new use for gene phylogenies is to study population structure within species. One of the most exciting results of using gene phylogenies is the detection of recombination within species that were assumed to be asexual, by comparing different gene phylogenies (Burt et al., 1996, Geiser et al., 1998).

In this thesis, sequence data have been used to infer phylogenetic relationships i) between biological species and ii) between individuals within biological species.

Sexual incompatibility, cause or consequence of speciation?

Speciation is the process of becoming a species (Otte and Endler, 1989). The meaning of the term speciation therefore depends on the species concept that is applied. Since the basal species concept applied in this thesis is the biological species concept based on sexual intercompatibility, the definition of speciation in this thesis is the origin of sexual interincompatibility between populations.

Reproductive isolation is usually divided into two components, prezygotic and postzygotic isolation, depending on the stage where isolation takes place (Endler, 1989). For heterothallic Agaricales, reproductive isolation could theoretically be divided into three stages:

1. premating (no formation of a dikaryon);
2. postmating but prezygotic (formation of dikaryon, but no spore formation);
3. postzygotic (spore formation, but spores have lower viability).

Since the application of a biological species concept for Agaricales is usually only involving intercompatibility, postmating and postzygotic isolation mechanisms are not tested.

Therefore, reproductive isolation between two strains in this thesis is equated with sexual interincompatibility between those strains.

Relatively few studies have explicitly addressed the evolutionary origin of sexual interincompatibility in Basidiomycetes (Vilgalys; 1991, Vilgalys and Sun, 1995; Garbelotto *et al.*, 1998). Most models of speciation can be classified as belonging to one of two main classes. One class of models (**divergence-first**) assumes that the origin of reproductive barriers between species is preceded by a process of gradual genetic divergence. The classical allopatric mode of speciation (Mayr, 1963) belongs to this class. Within Basidiomycota evidence has been found in support of this class of models, interincompatibility as a *consequence* of genetic divergence (*Pleurotus*, Vilgalys and Sun, 1995; *Exidiopsis plumbescens*, Wells and Wong, 1989).

The genetic basis of sexual interincompatibility has been described by relatively simple models in some cases (Chase and Ullrich, 1990a+b). These simple models have led to hypotheses about sexual incompatibility as a *cause* of divergence (Worrall, 1997, Hallenberg, 1991, Brasier, 1987). Although agreement seems to exist on the fact that genetic reproductive barriers can arise easily between populations of Basidiomycota (Bresinsky *et al.*, 1987), the idea of **incompatibility-first** models is still controversial. Cases of so-called ABC-relationships, where two sympatric populations are interincompatible, but both of them are intercompatible with a third allopatric population (Chamuris, 1991; Hallenberg, 1991; Petersen and Ridley, 1996; Garbelotto *et al.*, 1998), have been interpreted as evidence for this incompatibility-first model, or at least for reinforcement, *i.e.* the evolution of interincompatibility in sympatry after initial genetical divergence in allopatry.

However, the distinction between these two classes, viz. divergence as a *cause* of interincompatibility or divergence as a *consequence* of interincompatibility seems rather artificial. The two classes of causal relationships can better be considered as the extremes of a continuum.

The role of ecological specialisation in speciation has been stressed, both in theoretical models (Maynard Smith, 1966; Diehl and Bush, 1989; Kondrashov and Kondrashov, 1999; Dieckmann and Doebeli, 1999) as well as in case studies, usually concerning host switches in parasitic relationships (*e.g. Heterobasidion annosum*, Garbelotto *et al.*, 1998). For

ectomycorrhizal fungi, host tree switching could theoretically play this role in ecological specialisation (Kretzer *et al.*, 1996).

Aims and outline of this thesis

In this thesis the results are described of a study on the taxonomy of and speciation in the *Hebeloma crustuliniforme* complex. The aims of this study were:

1. To apply a biological species concept by defining InterCompatibility Groups (ICGs) and to look for cases of partially reproductively isolated populations (Chapter 2);
2. To compare the application of a biological species concept with a morphological species concept (Chapter 6);
3. To study phylogenetic relationships between (biological) species (Chapter 3);
4. To study speciation (the origin of reproductive isolation) as a process (Chapter 4 and 5).

In Chapter 7 the results will be integrated and placed in a broader context. Furthermore, new questions that have arisen will be discussed and possibilities to study them in future studies will be proposed.

2

Intercompatibility tests in the *Hebeloma crustuliniforme* complex in northwestern Europe.

Duur K. Aanen and Thomas W. Kuyper

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Abstract

Sexual intercompatibility has been tested for members of the ectomycorrhizal *Hebeloma crustuliniforme* complex. Intercompatibility tests have been applied to 110 collections from northwestern Europe, for which monospore cultures were obtained. Crossing experiments indicated the existence of at least 20 InterCompatibility Groups (ICGs). All ICGs were bifactorial heterothallic with multiple mating type alleles. A low level of partial intercompatibility between ICGs was observed in two species clusters. One isolate was completely compatible with members of two ICGs (and partly compatible with one member of a third ICG) and could therefore not be assigned to an ICG. A large majority of these 20 ICGs could not be recognized by morphological characters, supporting claims that many basidiomycete morphospecies contain cryptic biological species. Possible explanations for this large number of ICGs that are not morphologically, ecologically or geographically delineated are discussed. A simplified strategy for executing intercompatibility tests with a high number of isolates is described. This procedure could substantially reduce the number of necessary matings.

Introduction

Species of the genus *Hebeloma* (Fr.) Kumm. (Agaricales, Basidiomycotina) form ectomycorrhizas with many tree species under a wide range of environmental conditions. According to Molina et al (1992) members of this genus have a broad host range with apparently little specificity. However, the genus is considered taxonomically difficult and the taxonomic status of a number of the recognized taxa is uncertain, making it difficult to

evaluate this claim about lack of specificity. Within the genus, one of the best known names is *H. crustuliniforme* (Bull.) Quél., a name regularly encountered in the mycorrhizal literature. However, it is likely that this name is used for a species complex of taxa with relatively pale (but sometimes more brown-tinged), veilless carpophores with weeping lamellae. This complex also includes species such as *H. alpinum* (J. Favre) Bruchet, *H. fragilipes* Romagn., *H. helodes* J. Favre, *H. leucosarx* P.D. Orton, *H. longicaudum* (Pers.: Fr.) Kumm., *H. pusillum* J. Lange, and *H. velutipes* Bruchet (Bruchet 1970, Vesterholt 1993, 1995). For the purpose of this paper we will refer to the *H. crustuliniforme* complex.

As part of a study on the taxonomy of and speciation in the *H. crustuliniforme* complex, sexual intercompatibility has been tested. Sexual intercompatibility has been widely used in mycology to delimit intercompatibility groups in the last decades, especially for saprotrophic basidiomycetes (Boidin 1986, Petersen 1995a). However, for ectomycorrhizal fungi the use of sexual intercompatibility to delimit species has been limited to a few species complexes or genera (for example *Laccaria* B. & Br. - Fries 1983, Fries and Mueller 1984, Mueller 1991, Mueller and Gardes 1991 Raffle et al 1995, *Paxillus* Fr. - Fries 1985, *Suillus* Adans - Fries and Neumann 1990). The main reasons for the relatively small impact of sexual intercompatibility in the taxonomy of ectomycorrhizal fungi are the problematic spore germination and cultivation under *in vitro* conditions.

Within intercompatibility groups mating is regulated by mating type incompatibility (homogenic incompatibility). This system, which prevents mating when one or more mating alleles are identical, affects the balance between outbreeding and inbreeding by very strongly promoting outbreeding. Interincompatibility and intercompatibility (rather than intersterility and interfertility: Boidin 1986) imply pairing reactions regulated by factors other than mating type factors. This phenomenon has also been described as heterogenic incompatibility (Burnett 1975) in case of cryptic species within morphospecies. From an evolutionary point of view these interincompatibility factors affect the degree of genetic isolation and the formation of new species. An InterCompatibility Group (ICG) is defined as a group within which all combinations are intercompatible and between which there is no intercompatibility. Assuming that dikaryons can form fertile sporocarps, such ICGs can be equated with biological species under the biological species concept. However, because spore formation is not tested, we prefer to use the term InterCompatibilty Group (ICG).

Although interincompatibility is often used qualitatively as a criterion to delimit ICGs, the phenomenon can be better considered quantitative.

Several examples have been found of interincompatibility reactions within ICGs independent of mating type factors and of occasional intercompatibility reactions between taxa that are to a very large extent interincompatible (Petersen and Ridley 1996, Chamuris 1991, Mueller and Gardes 1991, Chase and Ullrich 1990 a,b, Fischer 1996, Petersen 1992). Such cases can be considered as indications for incipient or recent speciation events. Such cases have been hypothesized to contribute to our understanding of patterns of speciation and to support models of sympatric speciation, especially in cases where sympatric populations are interincompatible, whereas both of them are compatible with allopatric populations. Such triangular relationships (so-called ABC inter(in)compatibility reactions) have been reported for *Armillaria* (Fr.) Staude (Anderson et al 1980), *Trichaptum* Murrill (Macrae 1967), and *Amylostereum* Boidin (Boidin and Lanquetin 1984).

The main goal of our study was to delineate reproductively isolated groups (ICGs) within the *H. crustuliniforme* complex. Studies on phylogenetic relationships among ICGs and among populations within ICGs will be the subject of a separate paper. Comparisons between ICGs and morphological species and the relationship between ICGs and host specialisation also will be treated separately.

Material and Methods

Media

The medium used for spore germination was the medium described by Fries (1978), further referred to as Fries medium. Intercompatibility tests were either performed on Fries medium or on a diluted malt extract medium [5 g/l maltextract agar (Merck) and 10 g/l agar]. For most isolates radial growth was found to be highest on this latter medium, although biomass increment was very low.

Collection

Carpophores belonging to the *H. crustuliniforme* complex were collected in a variety of habitats, with a variety of host trees in northwestern Europe in 1994, 1995 and 1996.

Only carpophores growing closely together were considered to belong to the same mycelium and were given one collection number. In cases of doubt, only one carpophore was collected. Table 2-I presents data on all collections that have been used in this study. For each collection, dikaryotic mycelium was regenerated from excised sporocarp context tissue and monokaryons were obtained using spore germination. Sporocarps were dried and preserved in the herbarium of the Biological Station Wijster (WBS) for future reference. Data on ecology (including potential host trees), and macro- and micromorphology for each collection were recorded. Sporocarps, dikaryotic tissue, spores, and monokaryons received the same stock number. Within a stock, each monokaryon was given an additional individual number. A selection of fungal cultures are preserved at the Centraalbureau voor Schimmelcultures, Baarn (CBS).

Spore germination

Generally, several spore prints per collection were directly deposited on agar plates containing Fries medium. Exposure time of the agar plates was varied from 15 min to five h to obtain different spore densities. Agar plates were incubated at 20 C for several wk. As soon as monokaryons were visible by eye (usually starting after two wk), they were isolated and transferred to agar plates containing the same medium. After one or two wk, the mycelia were again transferred to new plates to obtain mycelia without spores. Up to 25 monokaryotic mycelia were isolated per collection in this way, but usually less. Mycelia were examined microscopically (with an inverted microscope) for the absence of clamp connections as an indication of monokaryotic state, before being used in intercompatibility tests.

Some additional methods were used to obtain spore germination. Spore prints were made on empty Petri dishes. These spore prints were stored in a refrigerator (4 C) for extended periods (up to four months). Spore prints were suspended in 0.5 to 1 mL sterilized water with a drop of sterile Tween 20. Dilutions were made to obtain spore concentrations of 10^5 to 10^6 spores mL^{-1}. From these suspensions 0.1 mL was plated out on agar plates containing Fries medium and the surfaces of the plates were air dried for 10 min. Finally, to induce spore germination, dikaryotic mycelia of the same collection or birch tree seedlings were placed in the center of the agar plates (Fries and Birraux 1980, Ali and Jackson 1988).

To attach the roots of the birch seedlings on the agar surface, roots were covered with sterilized uncoated cellophane.

Intercompatibility tests

Intercompatibility tests were performed by placing two pieces of mycelium taken from the margin of an actively growing monokaryon on a five cm Petri plate, containing malt extract agar or Fries medium two mm apart. Petri plates were incubated at 20 C. After three wk of contact, mycelium from contact zones was inspected microscopically through the bottom of the Petri dishes (at X 320 with an inverted microscope) for the presence of clamp connections, indicating dikaryotization. Mycelium from the margins of the colonies was also inspected for the presence of clamp connections as an indication of unilateral or bilateral nuclear migration. The density and shape of clamp connections were also inspected and abnormalities were noted. After an additional three wk plates were inspected again. Occasionally, slides were prepared and studied microscopically at times 400 or 1000 for the presence of clamp connections.

Positive intercompatibility reactions were classified as one of three types: (i) development of dikaryotic mycelium only in contact zone followed by outgrowth (indicated by O in tables), (ii) unidirectional nuclear migration into one of the monokaryons (indicated by U if the upper monokaryon in the table was dikaryotized and with L if the left monokaryon in the table was dikaryotized) and (iii) bidirectional nuclear migration resulting in a completely dikaryotic mycelium (indicated with B).

To determine the nuclear status of clampless and clamp-bearing mycelia, the numbers of nuclei per cell of some clampless and clamp-bearing mycelia were determined by staining mycelial fragments with the fluorochrome 4',6-diamidino-2-phenylindole (DAPI: 0.5µg mL^{-1}) and observation under epifluorescence microscopy.

Mating type tests were performed to recover all mating types from one collection of each ICG that was found.

The strategy to find ICGs was as follows. First, the maximum possible number of ICGs was reduced from 110 to 27. The steps to achieve this reduction were as follows. From the first 34 collections, one monokaryon was selected and these 34 monokaryons were paired in most possible combinations. The results obtained by this initial pairing indicated a

maximum of 16 ICGs. From the 16 ICGs found at this stage, one monokaryon of each collection was chosen as a preliminary tester for new collections. Of the 76 new collections tested in this way, 50 could be unambiguously placed into one of the existing ICGs. The 26 monokaryons that were not compatible with any of the 16 preliminary tester strains were subsequently paired in all possible combinations. This pairing provided evidence for a maximum of 11 additional ICGs. Through both strategies the maximum possible number of ICGs had been reduced to 27 (16 from the first two years + 11 from the third year).

Second, a preliminary division of the ICGs into four clusters was made on the basis of morphological characters. Four clusters were recognized. The clusters comprised the species complexes around i) *H. crustuliniforme* (Bull.) Quél., incl. *H. alpinum* (J. Favre) Bruchet (spores ± indextrinoid; cheilocystidia (sub)capitate; stipe thick-set, floccose; pileus whitish to yellowish buff); ii) *H. helodes* J. Favre, incl. *H. pusillum* J. Lange (spores ± indextrinoid; cheilocystidia (sub)capitate; stipe rather slender, pruinose to subfloccose; pileus yellow-brown to red-brown); iii) *H. leucosarx sensu auct. neerl.* (spores ± indextrinoid; cheilocystidia (sub)capitate; stipe short and rather thick-set, slightly floccose; pileus orange-brown to red-brown); iv) *H. velutipes* Bruchet (spores ± dextrinoid; cheilocystidia cylindrico-clavate, never (sub)capitate; pileus yellowish to orange-brown).

One monokaryon of each collection was chosen and all combinations between those monokaryons within each cluster were made to determine the exact pattern of compatibility and to find cases of partial compatibility. This reduced the total number of ICGs to 22 at most.

This scheme resulted in 13 groups that consisted of three or more fully compatible collections, and nine groups that consisted of two or one collections. When a group consisted of three or more fully compatible collections, other isolates that belong to this ICG must necessarily form a dikaryon with at least one of these isolates. For that reason, we could not have missed isolates that belonged to such a group, even when only one monokaryon of an isolate has been tested against the three fully compatible collections. For groups consisting of less than three fully compatible isolates, however, this procedure could have missed single isolates that belong to such an ICG, because they potentially shared one or more mating factors and therefore were unable to form dikaryons. For those ICGs only we had to determine all mating types for at least one of the collections. After this had been done, intercompatibility tests were performed between all recovered mating types for the

members of these groups. This did not change our conclusions with regards to the maximum number of ICGs, which remained 22.

Results

Spore germination

From 110 collections monospore cultures were obtained. The most successful way to obtain monokaryons appeared to be by collecting spores from fresh sporocarps on Fries medium. We were unable to obtain monokaryons for only 6 % of the collections that were tested this way. The percentage germination per collection was usually very low. Use of spore prints stored for some time at 4 C was less successful. This method worked for only some isolates. These isolates appeared to belong to ICG 16, 17, or 18 (= morphological cluster 4). Dikaryotic mycelium of the same collection and birch tree seedlings sometimes had a slightly stimulating effect, although this effect was not large.

Intercompatibility tests.

Usually after three wk results of intercompatibility tests could be scored. Nuclear staining with DAPI of some isolates revealed that clampless mycelia had one nucleus per cell, whereas clamp-bearing mycelia had two nuclei per cell. The results of mating type tests indicate a heterothallic, tetrapolar mating system. In negative combinations, common AB, common A, and common B combinations could not be recognized on morphological grounds. We therefore only looked for pairs of fully compatible monokaryons for a collection (Murphy and Miller 1997, Fries and Mueller 1984) and arbitrarily assigned the numbers I, II, III and IV to the mating types, where I and II are compatible as III and IV are. To find the two pairs, we first paired five or 10 monokaryons in all possible combinations. In this way we usually found at least one pair of compatible monokaryons and at least one monokaryon that was not compatible with any of this pair. This monokaryon was tested with additional monokaryons to look for the opposite member of the second pair. The results of the search for pairs of compatible mating types for one collection per ICG are summarized in Table 2-II.

InterCompatibility Groups.

When analysing the 110 collections according to the procedure described, the maximum number of ICGs turned out to be 22 (Table 2-I). On the basis of morphological criteria, four morphological clusters have been recognized. These clusters were provisionally equated with morphospecies, but we do not claim that these clusters can be maintained as morphospecies in a final revision of this complex (Aanen et al unpubl).

Cluster 1: H. crustuliniforme - H. alpinum.-- This cluster was found to consist of five ICGs (Table 2-III). However, four ICGs, 1- 4, showed levels of partial intercompatibility ranging from 0.9 to 8.3 %. Isolate 9605-5 was completely intercompatible with all members of ICGs 3 and 4 and with one member (9671-2) of ICG 2. This collection (9605) could therefore not inequivocally be assigned to one ICG (indicated in Tables 1 and 3). All other cases of partial intercompatibility involved situations in which two isolates could clearly be placed in two different ICGs, but were intercompatible (for example the combination between 9613-9, which belongs to ICG 4, and 9680-1, which belongs to ICG 3). The compatible combinations between ICGs 1 and 2 did not show nuclear migration (indicated with an O), whereas combinations between 3 and 4 sometimes did (see discussion). Moreover, some of the combinations between ICG 1 and 2 showed reduced growth rate and aberrant clamp connections, whereas the combinations between ICG 3 and 4 and the single combination between ICG 2 and 4 did not (indicated in Table 2-III).

Cluster 2: H. pusillum - H. helodes.-- The relatively broad circumscription of this morphological cluster was adopted because all forms intermediate between very slender to fairly robust and from pale-colored to (dark) brown were encountered. Within this morphological cluster at most 12 ICGs could be distinguished (Table 2-IV). However, strains 9692-1 and 9694-1 have never formed a dikaryon, not in the intercollection tests, nor in the within collection tests. We can therefore not exclude the possibility that these strains are 'incompetent' (Petersen, pers comm). We therefore at this point do not conclude that these two collections represent other ICGs. All the found ICGs were completely isolated and no cases of partial intercompatibility were found. A relatively large proportion of the pairings within ICG 9 (33%) did not result in dikaryons. Growth rate of several monokaryons of that ICG was also remarkably low. We did not determine whether failure to dikaryotize was due to shared mating type alleles or to factors other than mating type.

Cluster 3: H. leucosarx sensu auct. neerl.-- This cluster consisted of two ICGs that were completely isolated (Table 2-V). Within ICGs all combinations were intercompatible.

Cluster 4: H. velutipes.-- In this cluster, three ICGs were found, two of which, 16 and 17, were partially compatible. Usually in positive combinations between these ICGs only contact zones had clamp connections, whereas combinations within ICGs often showed nuclear migration in one or both directions (Table 2-VI). Moreover, hyphal morphology of such illegitimate combinations often was aberrant: clamp connections were more dispersed and hyphae irregularly shaped. Finally, growth of intercompatible combinations between ICGs was reduced.

Discussion

Spore germination.

The best results for spore germination were obtained by incubating spores freshly collected on Fries medium at 20 C. Worse results were obtained with spores that had been stored at 4 C for some time. Whether this is due to a loss of germinability or to the treatment after storage has not been established. As the main goal of this study was to obtain monokaryons and not to investigate patterns and mechanisms of spore germination, no quantitative estimates were made of different spore treatments.

Intercompatibility tests.

We had two reasons to perform intercompatibility tests according to the scheme as outlined below: i) collecting was done during different years, but initial intercompatibility tests were already performed before the collecting was finished. When additional collections were made in subsequent years, not all test had to be done because of the available results of the initial tests and ii) in order to reduce the number of intercompatibility tests. The total number of matings performed amounted to about 7000. This contrasts conspicuously with the 97580 tests that would have been necessary if we would have searched for all mating types for each collection and would have tested all possible combinations between all mating types. At least 660 [=110 x 6] tests are necessary to recover all mating types, and 95,920

[((4 x 110)-1) x (4 x 55) - 110 x 6] tests are necessary to test mating between monokaryons. Our protocol could therefore well reduce the number of tests that are necessary by more than 90%.

Members of this species complex have a heterothallic tetrapolar (bifactorial) mating system with multiple mating alleles. Other species of the genus *Hebeloma*, many of them not belonging to the *H. crustuliniforme* complex, such as *H. cylindrosporum* Romagn., *H. anthracophilum* R. Maire, *H. edurum* Métrod ex M. Bon, *H. longicaudum* (Pers.: Fr.) Kumm., *H. radicosum* (Bull.: Fr.) Ricken, *H. sinapizans* (Fr.) Gillet, *H. subsaponaceum* P. Karst., and *H. truncatum* (Schaeff.: Fr.) Kumm. have also been shown to possess a heterothallic bifactorial mating system with multiple mating types (Lamoure 1989, Meyselle et al 1991). Within genera, and even within species mating systems can vary (Hallenberg 1991, Petersen 1995b), but all species of the genus *Hebeloma* tested so far appear to have a bifactorial heterothallic mating system.

Although we did not explicitly address the question of the number of A and B alleles in these ICGs, the results of our crosses show that most ICGs are extensively multi-allelic, similar to results reported for other higher basidiomycetes (Doudrick and Anderson 1989, Raffle et al 1995, Kropp and Fortin 1988, Fries and Mueller 1984, Boidin 1986), resulting in outbreeding potentials usually higher than 90%.

Murphy and Miller (1997) summarized the number of mating types and reported that for tetrapolar species the number of A factors range from 5 to 339 and of B factors from 5 to 240. As a consequence it is usually not necessary to recover all 4 mating types of individual collections, thereby substantially reducing the number of crosses that need to be executed (see Appendix). It appeared often very difficult to recover all 4 mating types, even though sometimes more than 20 monokaryons were tested. This suggests that not all mating types are equally frequent in the germinated spores. Fries and Mueller (1984) had previously also shown a significant deviation from an equal distribution of mating types in the recovered monokaryons of several species of *Laccaria*, but no explanation for this deviation has been proposed.

No false clamps or barrage zones, indicating common B or A factors, respectively, were observed. No false clamps or barrage zone formation were noted either in intercompatibility tests with *Laccaria bicolor s.l.* by Mueller and Gardes (1991).

InterCompatibility Groups

We found a large number of ICGs (20) within this species complex, confirming Hallenberg's (1991) suggestion that many fungal morphospecies contain cryptic biological species. However, in this complex the number of cryptic species seem to be higher than in various groups of saprotrophic fungi. We do not believe that this is an artifact determined by our *a priori* choice to work on a broader range of morphological groups, for the simple reason that the four morphological clusters are not always clearly delineated either and the species status of several of these might well be questioned. Ecological data (Table 1) suggest that the formation of cryptic species is not related to host switches or cospeciation, as many ICGs from clusters one, two and three form ectomycorrhizas with members of the Salicaceae (*Populus*, *Salix*). Biogeographical data furthermore suggest that some ICGs (1, 2, 3, 6, 8, 11, 12, 17 and 21) are widely distributed and not geographically isolated. We hypothesize that the *H. crustuliniforme* complex is evolutionarily young and that many ICGs in this complex have recently diverged. This hypothesis might explain the relative uniformity of both macro- and micromorphological characters. However, it raises new questions about factors that promote high rates of ICG evolution.

Patterns of partial compatibility

Several cases of partial compatibility were noted, both in cluster one and cluster four. In most cases, assignment of isolates to one ICG was possible. Only isolate 9605-5 could not be unequivocally assigned to one ICG. This phenomenon is similar to that found in Petersen and Ridley (1995) with a New Zealand strain of *Pleurotus*.

Isolates that were compatible with more than 50 % of the members of an ICG, were considered to belong to this ICG. The negative pairings were considered to be caused by shared mating alleles but usually no additional pairings were performed to test for this possibility. Due to this procedure, it might be possible that we underestimated the actual number of cases of partial intercompatibility.

Individual compatible combinations between members of the partially compatible ICG 1 and 2 and between members of the partially compatible ICG 16 and 17 showed signs of reduced compatibility. This was reflected by: (i) no or unidirectional nuclear migration, (ii)

reduced growth rate of dikaryon and (iii) aberrant morphology of hyphae. These observations show that these dikaryons are less vigorous than the combinations within ICGs. These observations also support Boidin's (1986) claim that intercompatibility should not be automatically equated with interfertility. On the other hand, most of the individual compatible combinations between the partially compatible ICG 3 and 4 did not show any sign of reduced compatibility and the single compatible combination between ICG 2 and 4 did not either, suggesting that these combinations have a higher persistence.

Partial compatibility between populations, called promiscuity by Petersen and Ridley (1996), might be significant from a micro-evolutionary point of view as indication of incipient speciation. Several examples of partial compatibility have been reported, often referring to allopatric populations that were intercompatible with two sympatric ICGs (*Amylostereum* - Boidin & Lanquetin 1984, *Heterobasidion* - Chase and Ullrich 1990a, b, Garbelotto et al 1998, *Armillaria* - Anderson et al 1980, *Pleurotus* - Petersen and Ridley 1996, *Melanotus* - Petersen 1992, *Trichaptum* - Macrae 1967). In some of our cases, however, such promiscuous collections were sympatric, indicating that allopatry is not a necessary condition for this phenomenon. Such instances of partial intercompatibility are very interesting for studies on the origin of interincompatibility since these populations are on the borderline of populations within ICGs and ICGs. This interface between population genetics and systematics is probably most informative for studies of speciation (Templeton, 1998).

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TABLE 2-I. Collections of the *H. crustuliniforme* complex used in this study.

No.	Locality	Date	Host tree	Morphological species	ICG
9503-2	Borgsjö, Medelpad, Sweden	19-IX-95	*Betula sp.*	*H. crustuliniforme*	1
9618-3	Sustenpass, Bern, Switzerland	7-IX-96	*Salix retusa, S. hastata*	*H. crustuliniforme/H.alpinum*	1
9621-3	Davos, Graubünden, Switzerland	8-IX-96	*Dryas octopetalis*	*H. crustuliniforme/H.alpinum*	1
9673-5	Eemshaven, Groningen, Netherlands	3-X-96	*Salix alba*	*H. crustuliniforme*	1
9674-9	Eemshaven, Groningen, Netherlands	3-X-96	*Salix alba*	*H. crustuliniforme*	1
9675-3	Eemshaven, Groningen, Netherlands	3-X-96	*Salix repens, S. alba*	*H. crustuliniforme*	1
9570-2	Lelystad, Flevoland, NL.	11-X-95	*Salix sp.*	*H. crustuliniforme*	2
9620-2	Davos, Graubünden, Switzerland	8-IX-96	*Dryas octopetalis*	*H. crustuliniforme/H.alpinum*	2
9626-1	Mollendruz, Vaud, Switzerland	21-IX-96	*Salix caprea*	*H. crustuliniforme/H.alpinum*	2
9627-1	Lac des Rouges Truites, Jura, France	22-IX-96	*Corylus avellana*	*H. crustuliniforme*	2
9671-2	Eemshaven, Groningen, Netherlands	3-X-96	*Salix repens*	*H. crustuliniforme*	2
9677-5	Eemshaven, Groningen, Netherlands	3-X-96	*Salix repens*	*H. crustuliniforme*	2
9682-9	Utrecht, Utrecht, Netherlands	13-X-96	*Salix sp.*	*H. crustuliniforme*	2
9683-6	Utrecht, Utrecht, Netherlands	13-X-96	*Salix sp.*	*H. crustuliniforme*	2
9684-1	Utrecht, Utrecht, Netherlands	13-X-96	*Salix sp.*	*H. crustuliniforme*	2
9685-1	Utrecht, Utrecht, Netherlands	13-X-96	*Salix sp.*	*H. crustuliniforme*	2
9511-1	Hilpoltstein, Bayern, Germany	24-IX-95	*Tilia sp.*	*H. crustuliniforme*	3
9672-1	Eemshaven, Groningen, Netherlands	3-X-96	*Salix repens, S. alba*	*H. crustuliniforme*	3

9679-1	Hoornaar, Zuid-Holland, Netherlands	15-X-96	*Populus sp.*	*?H. crustuliniforme*	3
9680-1	Hoornaar, Zuid-Holland, Netherlands	15-X-96	*Populus sp.*	*H. crustuliniforme*	3
9686-9	Utrecht, Utrecht, Netherlands	13-X-96	*Salix sp.*	*H. crustuliniforme*	3
9693-8	Hoornaar, Zuid-Holland, Netherlands	20-X-96	*Populus sp.*	*H. crustuliniforme*	3
9699-1	Elsloo, Limburg, Netherlands	25-X-96	*Populus sp.*	*H. crustuliniforme*	3
9602-4	Adelboden, Bern, Switzerland	5-IX-96	*Dryas octopetala*	*H. crustuliniforme/H.alpinum*	4
9605-1	Adelboden, Bern, Switzerland	5-IX-96	*Salix retusa*	*H. crustuliniforme/H.alpinum*	3/4*
9606-1	Adelboden, Bern, Switzerland	5-IX-96	*Salix retusa, Dryas octopetala*	*H. crustuliniforme/H.alpinum*	4
9607-5	Adelboden, Bern, Switzerland	5-IX-96	*Salix retusa*	*H. crustuliniforme/H.alpinum*	4
9608-4	Adelboden, Bern, Switzerland	5-IX-96	*Salix retusa*	*H. crustuliniforme/H.alpinum*	4
9609-2	Adelboden, Bern, Switzerland	5-IX-96	*Salix retusa, Dryas octopetala*	*H. crustuliniforme/H.alpinum*	4
9613-9	Adelboden, Bern, Switzerland	5-IX-96	*Salix foetida, S. retusa, Dryas octopetala*	*H. crustuliniforme/H.alpinum*	4
9614-2	Adelboden, Bern, Switzerland	5-IX-96	*Dryas octopetala*	*H. crustuliniforme/H.alpinum*	4
9615-2	Adelboden, Bern, Switzerland	5-IX-96	*Dryas octopetala*	*H. crustuliniforme/H.alpinum*	4
9616-3	Adelboden, Bern, Switzerland	5-IX-96	*Salix retusa*	*H. crustuliniforme/H.alpinum*	4
9617-9	Adelboden, Bern, Switzerland	5-IX-96	*Salix retusa*	*H. crustuliniforme/H.alpinum*	4
9546-8	Roden, Drenthe, Netherlands	5-X-95	*Quercus sp., Corylus avellana*	*H.crustuliniforme*	5
9581-1	Utrecht, Utrecht, Netherlands	5-XI-95	*Tilia sp.*	*H.crustuliniforme*	5
9689-7	Utrecht, Utrecht, Netherlands	13-X-96	*Tilia sp.*	*H. crustuliniforme*	5
9691-9	Rijswijk, Gelderland, Netherlands	20-X-96	*Quercus sp.*	*H. crustuliniforme*	5
9501-1	Amsterdam, Noord-Holland, Netherlands	9-IX-95	*Salix sp.*	*H. pusillum*	6
9645-1	Lac des Rouges Truites, Jura, France	24-IX-96	*Salix caprea and S. aurita*	*H. pusillum*	6

9630-1	Lac des Rouges Truites, Jura, France	22-IX-96	*Salix caprea*	*H.pusillum*	7
9654-1	Lac des Rouges Truites, Jura, France	26-IX-96	*Salix purpurea, Salix alba*	*H. pusillum*	7
9507-1	Vledder, Drenthe, Netherlands	20-IX-95	*Salix sp.*	*H.pusillum*	8
9508-2	Vledder, Drenthe, Netherlands	20-IX-95	*Salix sp.*	*H.pusillum*	8
9509-1	Vledder, Drenthe, Netherlands	20-IX-95	*Salix sp.*	*H.pusillum*	8
9644-2	Lac des Rouges Truites, Jura, France	24-IX-96	*Salix sp.*	*H. pusillum*	8
9648-2	Lac des Rouges Truites, Jura, France	24-IX-96	*Salix purpurea*	*H. pusillum*	8
9690-1	Meerkerk, Zuid-Holland, Netherlands	20-X-96	*Salix aurita, S. cinerea*	*H. pusillum*	8
9538-4	Beilen, Drenthe, Netherlands	4-X-95	*Salix sp.*	*H. helodes/pusillum*	9
9539-2	Wijster, Drenthe, Netherlands	4-X-95	*Salix repens*	*H. helodes/pusillum*	9
9571-1	Eemsterveld, Drenthe, Netherlands	11-X-95	*Salix sp.*	*H. pusillum*	9
9662-4	Wijster, Drenthe, Netherlands	11-X-96	*Salix repens*	*H. helodes/pusillum*	9
9663-6	Wijster, Drenthe, Netherlands	1-X-96	*Salix repens*	*H. helodes/pusillum*	9
9427-5	Beilen, Drenthe, Netherlands	21-IX-94	*Betula sp.*	*H. helodes*	10
9665-5	Beilen, Drenthe, Netherlands	3-X-96	*Salix aurita*	*H. helodes*	10
9573-1	Terschelling, Friesland, Netherlands	11-X-95	*Salix repens*	*H. helodes*	11
9651-2	Lac des Rouges Truites, Jura, France	26-IX-96	*Salix cinerea, Salix purpurea, Picea sp.*	*H. helodes*	11
9652-3	Lac des Rouges Truites, Jura, France	26-IX-96	*Picea sp., Fagus sp.*	*H. helodes*	11
9629-18	Lac des Rouges Truites, Jura, France	22-IX-96	*Salix purpurea*	*H. helodes*	12

9655-1	Lac des Rouges Truites, Jura, France	26-IX-96	*Salix purpurea*	*H. helodes*	12
9660-2	Beilen, Drenthe, Netherlands	1-X-96	*Salix sp.*	*H. helodes*	12
9661-14	Beilen, Drenthe, Netherlands	1-X-96	*Salix sp.*	*H. helodes*	12
9664-5	Beilen, Drenthe, Netherlands	1-X-96	*Salix aurita*	*H. helodes*	12
9696-1	Boerakker, Groningen, Netherlands	18-X-1996	*Populus sp.*	*H. helodes*	12
9692-1	Hoornaar, Zuid-Holland, Netherlands	20-X-1996	*Populus sp.*	*H. helodes*	?
9566-3	Lelystad, Flevoland, Netherlands	11-X-95	*Salix repens*	*H. leucosarx*	14
9567-1	Lelystad, Flevoland, Netherlands	11-X-95	*Salix repens*	*H. leucosarx*	14
9670-3	Eemshaven, Groningen, Netherlands	3-X-96	*Salix repens*	*H. leucosarx*	14
9678-5	Eemshaven, Groningen, Netherlands	3-X-96	*Salix repens*	*H. leucosarx*	14
9576-1	Lauwersmeer, Groningen, Netherlands	24-X-95	*Salix repens*	*H. leucosarx*	15
9624-1	Dwingeloo, Drenthe, Netherlands	18-IX-96	*Pinus sylvestris*	*H. leucosarx*	15
9668-3	Eemshaven, Groningen, Netherlands	3-X-96	*Salix repens*	*H. leucosarx*	15
9669-2	Eemshaven, Groningen, Netherlands	3-X-96	*Salix repens*	*H. leucosarx*	15
9676-8	Eemshaven, Groningen, Netherlands	3-X-96	*Salix repens*	*H. leucosarx*	15
9698-3	Boerakker, Groningen, Netherlands	18-X-1996	*Populus sp.*	*H. leucosarx*	15
9502-3	Wijster, Drenthe, Netherlands	15-IX-95	*Betula pubescens*	*H. velutipes*	16
9541-1	Roden, Drenthe, Netherlands	5-X-95	*Carpinus betulus*	*H. velutipes*	16
9542-1	Roden, Drenthe, Netherlands	5-X-95	*Fagus sp., Carpinus betulus*	*H. velutipes*	16
9543-1	Roden, Drenthe, Netherlands	5-X-95	*Fagus sp., Quercus sp., Corylus avellana*	*H. velutipes*	16
9544-1	Roden, Drenthe, Netherlands	5-X-95	*Quercus sp., Corylus avellana*	*H. velutipes*	16

9545-1	Roden, Drenthe, Netherlands	5-X-95	*Fagus sp.*	*H. velutipes*	16
9407-1	Terschelling, Friesland, Netherlands	7-IX-94	*Fagus sp.*	*H. velutipes*	17
9410-2	Terschelling, Friesland, Netherlands	7-IX-94	*Betula sp.*	*H. velutipes*	17
9432-10	Assen, Drenthe, Netherlands	4-X-94	*Quercus sp.*	*H. velutipes*	17
9504-1	Borgsjö, Medelpad, Sweden	18-IX-95	*Betula sp.*	*H. velutipes*	17
9516-1	Roth, Bayern, Germany	24-IX-95	*Picea sp., Pinus sylvestris*	*H. velutipes*	17
9522-1	Hilpoltstein, Bayern, Germany	25-IX-95	*Betula sp.*	*H. velutipes*	17
9523-1	Hilpoltstein, Bayern, Germany	25-IX-95	*Betula sp., Picea sp.*	*H. velutipes*	17
95242	Hilpoltstein, Bayern, Germany	25-IX-95	*Pinus sylvestris*	*H. velutipes*	17
9526-3	Roth, Bayern, Germany	25-IX-95	*Fagus sp.*	*H.velutipes*	17
9529-1	Roth, Bayern, Germany	26-IX-95	*Pinus sylvestris*	*H. velutipes*	17
9532-1	Kemnathen, Bayern, Germany	27-IX-95	*Quercus sp., Salix sp.*	*H.velutipes*	17
9534-2	Mauk, Bayern, Germany	28-IX-95	*Pinus sylvestris*	*H. velutipes*	17
9535-1	Mauk, Bayern, Germany	28-IX-95	*Pinus sylvestris*	*H.velutipes*	17
9540-1	Roden, Drenthe, Netherlands	5-X-95	*Carpinus betulus*	*H. velutipes*	17
9556-1	Havelte, Drenthe, Netherlands	8-X-95	*Quercus sp., Betula sp., Salix sp.*	*H. velutipes*	17
9623-5	Dwingeloo, Drenthe, Netherlands	18-IX-96	*Quercus sp., Betula sp.*	*H. velutipes*	17
9625-3	Wijster, Drenthe, Netherlands	20-IX-96	*Fagus sp.*	*H. velutipes*	17
9639-1	Lac de Rouges Truites, Jura, France	23-IX-96	*Picea sp.*	*H.velutipes*	17
9642-1	Lac de Rouges Truites, Jura, France	24-IX-96	*Salix sp.*	*H. velutipes*	17
9643-3	Lac de Rouges Truites, Jura, France	24-IX-96	*Fagus sp., Quercus sp., Carpinus betulus*	*H.velutipes*	17
9647-4	Lac de Rouges Truites, Jura, France	24-IX-96	*Salix purpurea*	*H. velutipes*	17
9653-2	Lac de Rouges Truites, Jura, France	26-IX-96	*Picea sp., Salix cinerea*	*H. velutipes*	17

9527-2	Roth, Bayern, Germany	26-IX-96	*Pinus sylvestris*	*H. incarnatulum/H. velutipes*	18
9557-10	Havelte, Drenthe, Netherlands	8-X-95	*Quercus sp.*	*H. helodes*	19
9574-8	Culemborg, Utrecht, Netherlands	15-X-95	*Quercus sp.*	*H. helodes*	20
9688-4	Utrecht, Utrecht, Netherlands	13-X-96	*Quercus sp.*	*H. helodes*	20
9650-10	Tourbierre de Bellefontaine, Jura, France	26-IX-96	*Betula sp.*	*H. crustuliniforme*	21
9666-5	Rensumaborg, Groningen, Netherlands	3-X-96	*Tilia sp.*	*H. crustuliniforme*	21
9694-1	Hoornaar, Zuid-Holland, Netherlands	20-X-96	*Betula sp.*	*H. helodes*	?

* Strain 9605-1 could not be unambiguously assigned to either ICG 3 or 4 because it was fully intercompatible with all collections of both.

TABLE 2-II. Summary of tester strains of fully compatible monokaryons for one collection of each ICG.

ICG	collection	set 1* I, II	set 2 III, IV
1	9674	2, 3	5, 11
2	9682	4, 10	7, 15
3	9672**	1, 10	3
4	9617	1, 6	9, 12
5	9546	3, 5	7, 8
6	9645	1, 15	4, 19
7	9654**	1	
8	9690	1, 10	7, 14
9	9538	2, 7	3, 4
10	9665	1, 3	2, 9
11	9651	1, 6	4, 15
12	9661	3, 6	4, 9
?	9692**	2, 3	1
14	9678**	5	
15	9676*	2, 8	7
16	9502	1, 8	3, 5
17	9524	2, 5	3, 4
18	9527**	2, 5	4
19	9557**	10, 12	5
20	9688	1, 3	2, 4
21	9650	1, 2	4, 10
?	9694**	1	

* The isolates numbered with 1 are compatible with those numbered with 2, and the same accounts for those numbered with 3 and 4.

** not all mating types found

Table 2-III. Intercompatibility tests within morphological cluster 1 (*H. crustuliniforme-H. alpinum*)[a].

	9503	9673	9674	9675	9618	9621	9620	9570	9626	9627	9671	9677	9682	9683	9684	9685	9602	9606	9607	9608	9609	9613	9614	9615	9616	9617	9605-1[b]	9672	9680	9686	9693	9699	9511	9679	9546	9581	9689	9691
9503-2		B	-	B	B	-	-	-	-	O	-	-	-	-	O[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9673-5			B	B	B	B	-	-	-	O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9674-9				B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9675-3					B	B	-	-	-	-	O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9618-3						B	-	-	-	-	O[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9621-3							-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9620-2								-	U	U	B	B	B	L	-	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9570-2									O	B	O	B	B	B	U	L	O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9626-1										L	B	B	B	B	U	U	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9627-1											O	O	O	-	B	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9671-2													B	U	B	-	O	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-
9677-5														B	B	L	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9682-9														B	B	B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9683-6														B	U	B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9684-1															-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9685-1																-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9602-4																	-	O	B	U	U	B	-	U	L	B	L	-	-	-	-	-	-	-	-	-	-	-	-
9606-1																		-	U	O	O	U	U	U	B	-	L	-	-	-	-	-	-	-	-	-	-	-	-
9607-5																			L	O	B	L	L	B	L	B	O	B	O[c]	-	-	-	-	-	-	-	-	-	-
9608-4																				B	B	B	-	U	-	B	B	B	-	-	-	-	-	-	-	-	-	-	-
9609-2																					B	B	L	U	L	B	B	B	-	-	-	-	-	-	-	-	-	-	-
9613-9																						O	U	U	O	B	B	B	-	B	-	-	O	L	-	-	-	-	-
9614-2																							-	U	L	B	B	B	-	-	-	-	-	-	-	-	-	-	-
9615-2																								-	-	-	B	B	-	-	-	-	-	-	-	-	-	-	-
9616-3																											-	B	-	-	-	-	-	-	-	-	-	-	-
9617-9																												B	-	-	-	-	-	-	-	-	-	-	-
9605-1[b]																													B	B	B	B	B	O		-	-	-	-
9672-1																														B	B	B	B	B	-	-	-	-	-
9680-1																															L	B	B	B	-	-	-	-	-
9686-9																																B	B	B	L	-	-	-	-
9693-8																																	B	B	B	-	-	-	-
9699-1																																		B	B	-	-	-	-
9511-1																																			-	-	-	-	-
9679-1																																							
9546-8																																				B	B	U	O
9581-1																																					U	O	O
9689-7																																							
9691-9																																							

[a] The meaning of the symbols used is: B dikaryotization of both partners in Table, L dikaryotization of left partner, U dikaryotization of upper partner and O only dikaryotic outgrowth in contact zone of monokaryons.

Table 2-IV. Intercompatibility tests within morphological cluster 2 (*H. helodes-H. pusillum*)[a].

	9501	9645	9630	9654	9507	9508	9509	9644	9648	9690	9538	9539	9571	9662	9663	9573	9651	9652	9629	9655	9660	9661	9664	9696	9427	9665	9692	9694	9557	9574	9688	9650	9666
9501-1		B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9645-1			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9630-1			B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9654-1				-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9507-1					B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9508-2						B	L	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9509-1							B	B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9644-2								B	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9648-2									-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9690-1										-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9538-4											U	B	U	U	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9539-2												B	B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9571-1													B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9662-4														B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9663-6															-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9573-1																B	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9651-2																	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9652-3																		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9629-18																			-	B	B	B	U	-	-	-	-	-	-	-	-	-	-
9655-1																				B	B	B	B	-	-	-	-	-	-	-	-	-	-
9660-2																					B	B	B	-	-	-	-	-	-	-	-	-	-
9661-14																						B	-	-	-	-	-	-	-	-	-	-	-
9664-5																							B	-	-	-	-	-	-	-	-	-	-
9696-1																								-	-	-	-	-	-	-	-	-	-
9427-5																									B	-	-	-	-	-	-	-	-
9665-5																										-	-	-	-	-	-	-	-
9692-1																											-	-	-	-	-	-	-
9694-1																												-	-	-	-	-	-
9557-10																													-	-	-	-	-
9574-8																														B	-	-	-
9688-4																																	
9650-10																																B	
9666-5																																	

[a] The meaning of the symbols used is: B dikaryotization of both partners in Table, L dikaryotization of left partner, U dikaryotization of upper partner and O only dikaryotic outgrowth in contact zone of monokaryons.

Table 2-V. Intercompatibility tests within the morphological cluster 3 *(H. Ieucosarx sensu auct. Neerl.)*[a]

	95 66	95 67	96 70	96 78	95 76	96 24	96 68	96 69	96 76	96 98
9566-3		B	B	B	-	-	-	-	-	-
9567-1			B	B	-	-	-	-	-	-
9670-3				U	-	-	-	-	-	-
9678-5					-	-	-	-	-	-
9576-1						B	B	B	B	L
9624-1							L	O	B	B
9668-3								B	B	B
9669-2									B	B
9676-8										B
9698-3										

[a] The meaning of the symbols used is: B dikaryotization of both partners in Table, L dikaryotization of left partner, U dikaryotization of upper partner and O only dikaryotic outgrowth in contact zone of monokaryons.

Table 2-VI. Intercompatibility tests within the morphological cluster 4 *(H. velutipes)*[a].

	9502	9541	9542	9543	9544	9545	9407	9410	9432	9504	9516	9522	9523	9524	9526	9529	9532	9534	9535	9540	9556	9623	9625	9639	9642	9643	9647	9653	9527
9502-3		B	B	U	B	B				-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9541-1			B	B	B	B				-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
9542-1				B	B	B				-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9543-1					B	B				-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
9544-1						B				-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9545-1										-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
9407-1								B	B																				
9410-2									B																				
9432-10																													
9504-1										B	L	B	L	B	B	B	L	B	B	B	B	B	B	B		B	B	B	-
9516-1											B	B	B	B	B	B	L	B	B	B	B	-	B	B		B	B	B	-
9522-1												U	B	U	B	B	O	O	O	B	B	U	B	U		O	U	B	
9523-1													L	B	B	B	L	O	B	B	U	-	B	B		-	B	B	-
9524-2														B	U	-	O	B	B	B	B	B	B	U	B	B	B	U	-
9526-3															B	L	L	B	B	B	B	B	-	-		B	B	B	
9529-1																L	L	B	B	B	B	B	-	B		L	B	B	-
9532-1																	O	B	B	-	B	U	U	U		O	U	B	
9534-2																		B	B	B	U	U	B	U		U	B	U	
9535-1																			B	B	B	B	B	-		L	B	B	-
9540-1																				B	B	B	B	B		B	B	B	-
9556-1																					B	B	B	B		B	B	B	
9623-5																						B	B	B		L	B	B	-
9625-3																							B	B		L	B	B	-
9639-1																										L	B	-	-
9642-1																													-
9643-3																											B	-	-
9647-4																												B	-
9653-2																													-
9527-2																													

[a] The meaning of the symbols used is: B dikaryotization of both partners in Table, L dikaryotization of left partner, U dikaryotization of upper partner and O only dikaryotic outgrowth in contact zone of monokaryons.

3

Phylogenetic relationships in the genus *Hebeloma* based on ITS 1 and 2 sequences, with special emphasis on the *Hebeloma crustuliniforme* complex.

Duur K. Aanen, Thomas W. Kuyper, Teun Boekhout and Rolf F. Hoekstra

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Abstract

Phylogenetic relationships within the genus *Hebeloma* (Cortinariaceae, Agaricales) were determined, based on nuclear ribosomal ITS sequences, using cladistic methods. Special emphasis was on phylogenetic relationships within the *H. crustuliniforme* complex. In total 52 sequences were analysed, representing 51 collections and 39 taxa. *Agrocybe praecox* and two species of *Alnicola* were used as outgroups. The genus *Hebeloma* appears to be monophyletic. Several well-supported clades could be recognized. However, many of the basal relationships are unresolved or only weakly supported. Alternative topologies could not be rejected. It is therefore impossible to derive a revised infrageneric classification of *Hebeloma* at this stage. The *H. crustuliniforme* complex appears paraphyletic, consisting of two clades with three and 17 InterCompatibility Groups, respectively. In the second clade many of the phylogenetic relationships are also unresolved, reflecting a high rate of recent speciation events. Most of the species in this clade form ectomycorrhizae with members of the *Salicaceae*. The taxon that is basal to this clade, however, is not associated with these hosts. The host tree switch to *Salicaceae* has obviously been followed by extensive and rapid speciation.

Introduction

Hebeloma (Fr.)Kumm., a genus in the Cortinariaceae (Agaricales), occurs worldwide in the temperate zone. Species of *Hebeloma* form ectomycorrhizae (Hacskaylo and Bruchet 1972) but a few species simultaneously decompose animal wastes (Sagara 1995). Many species are associated with a number of tree species, and some are pioneer species, among the first ectomycorrhizal fungi to appear during succession (Gryta et al 1997).

Species delimitations and relationships between species traditionally have been based on morphological characters (Bruchet 1970, Boekhout 1982, Singer 1986, Vesterholt 1989). However, species identification is difficult and species concepts and infrageneric classifications are controversial (Table 3-I, Chapter 1). Relatively few morphological characters have been used in existing taxonomic treatments. A submembranaceous ring, unique to *H. radicosum* (Bull.: Fr.) Rick., has been used to delimit subgenus *Myxocybe*, and the presence of a reddish spore print uniquely characterizes *H. sarcophyllum* (Peck) Sacc., which has been assigned to section or subgenus *Porphyrospora* (Bruchet 1970; Singer 1986). Of the remaining species, those with a velum partiale have been placed in section *Indusiata* (Vesterholt 1989), and those without a velum partiale in section *Hebeloma* (formerly called sect. *Denudata*, see Kuyper and Vesterholt 1990). The latter group is fairly heterogeneous and various attempts have been made to arrive at more homogeneous groupings. Vesterholt (1989) proposed that the species with a rooting stipe of section *Hebeloma* should be transferred to *Myxocybe*. Boekhout (1982) recognized two groups within the group without a velum partiale, i) species with strongly ornamented, usually dextrinoid spores with a loosening perispore, cylindrical cheilocystidia, and the potential of having a rooting stipe (section *Anthracophila*), and ii) species with less ornamented spores without a loosening perispore, the presence of weeping lamellae, and a non rooting stipe (section *Denudata*; section *Denudata* subsect. A in Bruchet (1970)). This latter group here is called the *H. crustuliniforme* complex. Although the *H. crustuliniforme* complex is generally considered a homogeneous grouping (Bruchet 1970), Boekhout (1982) noted that two separate subgroups within that complex could be recognized, exemplified by *H. velutipes* Bruchet (subsection *Attenuatocystis*; dextrinoid spores; slenderly clavate cheilocystidia) and *H. crustuliniforme* (Bull.) Quél. (subsection *Denudata*; non-dextrinoid spores; capitate cheilocystidia).

The scarcity of qualitative characters that can be used unambiguously and the occurrence of diffuse transitions between quantitative character states has led to infrageneric divisions that are contradictory to some extent. Phylogenies inferred from molecular sequences might help to resolve infrageneric classification and provide clues to the evolution of morphological characters (Berbee and Taylor 1995, McLaughlin et al 1995, Hibbett et al 1997). Knowledge about phylogenetic relationships furthermore provides an opportunity to study the evolution of host specificity (*e.g.* Kretzer et al 1996). *Hebeloma* species are generally considered to be generalists, *i.e.* with wide host ranges (Molina *et al.*, 1992, Smith and Read, 1997). However, claims on (lack of) host specificity for *Hebeloma* species are difficult to judge, since the delimitation of species is problematical.

This study is part of a project on taxonomy and speciation in the *Hebeloma crustuliniforme* complex. We use the biological species concept, based on sexual intercompatibility (Petersen 1995, Boidin 1986) as the criterion to delimit species within this complex. Aanen and Kuyper (1999) reported 20 InterCompatibility Groups (ICGs) within this *H. crustuliniforme* complex, with some instances of partial intercompatibility between ICGs. As a first step to study the evolution of incompatibility between closely related ICGs, we determined phylogenetic relationships among all ICGs of the *H. crustuliniforme* complex and the other main groups within the genus *Hebeloma*.

The main questions addressed in this study are: i). Is *Hebeloma* monophyletic? ii). Is the *H. crustuliniforme* complex monophyletic? iii). What are the major monophyletic groups in the genus *Hebeloma*? iv). Does the molecular phylogeny support the various infrageneric divisions based on morphological characters? v). How rapidly do host switches (evolution of host specificity) occur compared to speciation?

Materials and Methods

Taxon sampling

In Table 3-I data on all collections used in this study are summarized. For all 20 InterCompatibility Groups (ICGs) in the *H. crustuliniforme* complex, at least one representative was chosen. In Aanen and Kuyper (1999) two collections could not be assigned to any of the 20 ICGs (9692 and 9694), because these collections were not compa-

TABLE 3-I. Material examined

Morphospecies	ICG	Collection[a]	Collection site	Host genus	GenBank
H. crustuliniforme **complex**					
H. crustuliniforme (Bull.) Quél.	1	DKAm503-2	Borgsjö, S.	*Betula*	AF124668
	1	DKAm618-3	Sustenpass, CH	*Salix*	AF124671
	1	DKAd621	Davos, CH	*Dryas*	AF124665
	1	DKAd673	Eemshaven, NL	*Salix*	AF124672
H. crustuliniforme	2	DKAd627	Jura, F.	*Corylus*	AF124696
	2	DKAm570-2	Lelystad, NL.	*Salix*	AF124716
H. crustuliniforme	3	DKAd680	Hoornaar, NL	*Populus*	AF124708
H. crustuliniforme	4	DKAd602	Adelboten, CH	*Dryas*	AF124694
H. crustuliniforme	5	DKAm581-1	Utrecht, NL	*Tilia*	AF124683
H. pusillum J. Lange	6	DKAd645	Jura, F	*Salix*	AF124702
H. pusillum	7	DKAd654	Jura, F	*Salix*	AF124706
H. pusillum	8	DKAd509	Vledder, NL	*Salix*	AF124681
H. pusillum	12	DKAd629	Jura, F	*Salix*	AF124697
H. helodes J. Favre	9	DKAd538	Beilen, NL	*Salix*	AF124687
	9	DKAd539	Wijster, NL	*Salix*	AF124688
H. helodes	10	DKAd665	Beilen, NL	*Salix*	AF124674
H. helodes	11	DKAd651	Jura, F	*Salix, Picea*	AF124704
	11	DKAm573-1	Terschelling, NL	*Salix*	AF124705
H. helodes	?	DKAd692	Hoornaar, NL	*Populus*	AF124710
H. helodes	19	DKAd557	Havelte, NL	*Quercus*	AF124690
H. helodes	20	DKAd688	Utrecht, NL	*Quercus*	AF124709
H. helodes	21	DKAd650	Jura, F	*Betula*	AF124703
	21	DKAd666	Groningen, NL	*Tilia*	AF124707
H. helodes	?	DKAd694	Hoornaar, NL	*Betula*	AF124711
H. lutense Romagn.[d]	14	DKAm566-3	Lelystad, NL	*Salix*	AF124678
H. lutense	15	DKAd624	Dwingeloo, NL	*Pinus*	AF124666
H. velutipes Bruchet	16	DKAd502	Wijster, NL	*Betula*	AF124679
H. velutipes	17	DKAd504	Borgsjö, S	*Betula*	
		with m504-1			AF124676
		and m504-2			AF124677
	17	DKAd535	Mauk, D	*Pinus*	AF124667
	17	DKAd540	Roden, NL	*Carpinus*	AF124685
	17	DKAd642	Jura, F	*Salix*	AF124686
H. incarnatulum Smith[c]	18	DKAd527	Roth, D	*Pinus*	AF124684

H. bulbiferum Maire	n.d.[b]	PR21860	F		AF124673
Other species					
H. sacchariolens Quél.	n.d.	DKAd552	Rheebruggen, NL	*Salix*	AF124689
H. tomentosum (Mos.) Gröger & Zschieschang	n.d.	DKAd506	Vledder, NL	*Salix*	AF124680
H. cavipes Huijsman	n.d.	LY66BR106	F	?	AF124670
H. hiemale Bres.	n.d.	LY66BR104	F	?	AF124669
H. birrus (Fr.) Gillet	n.d.	DKAd580	Callantsoog, NL	*Quercus*	AF124693
H. danicum Gröger	n.d.	LY64BR38	F	?	AF124675
H. edurum Métrod	n.d.	DKAd637	Jura, F	*Pinus, Picea*	AF124698
H. cylindrosporum Romagn.	n.d.	Am6100	Dwingeloo, NL	*Pinus*	AF124695
H. sinapizans (Fr.) Gillet	n.d.	DKAd514	Roth, D	*Picea, Pinus*	AF124682
H. truncatum (Schaeff.: Fr.) Kumm.	n.d.	DKAd641	Jura, F	*Picea*	AF124701
H. circinans Quél.	n.d.	DKAd638	Jura, F	*Picea*	AF124699
H. collariatum Bruchet	n.d.	DKAd565	Lelystad, NL	*Salix*	AF124691
H. mesophaeum (Pers.) Quél.	n.d.	DKAd572	Terschelling, NL	*Salix*	AF124692
H. sarcophyllum Peck	n.d.	LY65BR25	F	?	AF124715
H. radicosum (Bull.: Fr.) Rick.	n.d.	DKAd640	Jura, NL	*Quercus*	AF124700
Outgroups					
Agrocybe praecox (Pers.: Fr.) Fay	n.d.	CBS108.59		?	AF124713
Alnicola bohemica (Velen.) Kühn. & Maire	n.d.	Ad701	Beilen, NL	*Salix*	AF124712
A. escharoides (Fr.: Fr.) Romagn.	n.d.	MdM29	Beilen, NL	*Alnus*	AF124714

[a] Isolates beginning with DKA collected by D.K. Aanen; cultures deposited at CBS, excicates at Wageningen UR. DKA followed by m are monosporous isolates, DKA followed by d, sporocarp regenerants; PR21860: collected by R. Kühner, and isolates beginning with LY are cultures from Université Claude-Bernard Lyon, kindly provided by Dr R. Marmeisse. Ad9701: sporocarps collected by Dr E.J.M. Arnolds, not in culture, in Wageningen UR. Am96100: collected by Dr. E.J.M. Arnolds; cultures deposited at CBS Baarn and excicates in Wageningen UR. MdM29: collected by M. Meyer zu Schlochtern; culture deposited at CBS Baarn and excicates in Wageningen UR.

[b]: n.d. =not determined

[c]: see Smith (1984).

[d]: *H. lutense* is identical with *H. leucosarx* sensu auct. Neerl.

tible with any of the 20 ICGs. In this study also sequences were determined of these two collections. As the tentative morphospecies within this group mostly consisted of several ICGs, most of the morphospecies were represented by more than one collection. To test for variation within ICGs we included several isolates of the ICGs 1, 2, 9, 11, 17 and 21. Several representatives of the other major lineages within the genus *Hebeloma* as recognized by Bruchet (1970), Boekhout (1982) and Vesterholt (1989) were included as well. As possible outgroups, two species of the proposed sister genus to *Hebeloma*, *Alnicola* (Kühner 1980, Singer 1986), viz. *Alnicola bohemica* and *A. escharoides*, were chosen. To test the status of *Alnicola* as a sister group of *Hebeloma*, *Agrocybe praecox* was chosen as an outgroup (J.-M. Moncalvo, personal communication). In all, 52 isolates were sequenced representing all 20 ICGs found within the *H. crustuliniforme* complex, 16 morphological species from the main groups recognized within *Hebeloma*, two species of the genus *Alnicola* and *Agrocybe praecox*. Most isolates were collected and identified by the first author, some by others (indicated in Table 3-I). Voucher specimens are preserved in Wageningen (WAG) and most cultures in Baarn (CBS; see Table 3-I for exceptions).

DNA isolation, PCR and sequencing

DNA was isolated from either dikaryotic cultures, obtained from young carpophores, or from monospore cultures. Prior to DNA isolation, fungal cultures were grown on Petri dishes for 3 to 5 weeks on a modified corn meal agar (17 gl^{-1}, Difco) medium with the following additions: 10.0 gl^{-1} saccharose, 7.0 gl^{-1} glucose, 1.0 gl^{-1} yeast extract, 0.1 gl^{-1} KH_2PO_4 and 6.0 gl^{-1} agar. The medium was covered with an uncoated-cellophane layer. DNA was extracted in duplo, mycelium was frozen in an Eppendorf tube in liquid nitrogen and ground with a pestle. Immediately after grinding, 300 µL extraction buffer (0.5 M NaCl, 10 mM Tris-HCl pH 7.5, 10 mM Na_2EDTA and 1 % SDS) was added and mixed with the ground mycelium. An incubation for 45 min at 65 C was followed by a phenol-chloroform extraction and an RNAse treatment. After a second phenol-chloroform extraction, the mixture was extracted with chloroform. Finally, DNA was precipitated with isopropanol, washed with 70% ethanol, and dissolved in 50 µL water.

DNA from *Alnicola bohemica* was isolated from a dried sporocarp according to Lee and Taylor (1990).

Primers ITS 1 and ITS 4 (White et al 1990) were used to amplify the nuclear ribosomal ITS1, ITS2 and the 5.8S rDNA. PCR reactions were performed in 100 µl volumes with 25 µL of a 10^3 or 10^4 dilution of the template DNA, following the recommendations of the manufacturer (Promega). The following cycling parameters were used: one cycle of: 3 min 92 C, 1 min 36 C and 3 min 74 C, followed by 44 cycles of: 1 min 92 C, 1 min 36 C and 3 min 74 C. The final cycle was 1 min 92 C, 1 min 36 C and 7 min 74 C. After amplification, 5 µL of the PCR reaction mixture was electrophoresed to check amplifications.

Amplified products were purified with the Quiaquick gel electrophoresis kit (Quiagen). PCR products were first run on an agarose gel (1% agarose), bands were cut out and subsequently cleaned according to the manufacturer's instructions.

Cleaned PCR products were sequenced with the Applied Biosystems Taq DyeDeoxy terminator cycle sequencing kit in a Perkin-Elmer thermal cycler. As sequencing primers ITS 1, ITS 3 and ITS 4 were used (White et al 1990). Sequencing reaction mixtures were analysed in an Applied Biosystems 373 DNA sequencer and the sequences were deposited in GenBank under accession numbers AF124665-AF124716 (Table 3-I). The aligned sequences have been deposited to TreeBASE (HTTP://herbaria.harvard.edu/treebase) as SN247 or are available from the first author upon request.

Borderlines between 18S rDNA, ITS1, 5.8 S, ITS2 and 28S were identified using *Heterobasidion annosum* (Fr.) Bref. sequences (Kasuga et al 1993).

Phylogenetic analysis

Sequences were aligned using ClustalV (Higgins et al 1992). The alignment was edited manually using a matrix created in PAUP* 4.0 (Swofford, test version 1998). Phylogenetic relationships were inferred from the aligned sequences using parsimony with PAUP. Since the alignment was ambiguous for some parts, alignment gaps were treated as missing data. All transformations were unordered and equally weighed. The ITS 1 and 2 data were combined in a single data set. To test whether a phylogenetic signal was present in the data, skewness of 100.000 random trees was determined, and the significance tested

(Hillis and Huelsenbeck 1992). The heuristic search option with 100 random addition sequences with multrees on and TBR branch swapping was used. Both the options 'collapse branches if maximum length is zero' and 'collapse branches if minimum length is zero' were tested. Clade stability was assessed by 1000 bootstrap replications (Hillis and Bull 1993), with settings: random addition, TBR branch swapping with multrees on and maxtrees 10.

Decay indices (Bremer 1988, Donoghue et al 1992) were calculated from PAUP tree files using the program Autodecay (Eriksson 1998) and PAUP. Other measures (tree length, sequence divergence, consistency and retention indices) were calculated using PAUP.
To determine the influence of differently weighing transitions and transversions, a separate analysis was performed. Weights were based on the approximate transition/transversion ratio, estimated using the Maximum Likelihood option in PAUP.

To examine alternatives to the maximum parsimony trees, a neighbor-joining (Saitou and Nei 1987) tree was generated, based on the Kimura-2 genetic distance, using PAUP.

To examine evolution of host tree preference and the evolution of some morphological characters, these characters were optimized on trees that we found using MacClade version 3.07 (Maddison and Maddison 1997). Character states were coded as unordered and reversible.

Some additional tests were performed with the following constraints: i) *Hebeloma crustuliniforme* complex monophyletic, ii) *Alnicola* monophyletic and iii) *H. crustuliniforme* and *H. velutipes* monophyletic. Constrained and unconstrained trees were compared by three criteria: tree length, Templeton's (1983) non-parametric test and the Kishino-Hasegawa test (Kishino and Hasegawa, 1989) under the parsimony criterion as implemented in PAUP.

To compare species richness in two different clades and to correct for differences in sample size, we used Simpson's index of concentration (SI, Simpson 1949). SI is defined as:
$SI = \Sigma ((n^2-n) / (N^2-N))$,
where n is the number of individuals in a species, and $N = \Sigma n$

Results

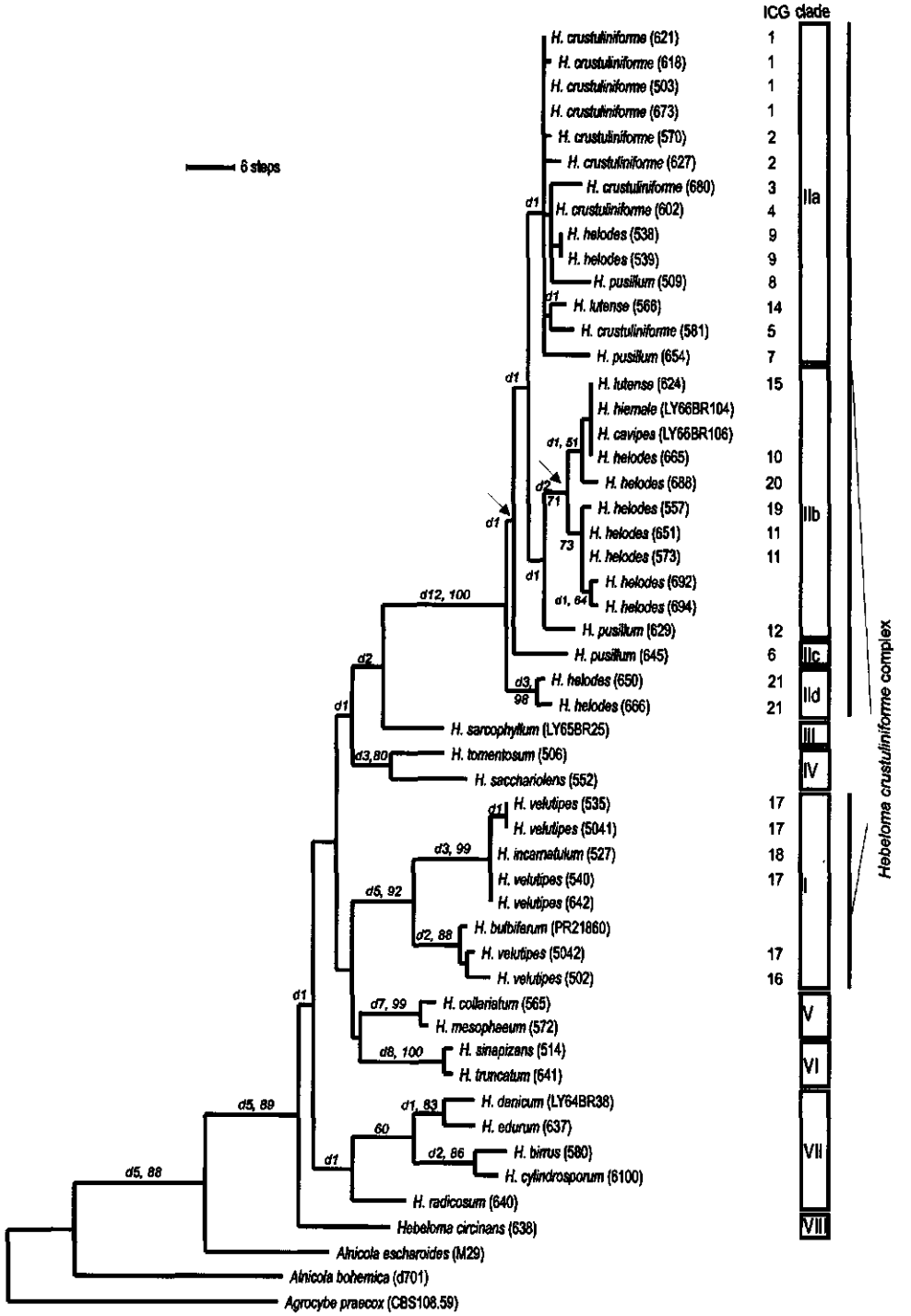
Sequences

The total number of nucleotides aligned ranged from 605 to 616, length of ITS 1 ranged from 229 to 239, of ITS 2 from 202 to 220 and the length of 5.8S rDNA was 159 base pairs. ITS 1 appeared to be slightly more variable than ITS 2 (47.6% variable sites in ITS 1 and 43.1% in ITS 2). Only one site of the 5.8S rDNA was variable. Maximum sequence divergence (absolute number of base pair differences) within the ingroup was 43 (7.1%), and between in- and outgroup 69 (11.4%). The total number of variable sites was 193, 97 of which were phylogenetically informative. Base composition of the entire region was approximately: A 0.23, C 0.23, G 0.22, and T 0.32.

The following sequences were identical: i) 618 and 621; ii) 503 and 673; iii) 624, 665, *H. hiemale*, *H. cavipes*; iv) 535 and 504-1; v) 573 and 651 and vi) 538 and 539. Only one of the identical sequences was used in the phylogenetic analysis. Infraspecific variation was very low in ICGs 1, 2, 9, 11 and 21 with less than 3 nucleotide differences. However, within ICG 17, the electropherogram of the sequence of dikaryon 504 showed a mixture of peaks at 20 positions. PCR products of two monokaryons (m504-1 and m504-2) of this same collection had single sequences that differed at these 20 positions. We used these 2 sequences in the phylogenetic analysis.

Phylogenetic relationships

Skewness, an indicator of phylogenetic signal to random noise, was significant (g1 = -0.287, $P << 0.01$). A total of 6527 trees of length 357 (including uninformative characters; 261, excluding uninformative characters) was found using the option 'collapse branches if maximum length is zero' (c.i. 0.63; 0.49, excluding uninformative characters; rescaled c.i. 0.51; r.i. 0.81). If the option 'collapse branches if minimum length is zero' was chosen, 272 trees were found, the strict consensus tree of which did not differ from the consensus tree of the 6527 trees. In Fig. 3-1 the tree with the highest likelihood of those trees is depicted. The main clades that can be recognized and that will be discussed are indicated.


ICG clade
6 steps
H. crustuliniforme (621) 1
H. crustuliniforme (618) 1
H. crustuliniforme (503) 1
H. crustuliniforme (673) 1
H. crustuliniforme (570) 2
H. crustuliniforme (627) 2
H. crustuliniforme (680) 3
H. crustuliniforme (602) 4
H. helodes (538) 9
H. helodes (539) 9
H. pusillum (509) 8
H. lutense (566) 14
H. crustuliniforme (581) 5
H. pusillum (654) 7
H. lutense (624) 15
H. hiemale (LY66BR104)
H. cavipes (LY66BR106)
H. helodes (665) 10
H. helodes (688) 20
H. helodes (557) 19
H. helodes (651) 11
H. helodes (573) 11
H. helodes (692)
H. helodes (694)
H. pusillum (629) 12
H. pusillum (645) 6
H. helodes (650) 21
H. helodes (666) 21
H. sarcophyllum (LY65BR25)
H. tomentosum (506)
H. saccharioiens (552)
H. velutipes (535) 17
H. velutipes (5041) 17
H. incarnatulum (527) 18
H. velutipes (540) 17
H. velutipes (642)
H. bulbiferum (PR21860)
H. velutipes (5042) 17
H. velutipes (502) 16
H. collariatum (565)
H. mesopheeum (572)
H. sinapizans (514)
H. truncatum (541)
H. denicum (LY64BR38)
H. edurum (637)
H. birrus (580)
H. cylindrosporum (6100)
H. radicosum (640)
Hebeloma circinans (638)
Alnicola escharoides (M29)
Alnicola bohemica (d701)
Agrocybe praecox (CBS108.59)
d1
d2
d3
d12, 100
d1, 51
d1, 64
71
73
98
d3,80
d3, 99
d5, 92
d2, 88
d7, 99
d8, 100
d5, 89
d1, 83
60
d2, 86
d5, 88
IIa
IIb
IIc
IId
III
IV
I
V
VI
VII
VIII
Hebeloma crustuliniforme complex

The transition-transversion ratio was estimated to be 2.36. Weighing transversions over transitions, using this value and doing the same heuristic search, we found 216 trees, the strict consensus of which did not positively differ from the tree using no weights. The neighbor-joining tree (Fig. 3-2) was highly similar to the strict consensus tree of the most parsimonious trees but differed in a few aspects. *H. circinans* is the basal taxon in the parsimony analysis, but not in the neighbor-joining tree. The trees further differed in the placement of *H. sarcophyllum* and the clade consisting of *H. sacchariolens* and *H. tomentosum*.

To determine the length difference between the most parsimonious trees and the neighbor-joining tree, the neighbor-joining tree was imported to PAUP; its length was 6 steps longer than the most parsimonious trees (l=363). We base the discussion on the strict consensus tree using equally weighing of transitions and transversions and using gap positions (with gaps as missing data).

Alnicola appears to be paraphyletic. *A. bohemica* is the sister group of all the other taxa, including *A. escharoides*. *A. escharoides* is the sister taxon of *Hebeloma*. Trees with *Alnicola* constrained as a monophyletic group are 7 steps longer than the most parsimonious trees without constraints, but can not be rejected by Templeton's non-parametric test or the Kishino-Hasegawa test ($p > 0.05$ in both cases).

Hebeloma appears to be monophyletic. Several well supported clades can be recognized within the genus *Hebeloma*. The basal status of *H. circinans* (clade VIII) within *Hebeloma* is not well supported, because the monophyly of the remaining taxa within *Hebeloma* is supported by a decay index of 1. The next basal phylogenetic relationships are not resolved in the strict consensus tree. *H. danicum* and *H. edurum* form a monophyletic group (bootstrap, bs 83 %, decay index, di 1) as well as *H. birrus* and *H. cylindrosporum* do (bs 86 %, di 2). These two groups together form a monophyletic group in 60 % of the

FIG. 3-1. Phylogenetic relationships in the genus *Hebeloma*, based on ITS sequences. Tree with the highest likelihood of 6527 most parsimonious trees of length 357 (c.i.=0.63; ci, excluding uninformative characters =0.49; rescaled c.i.=0.51; r.i.=0.81), found with PAUP* 4.0, using *Agrocybe praecox* as an outgroup. Two arrows mark the two single proposed deletion events for two indels of 3 bp (that were not used as characters). Bootstrap values higher than 50 % and decay indices (preceded by d) are indicated above branches. On the right, ICGs to which isolates belong are indicated. The basal clades that can be recognized are indicated as well. The *Hebeloma crustuliniforme* complex does not form a monophyletic group as indicated.

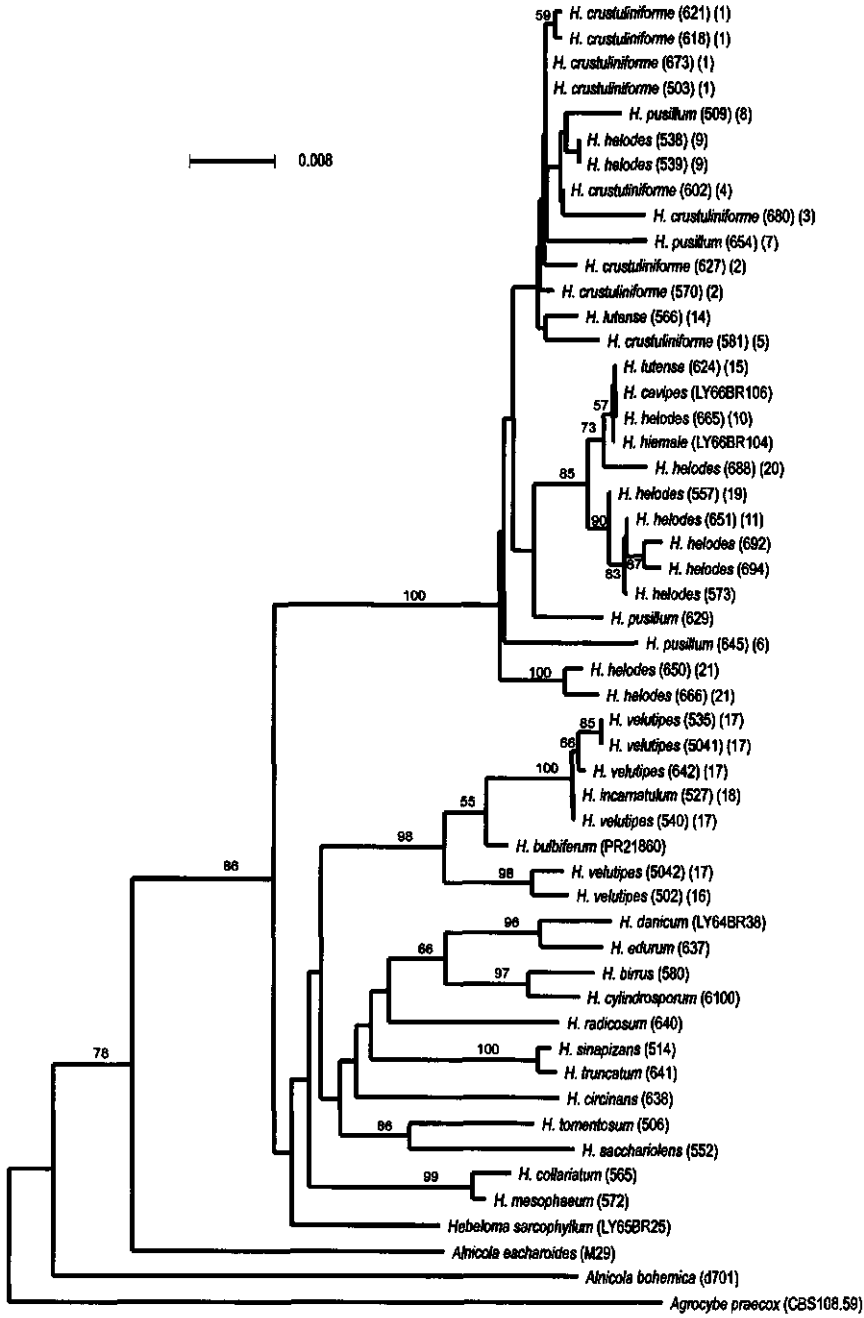


FIG. 3-2. Neighbor-joining tree, based on the Kimura-2 genetic distance. Bootstrap values higher than 50 % are indicated above branches .

bootstrapping replicates, but not in the strict consensus tree. Both groups together with *H. radicosum* form a monophyletic group in the strict consensus tree (clade VII), but this is not supported by high bootstrap values or a high decay index (di 1). *H. sinapizans* and *H. truncatum* form a monophyletic group, supported by a bootstrap value of 100 % and a decay index of 8 (clade VI). The two members of section *Indusiata*, *H. mesophaeum* and *H. collariatum*, form a monophyletic group supported by a high bootstrap value (99 %) and decay index (7) (clade V). The two species of the *H. sacchariolens* complex (Gröger and Zschieschang 1981), *H. tomentosum* and *H. sacchariolens*, form a monophyletic group (bs 80 %, d.i. 3; clade IV). The *H. crustuliniforme* complex consists of two distinct clades that do not form a single monophyletic group (Fig. 3-1).

One of these clades (clade I) consists of the two ICGs of *H. velutipes* (ICGs 16 and 17) and *H. incarnatulum* (ICG 18) and the monophyly of this group is supported by a 92 % bootstrap value and a decay index of 5. The other clade within the *H. crustuliniforme* complex (clade II) consists of the remaining 17 ICGs that were found by Aanen and Kuyper (1999). These belong to the morphological groups *H. crustuliniforme*, *H. alpinum*, *H. pusillum*, *H. helodes* and *H. lutense*. The monophyly of this group is also supported very strongly (100% bs, d.i. 12). Basal to clade II, *H. sarcophyllum* is found, weakly supported by a decay index of 2 (clade III).

Trees with clade I and II constrained to form a single monophyletic group are 2 steps longer than the most parsimonious tree without constraints but can not be rejected by Templeton's non-parametric test or the Kishino-Hasegawa test ($p > 0.05$ in both cases).

Phylogenetic relationships within the H. crustuliniforme *complex*

Clade I consists of two monophyletic groups, one of which contains *H. incarnatulum* (ICG 18; Smith 1984) and different individuals of ICG 17 of *H. velutipes*. The other branch consists of ICG 16 of *H. velutipes* and 1 monospore isolate of ICG 17 of *H. velutipes*. ICGs 16 and 17 are partially intercompatible, with 4 % of the combinations between those ICGs being compatible (Aanen and Kuyper 1999).

Clade II consists of closely related taxa as reflected by short branch lengths. The two isolates of ICG 21 form a monophyletic group (clade IId) that is the sister group of the rest of clade II. This is not supported by a high bootstrap value or decay index (di=1).

However, all members of clade II, except ICG 21, share a unique 3 base pair deletion. As indels were not used as characters in the phylogenetic analysis, this provides additional evidence that ICG 21 is the basal taxon within clade II (see Fig. 3-1, arrows). Within the group of the remaining ICGs, only the monophyly of a clade consisting of ICGs 10, 11, 13, 15, 19, 20 and 22 is supported by a decay index higher than 1 (di=2). The monophyly of this clade is further supported by a 3 base pair deletion unique to all taxa of this group.

Discussion

Infraspecific variation was absent or very low in ICGs 1, 2, 9, 11 and 21 of which we studied more than one collection. For none of these ICGs we could reject the hypothesis that collections from one ICG form a monophyletic group. Within ICG 17, however, we found considerable variation in ITS sequences, and the two main types even did not form a monophyletic group. Although generally a fairly good correspondance has been found between groupings based on mating criteria and groupings based on phylogeny (e.g. Vilgalys and Sun 1994), in several genera other than *Hebeloma* also paraphyletic ICGs have been found (*Pleurotus*, Vilgalys and Sun 1994; *Armillaria*, Piercey-Normore et al 1998). In the genus *Pleurotus* these paraphyletic ICGs consisted of monophyletic populations from different continents. Our sampling area was limited to northwestern and central Europe. By the inclusion of isolates from a larger geographic area we therefore may find even more cases of paraphyletic ICGs.

As this study focused on the *H. crustuliniforme* complex, infraspecific variation was not tested in the other groups.

While *Hebeloma* appeared monophyletic in our analysis, *Alnicola* turned out to be paraphyletic. Although trees with *Alnicola* constrained as a monophyletic group are 7 steps longer than the most parsimonious trees, these could not be rejected by both Templeton's and the Kishino-Hasegawa test. Clearly, for a better understanding of the evolutionary relationships between *Alnicola* and *Hebeloma* and within *Alnicola* a larger sample of the latter taxon needs to be studied. If *Alnicola* indeed turns out to be paraphyletic, at least two possibilities exist to define monophyletic entities: i) *Alnicola* can be split into monophyletic entities; ii) *Hebeloma* and *Alnicola* can be fused to a single monophyletic genus. The latter solution has been proposed by Kühner (1980).

Basal relationships between the major monophyletic groups that we have found are not well resolved. Alternative topologies with other major monophyletic groups as basal in the genus were usually only a few (less than 3) steps longer than the most parsimonious trees (data not shown) and could not be rejected. It is therefore not possible to translate this cladogram into a newly proposed infrageneric classification for *Hebeloma*. Recently, Hibbett & Donoghue (1998) wondered about the limited impact molecular phylogenetic studies had on the existing classifications. In our case, none of the existing classifications could clearly be rejected, and therefore we do not propose a new infrageneric classification. The existing divisions are contradictory to some extent. The groups with one or more special characters such as *H. radicosum*, *H. sarcophyllum*, and the veiled species are defined the same in all divisions and these groups indeed represent monophyletic entities. The remaining species, however, are grouped differently by the different authors and probably represent no monophyletic entities.

Our data provide some support for Vesterholt's (1989) proposal to extend circumscription of *Myxocybe* to include all rooting species and to Boekhout's (1982) suggestions that *H. velutipes* (clade I) and *H. crustuliniforme* (clade II) are not very closely related. Bruchet's (1970) suggestions on the other hand to have the morphospecies *H. crustuliniforme* and *H. velutipes* in one stirps can be rejected as trees with this constraint are 15 steps longer than unconstrained trees and rejected by Templeton's and the Kishino-Hasegawa test ($p < 0.01$ in both cases). Collections of *Hebeloma crustuliniforme* from the alpine zone, which are usually assigned to the species *H. alpinum* (Bruchet 1970) do not form a distinct group in this study (collections 618, 621, 602), thereby casting doubt on Bruchet's suggestion that *H. crustuliniforme* and *H. alpinum* belong to different stirpes. This is consistent with the different levels of intercompatibility found between those morphotaxa (Aanen and Kuyper 1999). Placement of *H. hiemale* and *H. cavipes* in the *H. sacchariolens* group (Bruchet 1970) should also be rejected in favour of Boekhout's suggestion that both species are close relatives of taxa in clade II of the *H. crustuliniforme* complex.

Evolution of some morphological characters

We have reconstructed the evolution of three morphological characters and one olfactory character on the 11 recognized clades (Fig. 3-3; tree with the highest likelihood).

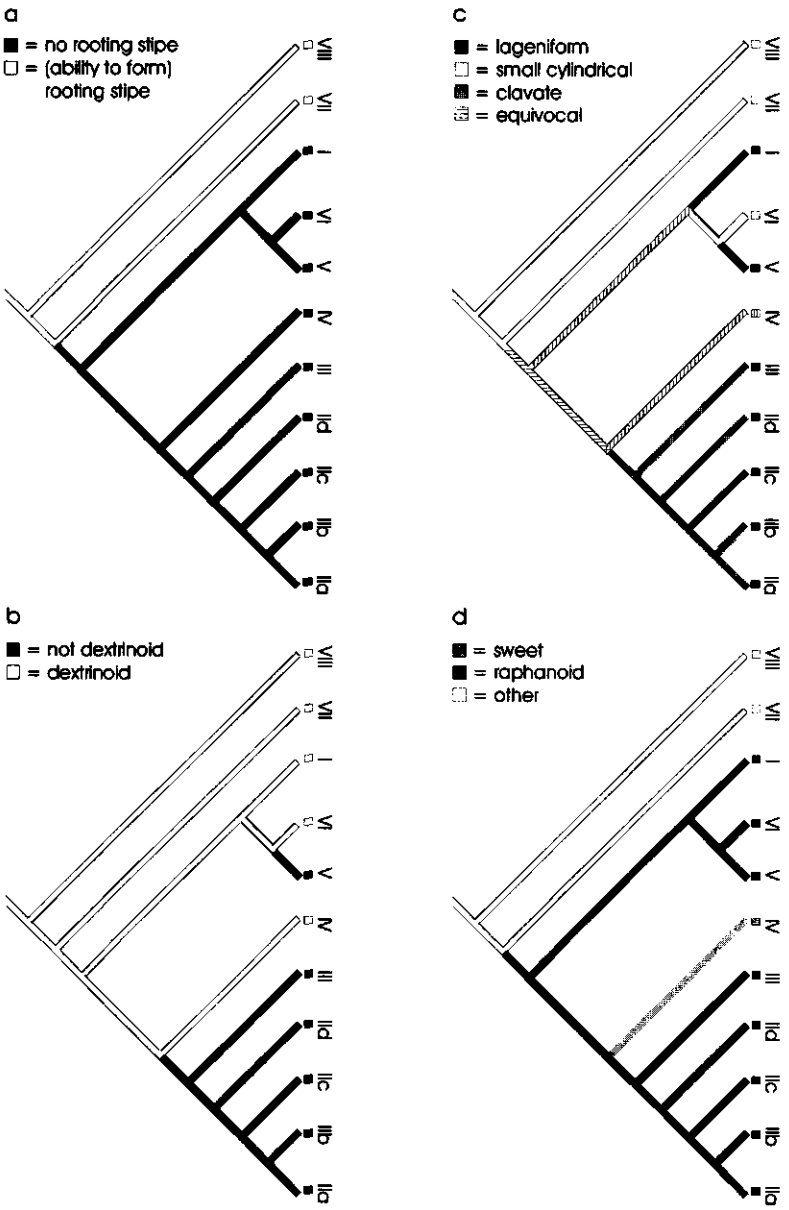


FIG. 3-3. Most parsimonious reconstruction of the evolution of four characters that have been used in infrageneric classification of the genus *Hebeloma* for the 11 clades recognized in Fig. 3-1. a. presence or absence of (potential to form) rooting stipe; b. dextrinoidy of spores; c. shape of cheilocystidia; d. smell of carpophores.

The presence of a rooting stipe (or rather: the ability to form a rooting stipe; Boekhout 1982) must be considered as a plesiomorphic character state, only present in the two basal clades VII and VIII. It has been lost once. The most parsimonious reconstruction of the character dextrinoidy of spores is that dextrinoid spores is the plesiomorphic state. Dextrinoidy has been lost twice: in clade V and in clade (II,III). Three basal types of cheilocystidia can be recognized in species of the genus *Hebeloma*, although the distinction between those types is not always unambiguous. The basal clades (VII and VIII) have small, cylindrical cheilocystidia. Clavate cheilocystidia have evolved twice: both in clade I and in the clade consisting of II and III. Species of clade IV can have both small, cylindrical cheilocystidia and clavate cheilocystidia. Lageniform cheilocystidia are uniquely found in clade V. Smell has been used as a character to delimit certain groups within the genus *Hebeloma*, even though it is difficult to assign states to this character objectively. The raphanoid smell has evolved in the lineage leading to the clade consisting of I, II, III, IV, V and VI. The sweet smell of clade IV is a synapomorphy for this clade. The two basal clades, VII and VIII have other smells like marzipan and cacao-like. The ecological habit as post-putrefaction fungi on nitrogen-rich substrates (Sagara 1995) or fire-place fungi with an ability to saprotrophic behaviour also characterizes the basal clade. The presence of veil remnants, which characterizes *H. mesophaeum* and *H. collariatum*, must be considered a synapomorphy. Many of the characters traditionally used in *Hebeloma* taxonomy are quantitative, with diffuse transitions. This makes it very difficult to objectively assign character states in many cases. More importantly, some of the character states that have been used for infrageneric classification represent plesiomorphic states. Dividing the genus on the basis of such character states inevitably leads to paraphyletic entities. These two factors, the difficulty in assigning character states and the plesiomorphic nature of important character states, have probably contributed to the contradictory opinions on *Hebeloma* infrageneric divisions.

Evolution of host preference

Many fungi that form ectomycorrhizae apparently have restricted host ranges to some degree. This varies from extremes such as *Suilloideae* that are almost exclusively associated with *Pinaceae* (Singer 1986, Kretzer et al 1996) to genera such as *Laccaria* that have not been considered to be host specific. However, Mueller (1992) reported that many species of

the genus *Laccaria* are either found with *Pinaceae* or *Fagaceae*. *Hebeloma* species have generally been considered to have broad host ranges (Molina *et al* 1992; Smith and Read 1997), but the uncertainty surrounding species recognition makes evaluation of this claim difficult. The ICGs recognized by Aanen and Kuyper (1999) did fall in two host preference groups. One group of ICGs preferentially grows with *Salicaceae* (> 50 %) and the other group of ICGs has a broad host range, but is rarely found with *Salicaceae* (< 10 %). The

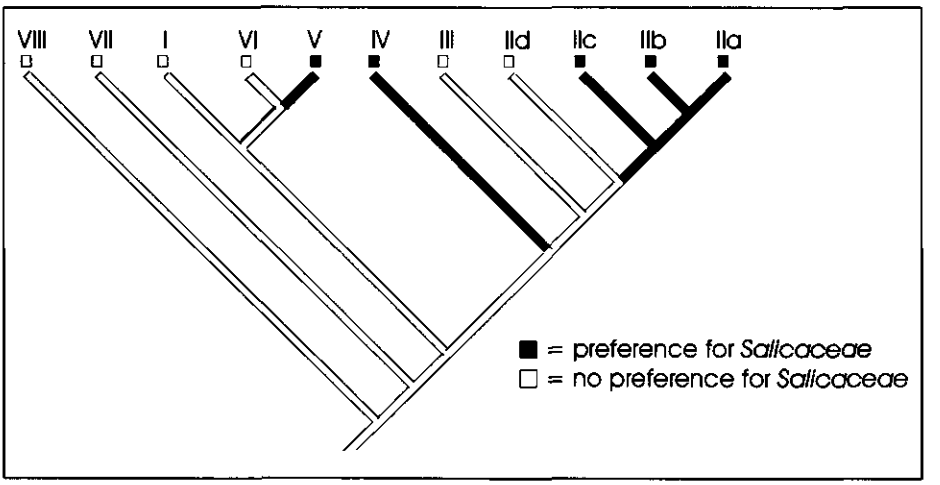


Fig. 3-4. Most parsimonious reconstruction of the evolution of host specificity in the genus *Hebeloma* for the 11 clades recognized in Fig. 3-1. Two states are recognized: a preference for *Salicaceae* (> 50%) or rarely found with *Salicaceae* (< 10 %).

Salicaceae group is exclusively found in clade II of the *H. crustuliniforme* group, the non-*Salicaceae* group is mainly found in clade I, but some ICGs are found in II as well. The morphospecies *H. crustuliniforme* (part of clade IIa) shows a strong preference for *Salicaceae*, thereby contradicting Smith and Read's (1997) statement that *H. crustuliniforme* occurs on a very wide range of plants. At the level of individual isolates, only 2 of the 29 isolates of clade I were found with *Salicaceae*, and 60 of the 81 isolates of clade II. Within the other groups of *Hebeloma*, *Salicaceae* preference is also found in the *H. mesophaeum* group and the *H. sacchariolens* group. We can only speculate about the cause of this difference in host preference between those groups. It has been shown that salicylic acid plays an important role as a signaling molecule in disease resistance in plants (for an

overview, see Durner et al 1997). A hypothesis to explain the distinction between species that can grow with *Salicaceae* and species that can not, is that some species are able to break the resistance associated with salicylic acid, whereas others are not.

A most parsimonious reconstruction of host preference, coded as a character with two states, *Salicaceae* preferring and non-*Salicaceae* preferring, indicated that a preference for *Salicaceae* has arisen at least three times independently within *Hebeloma* (Fig. 3-4). The basal taxon within clade II is clade IId (ICG 21), which has not been found with *Salicaceae*. The monophyletic status of the *Salicaceae*-specific group is supported by a synapomorphic 3 basepair deletion, which was not used as a character in the cladistic analysis, and can thus be considered as independent support for the position of ICG 21. The *Salicaceae* preferring clade consists of 16 ICGs, its non-*Salicaceae* preferring sister group of only one ICG. Interestingly, the two other groups in the genus *Hebeloma* that have *Salicaceae* preference (*H. mesophaeum* group and *H. sacchariolens* group), belong to the most species rich clades within the genus, judging from morphological studies (Vesterholt 1989, Gröger and Zschieschang 1981). In the non-*Salicaceae* preferring clade I of the *H. crustuliniforme* complex, which was also extensively studied for sexual intercompatibility, only 3 ICGs were found in a sample of 29 isolates. Maximum sequence divergence in this clade is in the same order of magnitude as in clade II (even slightly higher in I, 3.63 %, 2.81 % in II). Assuming that both clades are of roughly equal age, we can compare the magnitude of species (ICGs) diversity in both clades. To correct for differences in sample size, we used Simpson's index of concentration (SI, Simpson 1949) to compare species diversity in clade I and II. SI is much higher in clade I than in clade II, which means that species diversity is much higher in clade II than in I (SI = 0.072 in clade II, 0.61 in I). These findings suggest that the switch to *Salicaceae* in this genus is somehow correlated with species richness. In accordance with Kretzer et al (1996) we note that host switching is rare compared to speciation. This suggests that most speciation events are not directly caused by host switches.

Lack of phylogenetic resolution and rates of speciation

At two different taxonomic levels there is a lack of resolution in the estimation of phylogeny. First, many of the basal phylogenetic relationships are not resolved or are not

strongly supported. There are at least two possible explanations for this lack of resolution. Sites could be saturated, such that most sequence variation is noise, instead of phylogenetic signal. We do not think that saturation explanains the lack of resolution at the basal level, because sequence divergence within *Hebeloma* is low (maximum sequence divergence 43 basepair differences), possibly reflecting a relatively recent origin of species within the genus. Maximum sequence divergence within ITS 1 and 2 within other Basidiomycete genera is usually substantially higher (Liu et al 1997, Kretzer et al 1996, Hibbett et al 1995, Hallenberg et al 1996). A more plausible explanation is that this low level of resolution reflects a relatively short period during which the main groups in the genus have originated. Secondly, there is a lack of resolution in clade II of the *H. crustuliniforme* group. Most of the taxa in this group represent different ICGs, partially or completely reproductively isolated from each other. The lack of phylogenetic resolution at this level reflects a high rate of very recent speciation events.

Our results therefore suggest that there have been two periods of rapid speciation within the genus *Hebeloma*.

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4

The evolution of reproductive isolation in the ectomycorrhizal *Hebeloma crustuliniforme* aggregate (Basidiomycetes) in northwestern Europe: a phylogenetic approach.

Duur K. Aanen, Thomas W. Kuyper, Ted H.M. Mes and Rolf F. Hoekstra

Submitted.

Abstract

In order to reconstruct the evolution of reproductive isolation in the ectomycorrhizal *Hebeloma crustuliniforme* aggregate (Basidiomycetes), phylogenetic relationships were determined between strains that belong to a clade, consisting of nine InterCompatibility Groups (ICGs, biological species). Four of these nine ICGs are partially compatible, and belong to the *H. crustuliniforme* aggregate. This partial compatibility is organized hierarchically, with an intermediate level of compatibility between ICGs 3 and 4, and very limited compatibility between 2 and 3/4 and between 1 and 2. One strain is compatible with both ICGs 3 and 4. Both a nuclear phylogeny based on ribosomal IGS sequence data, and a mitochondrial phylogeny, based on a group-I intron located in the large subunit ribosomal RNA gene (LrRNA), were reconstructed. The level of compatibility was compared with the phylogenetic history of individuals belonging to this clade. For ICGs 2, 3 and 4 the level of incompatibility is positively correlated with the age of the most recent common ancestor. ICG 1 has a different position in the nuclear and the mitochondrial phylogenies. A possible explanation is that it has a hybrid origin, with different mitochondrial and nuclear donors. Overall, these results support the class of 'divergence-first' models of the evolution of (partial) incompatibility. However, we found evidence for genetic exchange between divergent populations as well. No clear pattern of ecological or geographical differentiation between ICGs was evident.

Introduction

Sexual intercompatibility (further referred to as compatibility in this study) has been used to delimit biological species (InterCompatibility Groups, ICGs) within Basidiomycetes (e.g. Boidin 1986; Petersen, 1995). For ectomycorrhizal fungi, the use of the biological species concept has been restricted to a few genera (*Laccaria*, Mueller and Gardes 1991; Fries and Mueller 1984; *Suillus*, Fries and Neumann 1990; *Paxillus*, Fries 1985; *Hebeloma*, Aanen and Kuyper, 1999). However, relatively few studies have explicitly addressed the question of the evolutionary origin of sexual incompatibility, which we define as speciation, in Basidiomycetes (Vilgalys 1991; Vilgalys and Sun 1995; Garbelotto *et al.* 1998). Most models of speciation can be classified as belonging to one of two main classes. One class of models assumes that the origin of reproductive barriers between species is preceded by a process of gradual genetic divergence. The classical allopatric mode of speciation (Mayr 1963) belongs to this class. Within Basidiomycetes evidence has been found in support of this class of models, incompatibility as a *consequence* of genetic divergence (*Pleurotus*, Vilgalys and Sun 1995; *Exidiopsis plumbescens*, Wells and Wong 1989). In this study we refer to this class of models as 'divergence-first' models.

The genetic basis of sexual incompatibility has been described by relatively simple models in some cases (Chase and Ullrich 1990a, 1990b). These simple models have led to hypotheses about sexual incompatibility as a *cause* of genetic divergence (Brasier 1987; Hallenberg 1991; Worrall 1997). Although agreement seems to exist that genetic reproductive barriers can arise easily between populations of Basidiomycetes (Bresinsky *et al.* 1987), the idea of 'incompatibility-first' models is still controversial. Cases of so-called 'ABC-relationships', where two sympatric populations are incompatible but both of them are compatible with a third allopatric population (Chamuris 1991; Hallenberg 1991; Petersen and Ridley 1996; Garbelotto *et al.* 1998), have been interpreted as evidence for this 'incompatibility-first' model, or at least for reinforcement, i.e. the evolution of incompatibility in sympatry after initial genetical divergence in allopatry. Phylogenetic relationships between populations could be used to discriminate between those different classes of models, because these classes predict different combinations of evolutionary history and compatibility. However, detailed knowledge about phylogenetic relationships is

usually lacking, especially between partially compatible populations (but see Garbelotto *et al.* 1998).

The role of ecological specialisation in speciation has been stressed, both in theoretical models (Maynard Smith 1966; Diehl and Bush 1989) as well as in case studies, usually concerning host switches in parasitic relationships (e.g. *Heterobasidion annosum*, Garbelotto *et al.* 1998). For ectomycorrhizal fungi, host tree switching can play this role in ecological specialisation (Kretzer *et al.* 1996; Aanen *et al.* 1999).

Sexual compatibility has been tested in the *Hebeloma crustuliniforme* complex to delineate biological species (ICGs; Aanen and Kuyper 1999). A total of 20 ICGs have been found in this group, some of which were only partially incompatible. These 20 ICGs formed two distinct monophyletic groups that did not form sister groups (Fig. 4-1; Aanen *et al.* 1999). One of these clades (clade II) consisted of 17 ICGs, the majority of which form ectomycorrhizae with members of the *Salicaceae*. The basal ICG of this clade (ICG 21) has not been found with *Salicaceae*, however, so the switch to *Salicaceae* appears to have preceded extensive speciation in this clade. Many of the phylogenetic relationships within this clade were not resolved, because of little sequence variation in the ribosomal ITS 1 and 2. One monophyletic group within clade II (clade IIa; the encircled clade in Fig. 4-1) consisted of nine ICGs, four of which were only partially incompatible. These four belonged to the morphospecies aggregate *H. crustuliniforme*, together with ICG 5 which was completely incompatible with these four. One isolate (605) could not unambiguously be placed into one of the ICGs: it was fully compatible with all members of ICGs 3 and 4. Of the other combinations between ICGs 3 and 4, 7 % was also compatible. Individual collections, however, could always be assigned to a single ICG. Isolate 605 was also the single isolate of 3/4 that was compatible with a member of ICG 2. Between ICGs 1 and 2 8 % of the combinations were compatible. However, these combinations usually showed signs of reduced performance indicated by lack of nuclear migration, aberrant morphology and reduced growth rate (Aanen and Kuyper 1999). Compatibility in this clade can thus be considered as a quantitative phenomenon with different levels of partial compatibility between populations. Both the lack of variation in ITS 1 and 2 sequences and partial compatibility between some ICGs, suggest a recent origin of sexual incompatibility within the *H. crustuliniforme* aggregate.

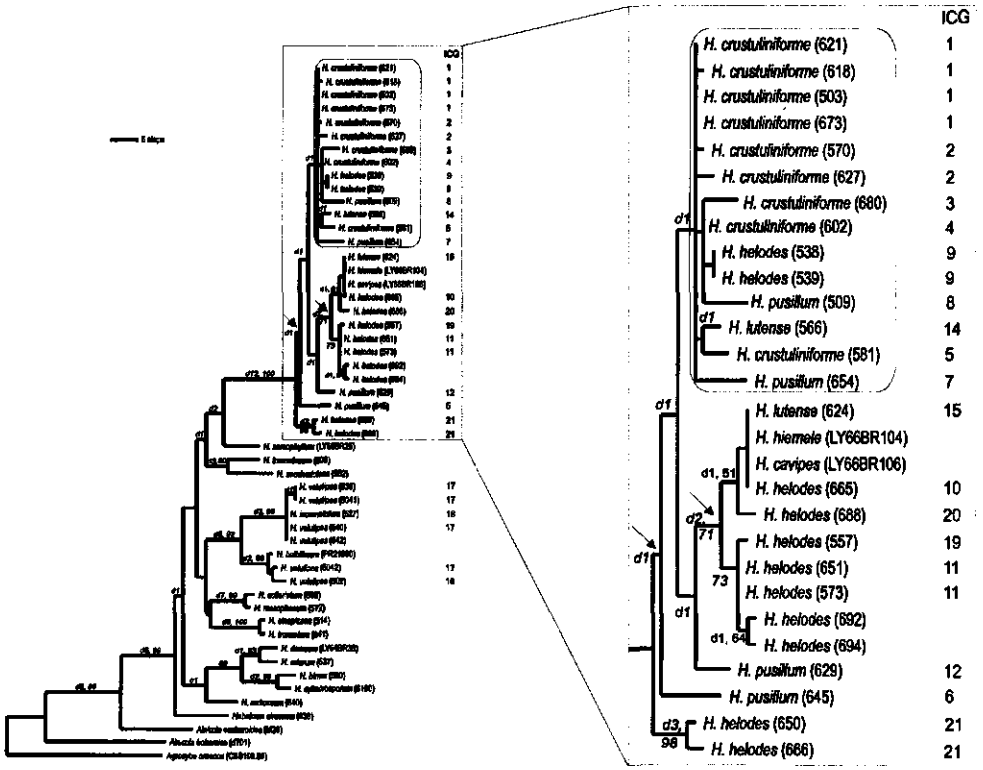

Fig. 4-1. Phylogenetic relationships in the genus *Hebeloma*, based on ITS sequences. Tree with the highest likelihood of 6527 most parsimonious trees of length 357 (c.i. =0.63, r.i.=0.81; excluding uninformative characters length=261, c.i.=0.49), found with PAUP, using *Agrocybe praecox* as an outgroup. Two arrows mark the two single proposed deletion events for two indels of 3 bp (that were not used as characters). Bootstrap values higher than 50 % and decay indices are indicated above branches. On the right, ICGs to which isolates belong are indicated. The enlarged clade is clade II, the encircled clade within clade II, clade IIa, which is the subject of this study.

The interface between population genetics and systematics is most informative for studies of speciation (Templeton 1998; Schilthuizen 1999; Coyne and Orr 1999). The more distant from the actual speciation event, the greater the expected genetic differences among the species, but the more difficult it becomes to infer which genetic differences were causally involved in speciation versus which were consequences of speciation (Templeton 1998). More importantly, at this interface between population biology and systematics examples exist where incompatibility is not qualitative ('all-or-nothing') but quantitative

with different levels of partial incompatibility (*Armillaria*, Anderson *et al.* 1980; *Heterobasidion annosum*, Chase and Ullrich 1990a+b; Garbelotto *et al.* 1998; *Hebeloma*, Aanen and Kuyper 1999). This provides the opportunity to compare different levels of compatibility with phylogenetic history of strains.

The aim of this study was to reconstruct the evolution of (partial) incompatibility in the *H. crustuliniforme* aggregate. We tried to find indications for either 'divergence-first' or 'incompatibility-first' models for the origin of incompatibility by reconstructing the phylogenetic history of strains belonging to ICGs of this aggregate that were partially or completely reproductively isolated.

For the class of 'divergence-first' models, genetical divergence is followed by complete incompatibility via intermediate stages of partial incompatibility. The most important prediction is a correlation between the level of compatibility and the relative age of the most recent common ancestor (MRCA). The 'incompatibility-first' model does not predict this correlation between the level of compatibility and the relative age of the MRCA. We furthermore considered the role of geographical and ecological differentiation in the evolution of incompatibility in the *H. crustuliniforme* aggregate.

Most studies on phylogenetic relationships between (biological) species do not include taxa that show partial compatibility (*e.g.* Piercey-Normore *et al.*, 1998; Vilgalys and Sun, 1994; Vilgalys *et al.*, 1996; Anderson and Stasovski, 1992). Only Garbelotto *et al.* (1998) explicitly addressed phylogenetic relationships between partially compatible strains of *Heterobasidion annosum*. In this study different levels of partial compatibility between some ICGs within the *Hebeloma crustuliniforme* aggregate were explicitly compared with phylogenetic history. The main questions that we addressed were:

i. Is there a positive correlation between the level of (partial) compatibility and the relative age of the most recent common ancestor (MRCA)?
ii. Does this correlation hold both for a nuclear and mitochondrial phylogeny?
iii. Do the data support 'divergence-first' or 'incompatibility-first' models?
iv. Do geographical and ecological specialisation play a role in speciation events in this aggregate?

Materials and Methods

Materials

For both the nuclear and mitochondrial phylogeny, single spore isolates of all ICGs belonging to clade IIa were used, except for two ICGs that belong to the morphospecies *H. pusillum* (ICGs 7 and 8; Table 4-I). As this study focused on the partially incompatible ICGs 1, 2, 3 and 4, three to five collections per ICG were used for these ICGs. Outgroups of clade IIa were based on the ITS phylogeny (Fig. 4-1; Aanen *et al.* 1999). For the nuclear phylogeny, two ICGs (15 and 20) of the sistergroup of clade IIa were used as outgroups. The sister group status of these two taxa was tested using another outgroup, ICG 21. For the mitochondrial phylogeny, one outgroup was used, viz. a representant of the sister group of clade IIa, ICG 20. In all, 23 isolates were used in the nuclear phylogeny and 21 in the mitochondrial. All isolates were collected and identified by the first author. Nomenclature of species follows Kuyper and Boekhout (1995). Voucher specimens are preserved in Wageningen (WAG) and a selection of the cultures in Baarn (CBS).

Choice of sequences, DNA isolation, PCR and sequencing

For methods of DNA isolation, PCR and sequencing, we refer to Aanen *et al.* (1999). As a nuclear sequence, the intergenic spacer (IGS; Anderson and Stasovsky 1992), located between 25S rDNA and 5S rDNA, was used. This sequence has been found to be somewhat more variable than the internal transcribed spacers 1 and 2 (ITS 1 and 2) (Erland *et al.* 1994). Primers 5SA and Cnl12 (Anderson and Stasovski 1992; Henrion *et al.* 1992) were used to amplify this region. Both primers were also used as sequencing primers.

To find a mitochondrial sequence with an appropriate level of variation, several mitochondrial ribosomal primer pairs that were found on the Bruns' lab homepage (HTTP://plantbio.berkeley.edu/~bruns/) were tested. The following pairs were used: MS1 and 2 (part of the small ribosomal subunit), ctb2 and 9 and CML5.5 and ML6 (both pairs part of the large subunit ribosomal RNA gene (LrRNA)). To test these different sequences, initially five sequences were determined from collections that belong to partially compatible ICGs. The level of variation found between these five sequences was used as a criterion to

TABLE 4-I. Material examined.

Morphospecies	ICG	collection number	collection site	host tree genus	genbank acc. no. igs	genbank acc. no. ctb
H. crustuliniforme	1	DKAm503-2	Borgsjö, S.	*Betula*	AF174437	AF174460
	1	DKAm618-3	Sustenpass, CH	*Salix*	AF174446	AF174468
	1	DKAm621-3	Davos, CH	*Dryas*	AF174448	AF174466
	1	DKA m673-5	Eemshaven, NL	*Salix*	AF174454	AF174472
H. crustuliniforme	2	DKAm570-2	Lelystad, NL.	*Salix*	AF174441	AF174462
	2	DKAm620-2	Davos, CH	*Salix*	AF174447	AF174469
	2	DKAm626-1	Mollendruz, CH	*Salix*	AF174450	AF174470
	2	DKAm627-1	Jura, F.	*Corylus*	AF174451	AF174471
	2	DKAm671-2	Eemshaven, NL	*Salix*	AF174453	AF174463
H. crustuliniforme	3	DKAm511	Hilpoltstein, D	*Tilia*	AF174438	AF174461
	3	DKAd680	Hoornaar, NL	*Populus*	AF174456	AF174473
	3	DKAm679-1	Hoornaar, NL	*Populus*	AF174455	AF174474
	3	DKAm686-9	Utrecht, NL	*Salix*	AF174457	AF174475
	3	DKAm699-1	Elsloo, NL	*Populus*	AF174459	AF174476
H. crustuliniforme	3/4	DKAm605-1	Adelboten, CH	*Salix*	AF174444	AF174465
	4	DKAm602-4	Adelboten, CH	*Dryas*	AF174443	AF174464
	4	DKAm617-9	Adelboten, CH	*Salix*	AF174445	AF174467
H. crustuliniforme	5	DKAm581-1	Utrecht, NL	*Tilia*	AF174442	-
H. helodes	9	DKAm538-4	Beilen, NL	*Salix*	AF174439	AF174477
H. lutense[a]	14	DKAm566-3	Lelystad, NL	*Salix*	AF174440	AF174478
Outgroups						
H. helodes	21	DKAm650-10	Jura, F	*Betula*	AF174452	-
H. helodes	20	DKAm688-4	Utrecht, NL	*Quercus*	AF174458	AF174479
H. lutense	15	DKAm624-1	Dwingeloo, NL	*Pinus*	AF174449	-

[a]: *H. lutense* is identical with *H. leucosarx sensu auct. Neerl.*

select one of those sequences for a broader analysis. Ctb 2 and 9 gave a PCR product of about 650 base pairs, containing an intron. These sequences showed both single nucleotide position variation as well as length variation. This sequence was therefore chosen as a mitochondrial marker for a broader analysis. Ctb 9 was used as a sequencing primer.

IGS1 sequences have been deposited in GenBank under accession numbers AF174437-AF174459 and CTB sequences under accession numbers AF174460-AF174479. The aligned sequences have been deposited to TreeBASE (HTTP://herbaria.harvard.edu/ treebase) as SN256-730 and SN256-731 or are available from the first author upon request.

Phylogenetic analysis

Sequences were aligned using Clustal V (Higgins et al 1992). The alignment was edited manually using a matrix created in PAUP* 4.0 (Swofford, 1998). Phylogenetic relationships were inferred from the aligned sequences using parsimony with PAUP* 4.0. Two analyses were performed, one with gaps as missing data, and one with gaps coded according to the coding scheme proposed by Hibbett *et al.* (1995). Under this coding scheme, nucleotide positions with single base indels are scored as characters, with gaps as fifth state; multiple-base indels are scored as binary characters; and single nucleotide positions aligned to multiple-base indel sites are scored as additional characters, with gaps as missing states. The goals of this coding scheme are that potentially informative indels are included in the analyses, that overweighing of single indels is avoided and finally that phylogenetically informative nucleotide variation in sequences aligned to gaps is preserved.

All transformations were unordered and equally weighed. The Branch and Bound option in PAUP was used to find most parsimonious trees. The option 'Collapse branches if minimum length is zero' was selected. Clade stability was assessed by 1000 bootstrap replications (Hillis and Bull 1993), using the option Branch and Bound. Decay indices (Bremer 1988; Donoghue *et al.* 1992) were calculated from PAUP tree files using the program Autodecay (Eriksson 1998) and PAUP. Other measures (tree length, sequence divergence, consistency and retention indices) were calculated using PAUP. To examine alternatives to the maximum parsimony trees, a neighbor-joining (Saitou and Nei 1987) tree was generated, based on the Kimura-2 parameter genetic distance, using PAUP. An additional test was performed with the constraint that ICGs 1, 2, 3, 4 and 5 (all belonging to

the morphospecies *Hebeloma crustuliniforme*) formed a monophyletic group in the nuclear phylogeny. Constrained and unconstrained trees were compared by three criteria: tree length, Templeton's (1983) non-parametric test and the Kishino-Hasegawa test (Kishino and Hasegawa, 1989) as implemented in PAUP.

To determine whether the two data sets were in conflict, a phylogenetic test designed to assess congruence between gene trees, the partition homogeneity test (Farris *et al.* 1995; Huelsenbeck *et al.* 1996), implemented in PAUP, was used for the 20 taxa that were common for both data sets. In this test, the observed sites from all genes for each individual are pooled and resampled without replacement to give an artificial data set in which sites have been swapped randomly among loci. Many such artificial data sets are produced. MP trees are then made for each newly sampled partition in each artificial data set. If the data sets have the same evolutionary history, the sums of the lengths of the gene trees for the observed and resampled data should be similar, but if they have different evolutionary histories, the sums of the tree lengths should be longer than that for the actual data, because of extra homoplasy in the data (Geiser *et al.* 1998). In this case, significance was assessed by comparing the summed tree length from the actual data (with gaps coded according to Hibbett *et al.* 1995) to those from 1000 artificial data sets.

Results

Distribution mitochondrial intron

For the first isolates tested, primers ctb 2 and 9 gave a product of approximately 650 base pairs. The *Suillus sinuspaulianus* sequence upon which primers ctb 2 and 9 were based (T.D. Bruns, pers. comm. 1998) was only 98 base pairs long, so an intron of around 550 base pairs was inferred to be present. Similarity searches indicated that this intron belongs to the class of group-I introns (Lambowitz and Belfort 1993). No long open reading frames, coding for endonucleases or maturases, were present in the intron. Unexpected results were found when using this primer pair for other isolates. First, the intron was absent from ICG 5, resulting in a pcr product of 98 base pairs, the sequence of which was identical with the partial LrRNA sequence of *Suillus sinuspaulianus*. Second, for ICGs 9, 14 and 20 no pcr products were obtained. Two possible explanations were: i) another insertion is present

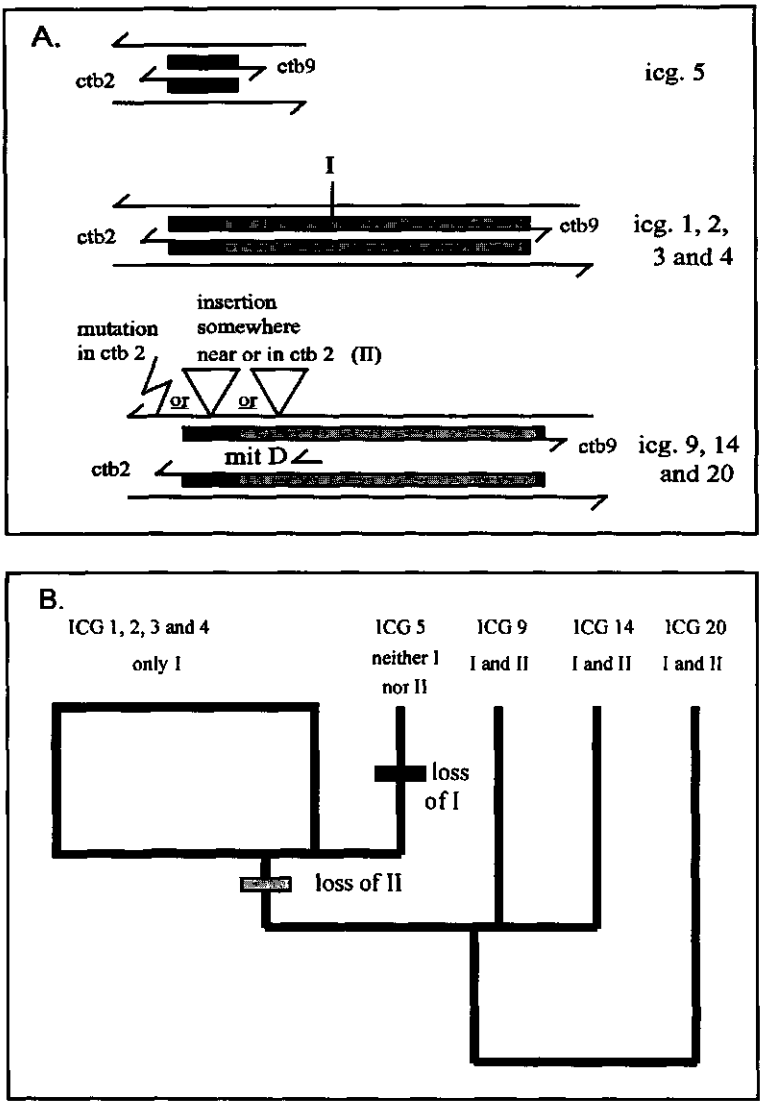

Fig. 4-2. Summary of gain and loss of intron(s) and/or mutation. 2A. A summary of the intron(s) and possible mutation found in the different ICGs. Using primers ctb2 and 9, no PCR products were obtained for ICGs 9, 14 and 20, but using mitD and ctb 9 PCR products were obtained for those ICGs. The possible explanations for these observations are illustrated. I is the insertion present in all ICGs except for ICG 5, II the mutation/insertion present in ICGs 9, 14 and 20.

2B. Most parsimonious explanation for the loss of the different introns and/or mutation, illustrated in Fig. 4-2a, using ICG 20 as outgroup. I is the insertion present in all ICGs except for ICG 5, II the mutation/insertion present in ICGs 9, 14 and 20.

somewhere in the mitochondrial DNA between ctb 2 and 9, making the sequence too long to be amplified under the applied pcr-conditions; ii) another insertion, or mutation is present in one of the two primer annealing sites ctb 2 or 9. In order to test whether the intron was present, several internal primers were tested in combination with either ctb 2 or 9. Primer mitD (GTTTATAAGAGAATATATCTA) located on the 5' side of the intron was used in combination with ctb9 and gave a product of 500 base pairs for ICGs 9, 14 and 20, which contains most of the phylogenetically informative variation. In Figure 2A, the possible hypotheses are presented that explain these results. Using ICG 20 as an outgroup, the most parsimonious explanation for the loss of the different introns and/or the mutation in the primer sites is given in Figure 2B.

Sequences

For the IGS, the total number of nucleotides used in the alignment ranged from 921 to 936. The length of the aligned sequences was 954. One region in the IGS sequences was excluded from the analysis because the alignment was ambiguous. The length of the aligned sequences used in the phylogenetic analysis was 906. Under gap=missing coding, 819 of these 906 were constant and 87 variable, 33 of which were parsimony informative. Maximum sequence divergence (absolute number of base pair differences) within the ingroup was 25 (2.7 %), and between in- and outgroup 27 (2.9 %). Under indel coding, the total number of characters used in the analysis was 914, 812 of these were constant, and 112 variable, 44 of which were parsimony-informative. Base composition of the entire region was approximately: A 0.32, C 0.23, G 0.22, T 0.23.

For the mitochondrial sequences all variation was within the intron. The following sequences were identical: i) m503-2, m618-3, m621-3 and m673-5 (ICG 1); ii) m679-1 and m686-9 (ICG 3); iii) m511-1, m680-1 and m699-1 (ICG 3) and of m602-4, m605-1 and m617-9 (ICG 4). For the sequences amplified with ctb2 and 9 (ICGs 1, 2, 3 and 4), the total number of nucleotides aligned ranged from 610 to 636. For the sequences amplified with MitD and ctb9 (ICGs 9, 14 and 20), this number ranged from 455 to 504. Two regions of the mitochondrial sequences were excluded from the analysis because the alignment was ambiguous. The length of the aligned sequences used in the phylogenetic analysis was 628. Under gap=missing coding, 608 of these 628 were constant and 21 variable, 13 of which

were parsimony-informative. Maximum sequence divergence (absolute number of basepair differences) within the ingroup was 13 (2.1 %), and between in- and outgroup 20 (3.2 %). Under indel coding, the total number of characters was 636, 602 of these were constant, and 34 variable, 23 of which were parsimony-informative. Base composition of the entire region was approximately: A 0.40, C 0.15, G 0.15, T 0.30.

Phylogenetic relationships

Nuclear phylogeny

Using the IGS sequences, under gap=missing coding one tree was found of length 94 (c.i.=0.92, r.i.=0.93; using only informative variation, length =43, c.i.=0.81). Coding gaps according to Hibbett *et al.* (1995) gave two trees of length 124 which only differed in the relationship between ICGs 14 and 5 (c.i.=0.92, r.i.=0.93; using only informative variation, length=57, c.i.=0.83). One of the two most parsimonious trees found with gap coding is shown in Figure 3 (topology similar to tree found with gap=missing coding). Using ICG 21 as an outgroup, ICGs 15 and 20 form a well supported monophyletic group, which is the sister group of group IIa. This relationship is in accordance with the ITS tree (Aanen *et al.* 1999). The monophyly of clade IIa is supported by a high bootstrap value (94%) and decay index (di=3). The neighbor-joining tree is almost identical with one of the two most parsimonious trees, except for the position of the clade consisting of 511 and 680 from ICG 3, which in the neighbor joining-tree is basal to a clade consisting of members of both 3 and 4 (neighbor-joining tree not shown).

The basal relationships of clade IIa are not resolved. One clade comprises ICG 9. The second clade consists of ICGs 1, 5 and 14. Within this well supported clade, ICG 1 forms a monophyletic group. The sister group of ICG 1 is 5. The sister group of 1 and 5 is ICG 14 (which belongs to the morphospecies *H. lutense*, whereas the two ICGs that constitute its sistergroup belong to the morphospecies *H. crustuliniforme*. The third clade within IIa consists of members of ICGs 2, 3 and 4. Within this clade, three monophyletic groups are found, the relationships between which are not resolved (a hard polytomy). One of these clades comprises all isolates of ICG 2. Two other clades comprise isolates of ICG 3, and isolates of ICGs 3 and 4, including isolate 605, respectively. Within the latter clade, one

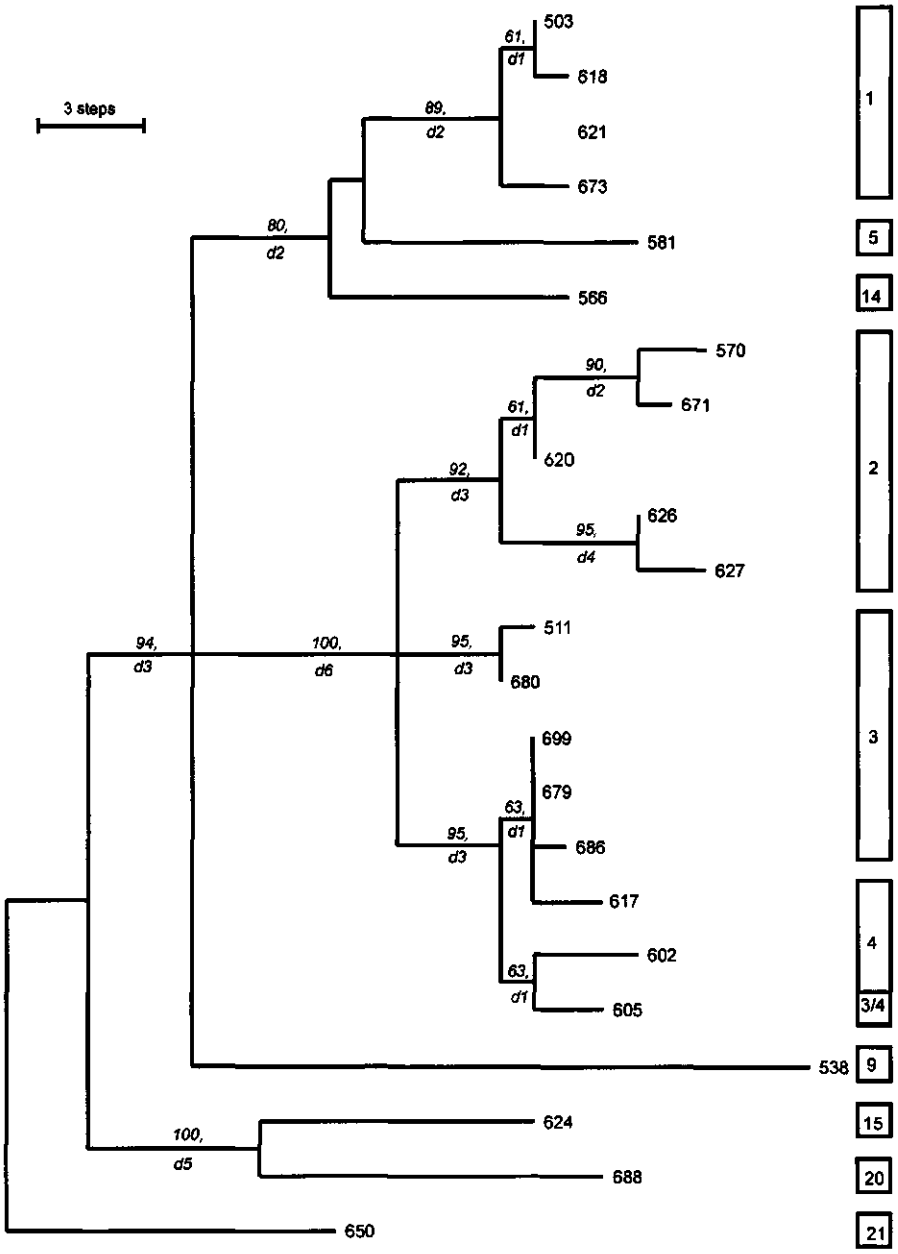

Fig. 4-3. One of two most parsimonious trees of length 124 (c.i.=0.92, r.i.=0.93, using all characters; using only parsimony-informative characters, length=57, c.i.=0.83) based on IGS1 sequences, using ICG 21 as outgroup. Indels were coded according to Hibbett *et al.* (1995). Bootstrap values higher than 50 % are indicated above branches. Decay indices (preceded by a d) are also indicated. On the right, ICGs to which isolates belong are indicated.

monophyletic group consists of one member of ICG 4 and 605, which was compatible with both 3 and 4. The sister group of this clade comprises three members of ICG 3 and one of ICG 4.

Mitochondrial phylogeny

When gaps were treated as missing data, 1 tree of 23 steps was found (c.i.=0.96, r.i.=0.98; using only informative sites, length=15, c.i.=0.93). The neighbor-joining tree did not differ from the most parsimonious tree. Using ICG 20 as an outgroup, the basal taxon within the ingroup is ICG 14. ICG 9 is the sister group of a clade comprising ICGs 1, 2, 3 and 4. Within the clade consisting of ICG 1, 2, 3 and 4, ICGs 1 and 2 together constitute a monophyletic group, as do 3 and 4. Within the clade consisting of 3 and 4, there is no resolution, because of a lack of variation. Within the clade with 1 and 2, 1 and 2 form monophyletic groups.

Using the gap coding scheme as described by Hibbett *et al.* (1995), three trees of length 41 (ci 0.85, ri 0.93; using only informative sites, length 30, ci 0.80) were found, which differ in the position of ICGs 14 and 9. The neighbor-joining tree did not differ from one of the most parsimonious trees. One of the most parsimonious trees under gap coding is depicted in Figure 4 (topology that is not in positive conflict with tree found with gap=missing coding). The only other difference with the 'gap missing' tree is the resolution within the clade consisting of ICGs 3 and 4. ICGs 3 and 4 in this tree form monophyletic groups.

Mitochondrial and nuclear phylogenies and compatibility compared

The mitochondrial and nuclear phylogenies were incongruent in several respects. To determine the significance of this conflict the partition homogeneity test was used for the 20 taxa that were common for both data sets. The actual length of the summed trees was 135 steps and this was shorter than 99.8 % of the artificial data sets, indicating that the gene trees have significantly different topologies (Fig. 4-5A). In Figure 6, the two phylogenies are compared, together with the pattern of compatibility, geographical origin and host tree genera. Strains of ICGs 1 and 2 form monophyletic groups in both the nuclear and mito-

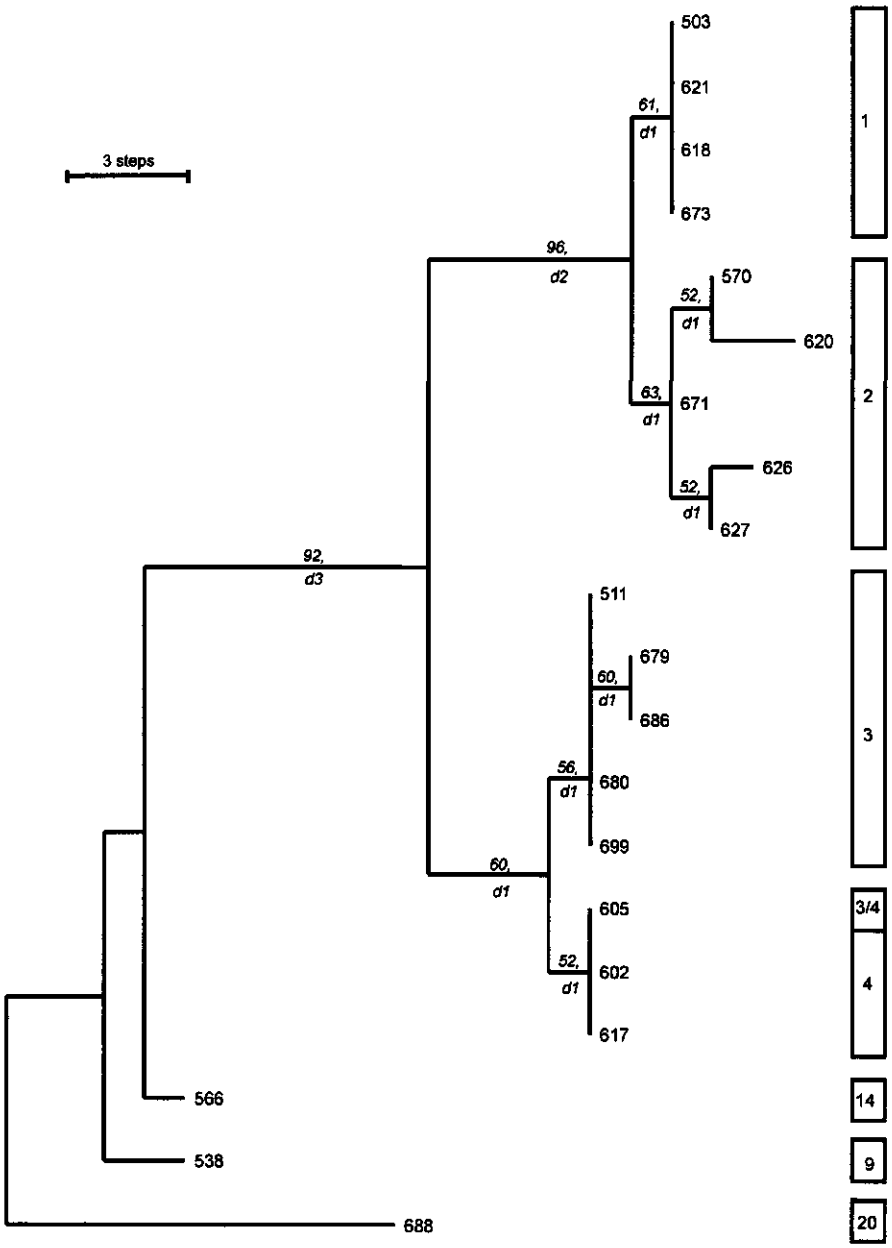

Fig. 4-4. One of three most parsimonious trees of length 41 (c.i.=0.85, r.i.=0.93, using all characters; using only parsimony-informative characters, length=30, c.i.=0.80) based on a group-I intron located in the mitochondrial large subunit ribosomal RNA gene (LrRNA), using ICG 20 as an outgroup. Indels were coded according to Hibbett *et al.* (1995). Bootstrap values higher than 50 % are indicated above branches. Decay indices (preceded by a d) are also indicated. On the right, ICGs to which isolates belong are indicated.

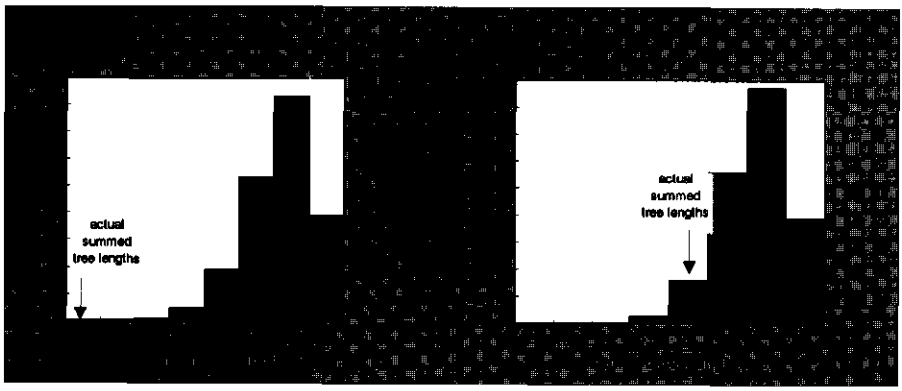

Fig. 4-5. Partition homogeneity test results. A: PHT for all 20 strains for which both data sets were available. B: PHT for the 16 strains other than ICG 1. See text for details.

chondrial phylogeny. In the mitochondrial phylogeny, the strains of ICG 3 form a monophyletic group and two strains of 4 form a clade with strain 605, that was compatible with both ICGs 3 and 4. In the nuclear phylogeny, however, strains of 3 and 4 form paraphyletic groups. The partially compatible 3 and 4 are sister groups in the mitochondrial phylogeny and belong to a hard polytomy in the nuclear phylogeny. The position of ICG 5 in the nuclear tree is not in contrast with the mitochondrial phylogeny, since we do not know its position there. In the nuclear phylogeny, ICGs 1, 2, 3, 4 and 5 form a paraphyletic group, but they form a monophyletic group with ICG 14. This is in contrast with the mitochondrial phylogeny, where 14 has a basal position relative to a clade consisting of 1, 2, 3 and 4 (and probably 5, according to the hypothesised gain and loss of the different introns/mutations, see Fig. 4-2B). However, a tree that forces the monophyly of ICGs 1, 2, 3, 4 and 5 in the nuclear tree is only 2 steps longer (gaps coded) and can not be rejected by the Kishino-Hasegawa test ($p=0.41$) or Templeton's non-parametric test ($p=0.69$).

The main difference between the two phylogenies is the position of ICG 1. In the mitochondrial phylogeny ICG 1 is the sister group of the partially compatible ICG 2, whereas in the nuclear phylogeny ICG 1 belongs to a clade consisting of ICGs 5 and 14, that is found basal to the clade consisting of 2, 3 and 4. We therefore also performed the partition homogeneity test on the data set without the four strains of ICG 1. Significance was assessed by comparing the summed tree length from the actual data to those from 1000 artificial data sets. The actual summed tree length of 123 was equal to or longer than 9.2 %

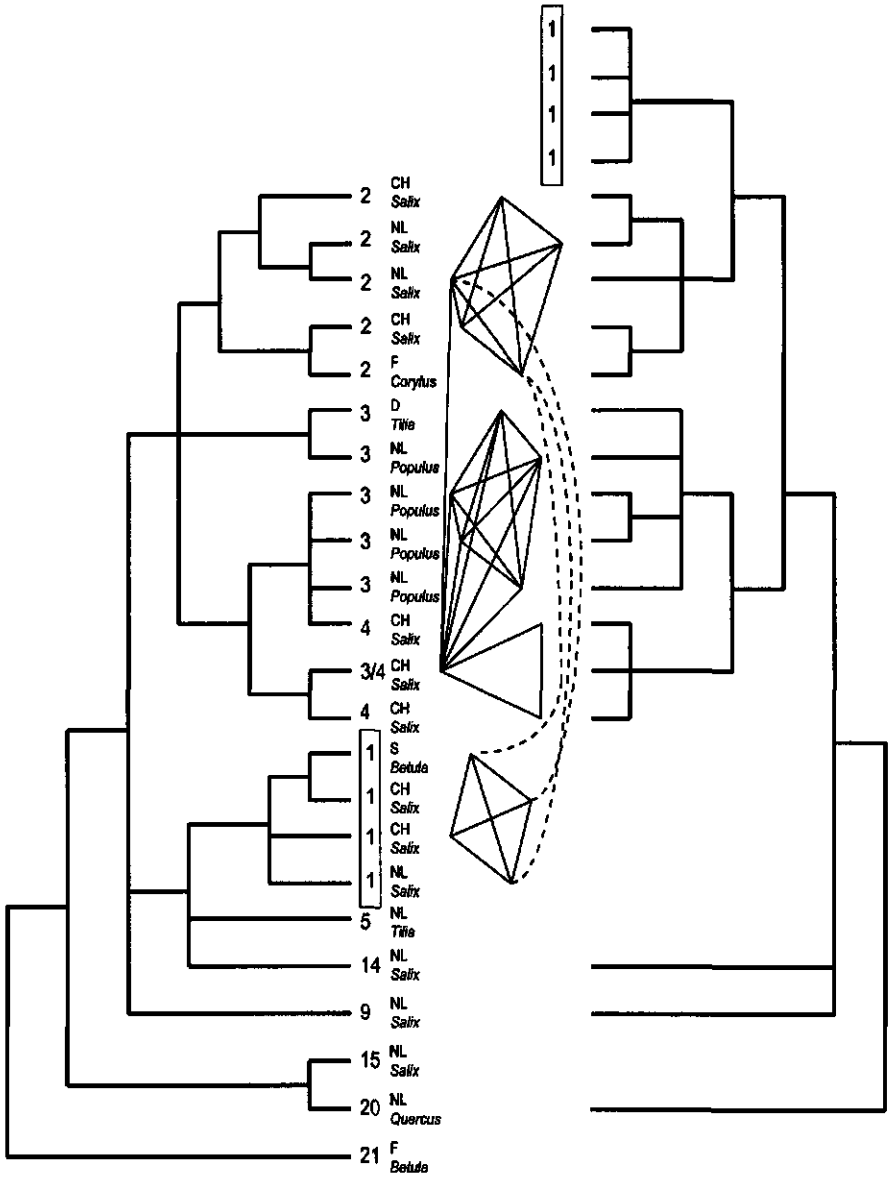

Fig. 4-6. Comparison between the nuclear and mitochondrial phylogeny. On the left the strict consensus tree of the two nuclear trees, on the right the strict consensus tree of the three mitochondrial trees. Corresponding taxa are placed next to each other except for ICG 1, which has different positions in the two phylogenies. Compatibility relationships between individual isolates are also depicted. Straight lines denote fully compatible pairings, not showing any sign of reduced performance, broken lines denote pairings showing signs of reduced performance. Geographical origin and host tree genera of individual isolates are also indicated.

of the artificial data sets, indicating that the gene trees for the taxa other than ICG 1 do not have significantly different topologies (Fig. 4-5B).

Discussion

Sexual compatibility

In a previous paper (Aanen and Kuyper 1999) we showed that there are some cases of partial compatibility between ICGs, viz. individual isolates usually could be placed into ICGs, but some combinations between ICGs were compatible as well. Isolate 605-1, however, was compatible with all members of both ICGs 3 and 4. This 'multi-ICG-compatibility' of a single isolate has also been observed in the genus *Pleurotus* (Petersen and Ridley 1995). Seven additional sib monokaryons of this 605-1 were also compatible with most isolates of both ICGs 3 and 4 (unpublished results).

We furthermore studied *individual* compatible combinations between ICGs 1 and 2, 3 and 4, and 2 and 3/4 and looked for evidence of partial compatibility at an *individual* level by subculturing dikaryotic area's from compatible pairings (unpublished observations). Generally, compatible combinations between ICGs 1 and 2 did show signs of reduced compatibility, viz. reduced growth and irregular or aberrant clamps. Moreover, in all compatible combinations between 1 and 2, no nuclear migration was observed and clamps were only found in the contact zone. The combinations between ICGs 3 and 4, however, did not show any sign of reduced compatibility, and usually also nuclear migration occurred. The single compatible combination between ICGs 2 and 3/4 (605) did not show any sign of reduced compatibility either and unilateral nuclear migration was observed. Furthermore, sib monokaryons of 605-1 were also compatible with sib monokaryons of 671-2, showing that the compatibility between 605 and 671 is not limited to the two monokaryons that were originally used (unpublished results).

Geographical and ecological differentiation

Generally, no clear pattern of geographical or ecological differentiation between ICGs nor between clades within ICGs was evident (Fig. 4-6). Members of ICGs 1 and 2 were

found adjacent to each other in the alpine zone with the same host tree species. Furthermore, members of 1, 2 and 3 were also found in the Netherlands next to each other with the same host trees.

However, geographic and/or ecological differentiation does occur between some closely related taxa to some degree. From the partially compatible ICGs 3 and 4, ICG 4 has been found exclusively in the alpine zone (as well as strain 605 that could not be placed into 3 or 4), whereas 3 has only been found in Western Europe. The alpine populations all fructify early in the autumn (end of August/beginning of September) whereas the non-alpine all fructify later (end September-November). This, together with the spatial isolation, will possibly contribute to genetical isolation between alpine and non-alpine populations.

ICG 5, which is completely incompatible with all other ICGs, is the only ICG of which no strains have been found with members of *Salicaceae*, whereas all other ICGs show a preference for this family of hosts. Within the group of *Salicaceae* specific ICGs also some evidence of different ecological specialisations has been observed. Most of the isolates of ICG 3 have been found with *Populus* species, whereas 1, 2 and 4 have been found mainly with *Salix* species.

Mitochondrial and Nuclear Phylogenies and the Level of Compatibility Compared

In sexual Agaricales, nuclei migrate bidirectionally after pairing, but mitochondria do not, resulting in dikaryotic mycelia that are mosaic for mitochondria from the two monokaryons. Heteroplasmy only occurs in the region where the hyphae of the two monokaryons meet. Recombination between different populations of mitochondrial genomes in a natural population of *Armillaria gallica* has been shown to occur (Saville *et al.* 1998). This implies that we cannot consider the mitochondrial sequences as strictly clonal; the frequency of recombination in Basidiomycete mitochondria in nature remains to be determined, however.

Generally, members of ICGs formed monophyletic groups. ICGs 1 and 2 formed monophyletic groups in the nuclear as well as in the mitochondrial phylogenies. ICGs 3 and 4 formed monophyletic groups in the mitochondrial phylogeny, but paraphyletic in the nuclear. Paraphyletic ICGs have been found in *Pleurotus* (Vilgalys and Sun 1994) and in a different group of *Hebeloma* as well (Aanen et al 1999). In *Pleurotus*, these paraphyletic

ICGs consisted of monophyletic populations from different continents. Future studies in the *H. crustuliniforme* aggregate should therefore include strains from a broader geographic area, to consider the possibility of finding other paraphyletic ICGs.

Several lines of evidence point to a relatively recent origin of incompatibility between 3 and 4. In the mitochondrial phylogeny ICGs 3 and 4 were sister groups and in the nuclear they were part of a hard polytomy. Moreover, ICGs 3 and 4 themselves did not form monophyletic groups in the nuclear phylogeny. The partial incompatibility between ICGs 2 and 3/4 is of an older origin than that between 3 and 4. This is evident from both the nuclear and the mitochondrial phylogeny. The level of compatibility between 3 and 4 (15%, including strain 605, which belongs to both 3 and 4) is higher than that between 2 and (3,4) (0.6 %). Therefore, for these three ICGs (2, 3 and 4) the level of incompatibility is positively correlated with the relative age of the MRCA. This is consistent with the class of 'divergence-first' models of the evolution of (partial) incompatibility.

The main difference between the nuclear and mitochondrial trees was the position of ICG 1. There are several possible explanations for this incongruence. One explanation is horizontal transmission of the mitochondrial intron sequences. At low taxonomic levels, good concordance was found between intron phylogenies and organismal phylogenies (Shinohara *et al.* 1996) but several unexpected cases of horizontal transfer have been documented (e.g. Cho *et al.* 1998; Nishida *et al.* 1998). However, since we did not find long open reading frames coding for endonucleases or maturases in this intron, it is probably not mobile (Cho *et al.* 1998; Shinohara *et al.* 1996). Therefore, horizontal transfer of the intron as an explanation for the found incongruence does not seem very plausible. A different explanation is that ancestral polymorphisms in the ribosomal DNA have been maintained with different lineage sorting in the different clades. The nuclear sequence we have used is part of the tandemly repeated rDNA cluster. Although this repeated array of sequences is generally believed to be homogenized by the process of concerted evolution (Elder and Turner 1995), ancestral polymorphisms can be maintained and the majority type of alleles in an array can fluctuate (O'Donnell and Cigelnik 1997). As a consequence, incongruence between phylogenies based on ribosomal sequences and phylogenies based on other sequences has been found (O'Donnell and Cigelnik 1997). One of the factors that may contribute to the maintenance of ancestral polymorphisms is that there are several repeats

dispersed on nonhomologous chromosomes, because concerted evolution is thought to be less efficient between chromosomal loci than within a chromosomal locus (O'Donnell and

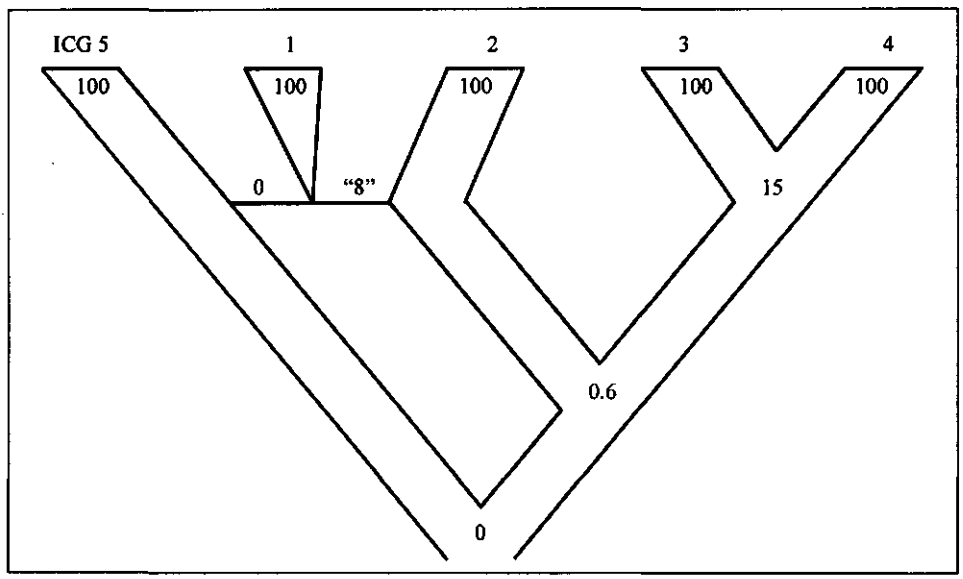

Fig. 4-7. A scenario for the evolution of partial compatibility in the *Hebeloma crustuliniforme* aggregate, based on evidence from nuclear and mitochondrial phylogenetic relationships and patterns of compatibility. On the tree the different levels of compatibility (%) within and between the different ICGs are indicated.

Cigelnik, 1997). However, basidiomycetes studied to-date present a unique rDNA locus (Selosse *et al.* 1996). Therefore, maintenance of ancestral polymorphisms is not a likely explanation for the different position of ICG 1 in the nuclear and mitochondrial phylogenies.

A different explanation for the incongruence between the nuclear and mitochondrial trees is that ICG 1 has a hybrid origin, with different donors of the mitochondrial and nuclear sequences. Under this scenario the mitochondrial donor of ICG 1 was the ancestral lineage leading to ICG 2, since ICG 1 is the sister group of 2 in the mitochondrial phylogeny; the donor of the nuclear sequence was the ancestral lineage leading to ICG 5, since 1 and 5 are sister groups in the nuclear phylogeny. The relative uniformity in both nuclear and mitochondrial sequences of ICG 1 (no differences in mitochondrial sequences, and maximally four base pairs difference in IGS sequences; maximum sequence divergence is higher in all other ICGs) suggests that its origin was relatively recent and/or accompanied

by a bottleneck. Phylogenies based on other molecular markers, both nuclear and mitochondrial, could be used to confirm the proposed hybrid origin of ICG 1. The partial compatibility found between ICGs 1 and 2 is in agreement with recent genetic exchange between ancestors of 1 and 2. ICG 5, the nuclear sister group of 1, lacks the mitochondrial intron, and therefore probably has not exchanged mitochondrial sequences with 1 and 2 recently, which is in agreement with the full incompatibility between 5 and 1 and 2.
A possible scenario for the evolution of (partial) incompatibility in the *H. crustuliniforme* aggregate is illustrated in Figure 7.

Overall, these results are partially consistent with the class of 'divergence-first' models of the evolution of (partial) incompatibility. However, we found evidence for genetic exchange between divergent populations as well.

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5

An extensive and widespread ITS polymorphism within an InterCompatibility Group of the ectomycorrhizal fungus *Hebeloma velutipes*.

Duur K. Aanen, Thomas W. Kuyper and Rolf F. Hoekstra

Submitted.

Abstract

Within InterCompatibility Group 17 of the morphospecies *Hebeloma velutipes* a dikaryotic strain was found with two divergent types of the ribosomal Internal Transcribed Spacer (ITS 1 and 2). These two types segregated in monokaryotic progeny of the same strain, showing that these different ITS types represent different alleles at homologous rDNA loci. RFLP analysis of more strains of ICG 17, representing 34 nuclei, showed that the polymorphism is widespread, with both types occuring in Europe as well as in America. Cladistic analyses of the two ITS sequences showed that they do not form a monophyletic group. One of the types belongs to a clade together with the single ITS type found in the partially compatible ICG 16 and the other to a clade together with the single ITS type found in the fully incompatible ICG 18.

RFLP analysis of the mitochondrial ribosomal SSU showed that there were fixed differences between the mitochondria of ICG 16 and 17. Several lines of evidence indicate that the ITS polymorphism in ICG 17 is not the result of actual hybridisation between 16 and 17. The polymorphism within ICG 17 must therefore be of a different origin. The lack of recombinants, both within the rDNA locus and between ITS 1 and 2, suggests that the two types have come together relatively recently.

These results clearly show the potential danger of using single ribosomal sequences for reconstructing species phylogenies and the potential problems for molecular identification of species.

Introduction

Species of the genus *Hebeloma* (Fr.) Kumm. (Agaricales, Basidiomycotina) form ectomycorrhizas with many tree species under a wide range of environmental conditions. The genus is considered taxonomically difficult and the taxonomic status of a number of the recognised taxa is uncertain. Within the genus, one of the best known names is *H. crustuliniforme* (Bull.) Quél., a name regularly encountered in the mycorrhizal literature. However, it is likely that this name is used for a species complex of taxa with relatively pale (but sometimes more brown-tinged), veilless carpophores with weeping lamellae. This complex includes species such as *H. alpinum* (J. Favre) Bruchet, *H. fragilipes* Romagn., *H. helodes* J. Favre, *H. leucosarx* P.D. Orton, *H. longicaudum* (Pers.: Fr.) Kumm., *H. pusillum* J. Lange, and *H. velutipes* Bruchet (Bruchet 1970, Vesterholt 1993, 1995). This complex was referred to as the *H. crustuliniforme* complex in previous studies (Aanen and Kuyper, 1999, Aanen *et al.*, 1999). Intercompatibility tests have shown the existence of at least 20 InterCompatibility Groups (ICGs) in this species complex (Aanen and Kuyper, 1999). Phylogenetic relationships within the genus *Hebeloma* based on a cladistic analysis of nuclear ribosomal ITS sequences showed that the *H. crustuliniforme* complex consists of two distinct clades (clade I and II) that probably are not sister groups (Aanen *et al.*, 1999). Clade I consists of the two ICGs of morphospecies *H. velutipes* (the partially compatible ICGs 16 and 17) and morphospecies *H. incarnatulum* Smith (ICG 18). Clade I is the subject of this study.

The ITS sequences were determined by direct sequencing of amplified PCR products. The electropherogram of one of the PCR products of a dikaryon (collection 504) of ICG 17 was found to have a double signal at 19 nucleotide positions. Two monokaryons of the same collection had single sequences that differed at these 19 positions. These two monokaryons belonged to two well supported monophyletic groups within clade I. One of these subclades consists of members of ICG 17 and 18, among which one of the types of 504, and the other of members of 16 and the other type of 504. Two types were thus found within ICG 17 that belong to different subclades.

The presence of two divergent ITS types within one biological species and even within a single dikaryotic strain raises several questions. Multigene families, such as the ribosomal DNA, often show a great similarity within a genome and within species, but accumulate

differences between species. The process of homogenisation of copies within a genome has been termed concerted evolution (Arnheim *et al.*, 1980). The two mechanisms most commonly proposed as responsible for homogenisation are gene conversion and unequal crossing over (Elder and Turner, 1995). However, the exact homogenising mechanism(s) is (are) not well understood. The polymorphism within ICG 17 shows that sequences within multigene families not always show a high level of sequence similarity.

The two ICGs found within the morphospecies *H. velutipes*, 16 and 17 were only partially incompatible, with 3.6 % of the combinations between 16 and 17 having clamp connections (Aanen and Kuyper 1999). Usually, individual compatible combinations between 16 and 17 showed reduced performance. This reduced compatibility was evident from i) aberrant clamp connections and hyphae ii) reduced growth and iii) no nuclear migration, but only dikaryotic outgrowth. Since ICGs 16 and 17 were partially compatible, it is tempting to explain the different types within strain 504 as a result of a direct hybridisation between ICG 16 and 17. However, strain 504 could be placed unambiguously in ICG 17 and all other strains tested could be placed unambiguously in a single ICG as well.

In this study we address the following questions: i) is the single case of mixed sequences that we found exceptional, or can we find more cases of mixed types; ii) do we find different types within nuclei or between nuclei; iii) if the different ITS types reside in different nuclei, is the distribution of both types in Hardy-Weinberg equilibrium; iv) do we find evidence for recombination between the two types; v) what are possible explanations for the existence of such divergent types in a single ICG and vi) can we find evidence for cytoplasmic exchange between 16 and 17?

We adress these questions by analysing ITS sequences of strains belonging to clade I in detail. Furthermore, we determine the frequencies of the different ITS types of ICG 16 and 17 by ITS RFLPs. In order to trace possible recombination between ITS 1 and 2, RFLPs that discriminate between the different types in both ITS 1 and 2 are studied. In order to study the inheritance of the different ITS types, we study RFLPs of monokaryotic progeny of one mixed dikaryon. Possible cytoplasmic exchange between ICG 16 and 17 is determined by studying sequences and RFLPs of part of the mitochondrial ribosomal small subunit.

Materials and Methods

Materials

Carpophores belonging to the morphospecies *H. velutipes* (members of the *H. crustuliniforme* complex with dextrinoid spores and cylindrico-clavate, non-capitate cheilocystidia, usually with a distinctly bulbous stipe) were collected in a variety of habitats, with a variety of host trees in Europe in 1994, 1995 and 1996. Only carpophores growing closely together were considered to belong to the same mycelium and were given one collection number. In cases of doubt, only one carpophore was collected. Table 5-I gives data on all collections that have been used in this study. For each collection, dikaryotic mycelium was regenerated from excised sporocarp context tissue and monokaryons were obtained using spore germination (Aanen and Kuyper, 1999). Sporocarps were dried and preserved in the herbarium of Wageningen (WAG), for future reference. Data on ecology (including potential host trees), and macro- and micromorphology for each collection were recorded. Sporocarps, dikaryotic tissue, and monokaryons received the same stock number. Within a stock, each monokaryon was given an additional individual number. Fungal cultures are preserved at the Centraalbureau voor Schimmelcultures, Baarn (CBS).

Two North American strains were obtained from D. McKay (Corvallis, Oregon). They were initially provided as *Hebeloma crustuliniforme*, but molecular data clearly show that they belong to the *H. velutipes* clade. As an outgroup the morphospecies *H. sinapizans* (Fr.) Gillet (strain 9514) was used.

DNA isolation and sequencing and phylogenetic analysis

Direct sequencing was performed and DNA was isolated from dikaryotic or monokaryotic cultures. For methods of DNA isolation, PCR and sequencing of the ITS 1 and 2 region, we refer to Aanen *et al.* (1999). In addition to the sequences already published elsewhere (d527 (ICG 18), d504, m504-1, m504-2, d535, d540, d642 (all ICG 17) and d502 (ICG 16); Aanen *et al.*, 1999) sequences were determined for two American strains (s166 and 7650). Since these were dikaryotic strains, we have not tested sexual intercompatibility relationships of these, and hence these strains could not be assigned to

Table 5-I. Data on the collections used in this study.

Collection number	Geographic origin	Collection date	Host tree genera	ICG	Sequence (s) or RFLP?*
9502	Wijster, Drenthe, Netherlands	15-IX-95	*Betula*	16	s, drflp
9541	Roden, Drenthe, Netherlands	5-X-95	*Carpinus*	16	drflp
9542	Roden, Drenthe, Netherlands	5-X-95	*Fagus, Carpinus*	16	drflp
9543	Roden, Drenthe, Netherlands	5-X-95	*Fagus, Quercus, Corylus*	16	drflp
9545	Roden, Drenthe, Netherlands	5-X-95	*Fagus*	16	mrflp
9407	Terschelling, Friesland, Netherlands	7-IX-94	*Fagus*	17	drflp
9432	Assen, Drenthe, Netherlands	4-X-94	*Quercus*	17	drflp
9504	Borgsjö, Medelpad, Sweden	18-IX-95	*Betula*	17	s, drflp
m504-1-2	,,	,,	,,	,,	s, mrflp
m504-3-10	,,	,,	,,	,,	mrflp
9516	Roth, Bayern, Germany	24-IX-95	*Picea, Pinus*	17	mrflp
9522	Hilpoltstein, Bayern, Germany	25-IX-95	*Betula*	17	mrflp
9524	Hilpoltstein, Bayern, Germany	25-IX-95	*Pinus*	17	mrflp
9526	Roth, Bayern, Germany	25-IX-95	*Fagus*	17	drflp
9529	Roth, Bayern, Germany	26-IX-95	*Pinus*	17	mrflp
9532	Kemnathen, Bayern, Germany	27-IX-95	*Quercus, Salix*	17	drflp
9534	Mauk, Bayern, Germany	28-IX-95	*Pinus*	17	mrflp
9535	Mauk, Bayern, Germany	28-IX-95	*Pinus*	17	s, drflp
9540	Roden, Drenthe, Netherlands	5-X-95	*Carpinus*	17	s, drflp
9556	Havelte, Drenthe, Netherlands	8-X-95	*Quercus, Betula, Salix*	17	mrflp
9623	Dwingeloo, Drenthe, Netherlands	18-IX-96	*Quercus, Betula*	17	drflp
9625	Wijster, Drenthe, Netherlands	20-IX-96	*Fagus*	17	drflp
9639	Lac de Rouges Truites, Jura, France	23-IX-96	*Picea*	17	drflp
9642	Lac de Rouges Truites, Jura, France	24-IX-96	*Salix*	17	drflp
9643	Lac de Rouges Truites, Jura, France	24-IX-96	*Fagus, Quercus, Carpinus*	17	drflp
9647	Lac de Rouges Truites, Jura, France	24-IX-96	*Salix*	17	drflp
9653	Lac de Rouges Truites, Jura, France	26-IX-96	*Picea, Salix*	17	drflp
9527	Roth, Bayern, Germany	26-IX-95	*Pinus*	18	s

	North American strains				
S166	Oregon, USA	15-X-71	*Pseudotsuga*	?	s
7650	Oregon, USA	13-X-83	*Pinus, Tsuga*	?	s
	outgroup				
9514	Roth, Bayern, Germany	24-IX-95	*Pinus, Picea*		s

* s=sequence, mrflp=rflp of a monokaryotic strain, drflp=rflp of dikaryotic strain.

ICGs. As sequencing primers ITS 1, ITS 3 and ITS 4 were used (White *et al.*, 1990). Sequences were deposited in GenBank. The aligned sequences are available from the first author upon request.

Sequences were aligned manually using a matrix created in PAUP* 4.0 (Swofford, test version, 1998). Phylogenetic relationships were inferred from the aligned sequences using parsimony with PAUP. Gaps were treated as missing data. The Branch and Bound option in PAUP was used to find most parsimonious trees. Clade stability was assessed by 1000 bootstrap replications (Hillis and Bull, 1993), with setting Branch and Bound. Decay indices (Bremer, 1988, Donoghue *et al.*, 1992) were calculated from PAUP tree files using the program Autodecay (version 4.0, Eriksson, 1998) and PAUP. Other measures (tree length, sequence divergence, consistency and retention indices) were calculated using PAUP.

Part of the mitochondrial small subunit was amplified using primers MS1 and MS2 (White *et al.*, 1990). For one strain of ICG 16 (d545) and one strain of ICG 17 (m524) the sequence of this PCR product was determined, using MS1 as sequencing primer.

RFLP analysis

Sequences belonging to clade Ia and Ib were compared to find RFLP markers. *Hae*III gave a difference in ITS I and *Hinf*I a difference in ITS 2. These enzymes were therefore used to study the ITS of a large sample of strains belonging to ICG 16 and 17. Furthermore,

10 monokaryotic progeny of dikaryon d504, which had a mixture of both ITS types, were analysed by ITS RFLP to study the inheritance of the polymorphism.

The mitochondrial sequences of 524 and 545 differed in three *Tru*9I restriction sites. *Tru*9I was therefore used to study the mitochondrial SSU of a large sample of strains belonging to ICG 16 and 17.

Between 1 and 2 μg of amplified DNA were digested for 3 h with 5 to 10 units of various restriction enzymes (*HaeIII*, *HinfI*, *Tru*9I, Pharmacia) according to the manufacturer's instructions. The restriction fragments were size fractionated using 1.5% agarose gel electrophoresis. Gels were stained with ethidium bromide, and photographed under u.v. light. A 100 bp ladder (Promega) was used as a size marker.

Results

ITS sequences

Borderlines between 18S rDNA, ITS1, 5.8 S, ITS2 and 28S were identified using *Heterobasidion annosum* (Fr.:Fr.) Bref. sequences (Kasuga *et al.*, 1993). The total length of ITS 1, 5.8S and ITS 2 was 611 nucleotides for all isolates, the length of the aligned sequences 613. The sequences of m504-1 and d535 were identical. The electropherograms of strains s166 and d504 showed mixed peaks at 17 and 19 positions, respectively. Using two monokaryons of d504, single sequences were found that differed at these 19 positions. Using *H. sinapizans* as an outgroup, and only the single sequences, one most parsimonious tree was found, which is depicted in Fig. 5-1. M504-2 and d502 form a monophyletic group (clade Ib) and m504-1 forms a clade with all other strains (clade Ia). Two main ITS types can therefore be recognised.

In Table 5-II the variable positions are given of the isolates sequenced. The total number of nucleotide positions showing variation, was 26. Clades Ia and b differed at 17 nucleotide positions. Ten of these 17 differences were in ITS1, seven in ITS2. The electropherogram of strain s166 has mixed peaks at all of these 17 positions. Since we had no monokaryons of this strain, no further analysis of this polymorphism was made. Nine of the 26 nucleotide differences were differences within clade Ia or b (indicated with * in Table 5-II).

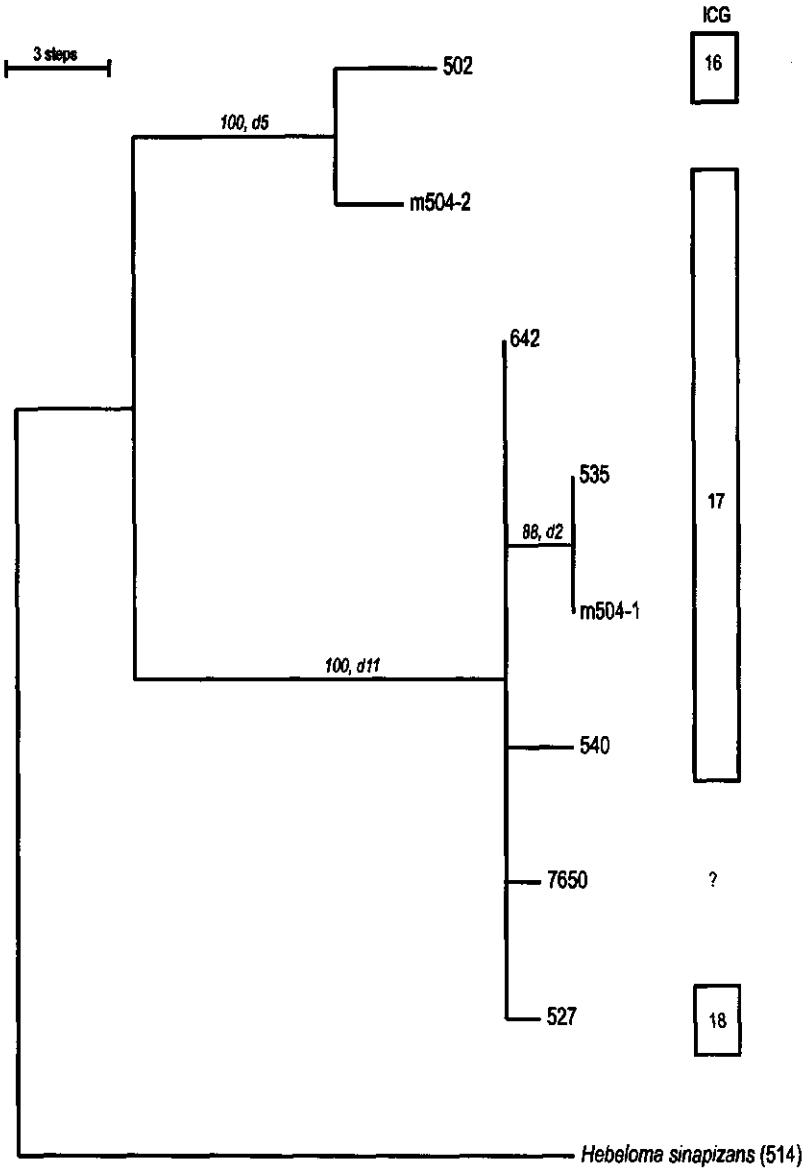

Fig. 5-1. Phylogenetic relationships between strains belonging to ICG 16, 17 and 18 of *Hebeloma velutipes*, based on ITS 1and 2 sequences. Single most parsimonious tree of length 48 (c.i. = 1.0, r.i. =1.0; length = 20 using only informative characters) using *H. sinapizans* as an outgroup. Indicated are bootstrap values and decay indices (preceded by d). On the right, ICGs to which isolates belong are indicated.

Table 5-II. Variable positions in ITS 1 and 2.

	ITS 1						ITS 2																				
Nucleotide	I	30	31	38	92	96	13	13	13	16	16	17	20	21	23	41	41	42	44	46	50	54	55	56	58	59	60
position	C				*		3	7	8	2	3*	8*	8	6	2	4	5	1	6	7	7*	5*	8*	5*	3	1	9*
Strain\	G					*																					
d502	16	T	C	G	T	G	T	A	T	G	G	-	T	C	C	C	T	C	C	G	T	G	T	G	C	C	C
m504-2	17	T	C	G	C	G	T	A	T	G	G	-	T	C	C	C	T	C	C	G	C	G	C	G	C	C	T
d504	17	TC	CT	G	C	G	TC	A	TC	G	G	-	TC	C	CT	C	TC	CT	CT	G	C	G	C	G	CT	C	TC
				A				G		A				A		A				A		C				G	
m504-1	17	C	T	A	C	G	C	G	C	A	G	-	C	A	T	A	C	T	T	A	C	C	C	G	T	G	C
s166	?	TC	CT	G	C	G	TC	A	TC	G	G	-	TC	C	CT	C	TC	CT	CT	G	C	G	C	G	CT	C	C
				A				G		A				A		A				A						G	
7650	?	C	T	A	C	G	C	G	C	A	T	-	C	A	T	A	C	T	T	A	C	G	C	G	T	G	C
d642	17	C	T	A	C	G	C	G	C	A	G	-	C	A	T	A	C	T	T	A	C	G	C	G	T	G	C
d535	17	C	T	A	C	G	C	G	C	A	G	-	C	A	T	A	C	T	T	A	C	C	C	G	T	G	C
d540	17	C	T	A	C	G	C	G	C	A	G	A	C	A	T	A	C	T	T	A	C	G	C	-	T	G	C
d527	18	C	T	A	C	A	C	G	C	A	G	-	C	A	T	A	C	T	T	A	C	G	C	G	T	G	C

-=indel in alignment

*=difference within clade Ia or Ib.

At position 216 (ITS1) there was a polymorphism discriminating clade Ia and b in the restriction site of the enzyme *Hae*III. Members of clade Ia had an A at this position and lack the restriction site, and members of Ib had a G and had the restriction site. For members of Ia, one band of approximately 680 base pairs was expected and for members of Ib, two bands, one of approximately 220 and one of 460. At position 591 (ITS2), a polymorphism was found, that discriminated between Ia and Ib, in a restriction site of *Hinf*I. For members of clade Ia, we expected two bands of approximately 340 base pairs. Members of clade Ib were expected to have bands of 340, 250 and 90 base pairs.

ITS RFLPs d504 and 10 monokaryons

PCR products of 10 monokaryotic progeny of dikaryon d504 were analyzed by ITS RFLPs, using the restriction enzyme *Hae*III. The results are shown in Fig. 5-1. No mixed patterns were observed, indicating segregation of both types in the monokaryons. Four monokaryons had type Ib and six type Ia.

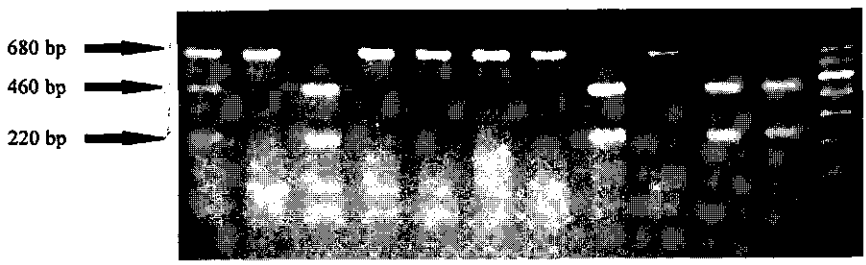

Fig. 5-2. RFLPs of ITS PCR-products digested with *Hae*III of strain d504 (lane 1) and 10 monokaryotic progeny of the same strain (lane 2-11). Lane 12: 100 bp ladder. The mixed pattern of d504 segregates in the monokaryotic progeny.

ITS RFLPs individuals of 16 and 17

In order to study the frequency of both types of ITS, RFLPs using the restriction enzymes *Hae*III and *Hinf*I were determined. Four additional strains of ICG 16 were studied (3 dikaryons and one monokaryon, giving a total of 7 nuclei) and 16 strains of ICG 17 (10

dikaryons and 6 monokaryons, giving a total of 26 nuclei). Strains that were classified as type Ia using *Hae*III, always were also classified as type Ia when using *Hinf*I. This indicates that we have not found recombinants between ITS 1 and 2. Monokaryons always had a single ITS type, dikaryons sometimes had mixed types.

Within ICG 16, type Ib was always found, within ICG 17 both type Ia and Ib were found. The total frequency of type Ib within ICG 17 was 35 % (12/34) and that of Ia 65 % (22/34) (using all data, also the sequences).

Of ICG 17 14 dikaryons were studied. Three heterokaryotic dikaryons were found: 9407, 9432 and 9504. We calculated the expected Hardy-Weinberg equilibrium frequencies of the different homokaryotic and heterokaryotic types and compared these expected values with the observed frequencies. In Table 5-III these calculations are summarised. There was a deficiency of heterokaryotic types, but the difference between observed and expected was not significant ($0.10 < p < 0.25$).

Table 5-III. Expected and observed heterodikaryotic types within ICG 17 and significance testing using the G test.

	Ia|a	Ia|b	Ib|b
expected frequency	0.419	0.457	0.124
expected numbers	5.86	6.39	1.74
observed numbers	8	3	3
$G=3.54$, $0.10<p<0.25$			

Mitochondrial sequences and RFLPs

The MS1-2 sequences of strains 9524 (ICG 17) and 9545 (ICG 16) differed at three positions. These three differences were in three *Tru*9I sites (TTAA). The predicted restriction pattern of *Tru*9I is very complicated with 12 bands for 524 and 13 for 545. RFLPs were determined of 16 strains of ICG 17 and 2 of ICG 16. Most of the bands could not be scored, because of the low resolution of the gel electophoresis and because of partial digestion. However, the largest bands could be scored easily, strain 524 having one of 182 base pairs and 545 one of 160. All 16 strains of ICG 17 had the 524 type and all 2 of ICG

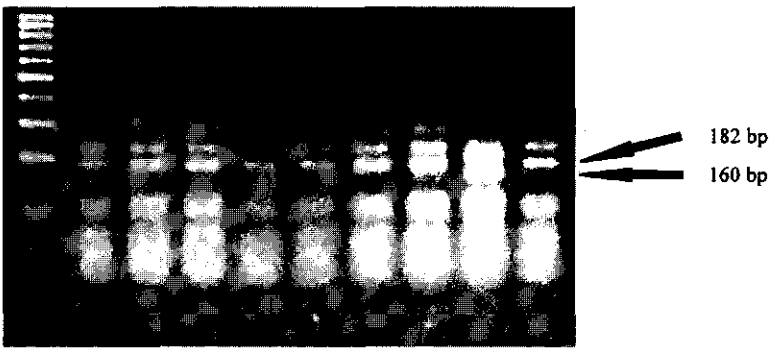

Fig. 5-3. RFLPs of MS1-2 PCR-products digested with *Tru*9I of different strains belonging to ICG 16 (lane 9) and strains belonging to ICG 17 (other lanes). Lane 1: 100 bp ladder. ICG 16 and 17 have different RFLP patterns.

16 the 545 type. We therefore conclude that a unique mitochondrial type is present within each of the two different ICGs (Fig. 5-2).

Discussion

Inheritance and nature of polymorphism

Several phenomena could explain the heterogeneity of the ITS found in ICG 17: i) allelic differences between multiple rDNA loci; ii) within-allele heterogeneity in ITS type, or finally iii) different alleles at homologous rDNA loci that reside in the different nuclei. RFLP analysis of 10 monokaryotic progeny of dikaryon d504, clearly showed that this last possibility is the explanation, *i.e.* the different ITS types reside on homologous chromosomes in the different nuclei of the dikaryon. In all ten cases analysed, a monokaryon received only one type of ITS. Furthermore, in the population analysis, monokaryons always had a single ITS type, whereas three dikaryons were found with mixed types.

Crossing over could result in monokaryons having mixed arrays of rDNA. No examples have been found of polymorphic ITS in monokaryons, suggesting that the amount of recombination is very low. Crossing over between ITS1 and 2 could result in nuclei

having ITS 1 of type Ia or Ib and ITS 2 of the other type. In all cases analysed, monokaryons having ITS 1 of type Ia or Ib had ITS 2 of the corresponding type, so no recombination between ITS 1 and 2 has been found. Taken together, these results show that recombination between these divergent types is rare.

Meiotic recombination within rDNA is thought to be suppressed in many eukaryotes including higher fungi (Petes and Pukkila, 1995). Iraçabal and Labarère (1994) found no recombination between different ribosomal types in the basidiomycete *Pleurotus cornucopiae* (Pers.) Rolland. However, recombination between divergent intergenic spacer (IGS) types in the ribosomal DNA has been shown in the basidiomycete *Laccaria bicolor* (Maire) P.D. Orton (Selosse *et al.*, 1996).

Even though recombination is rare, concerted evolution is expected to homogenise the rDNA repeats in the long run. This suggests that the two divergent types have come together relatively recently in evolutionary history. However, both in America as well as in widely separated localities in Europe the polymorphism has been found.

Origin of different types in ICG 17

In ICG 16, only one ITS type was present, type Ib. In the more common ICG 17, two types were found, Ia (65%) and Ib (35%). We have not found any indication of different host tree preference between strains with different ITS types. No geographical clustering could be detected either within Europe, both types occurred throughout Europe (Sweden, the Netherlands, southern Germany and France). The ITS regions of two American dikaryotic strains of *H. velutipes* were also sequenced. One of these also possessed a mixed type. This shows that the polymorphism is not limited to Europe. Since sexual intercompatibility was not tested between the American and the European strains, we do not know to which ICG the American strains belong.

Since ICG 16 which has type Ib is partially intercompatible with 17, it is tempting to conclude that the polymorphism in ICG 17 resulted from an actual hybridisation between ICG 16 and 17. Several lines of evidence point against this scenario. Although partial intercompatibility has been found between 16 and 17, resulting dikaryons had reduced performance, as was evident from aberrant clamp connections, aberrant hyphae, no nuclear migration and reduced growth. Moreover, individual isolates could always unambiguously

be assigned to a single ICG. The isolates of ICG 17 that were compatible with some strains of 16 (9540, 9556 and 9647) did not have the ITS type of ICG 16 (Ib). The strains of ICG 17 that had the ITS type Ib did show no compatibility with ICG 16. If hybridisation between ICGs 16 and 17 would occur we would expect to have isolates that can not be unambiguously assigned an ICG because of high levels of intercompatibility with both ICGs 16 and 17. Furthermore, both ICGs have unique mitochondria. Finally, ICG 16 has only one type of ITS (Ib) whereas we would expect both ITS types in the case of hybridisation between 16 and 17.

The polymorphism within ICG 17 must therefore be of a different origin, but we can not discriminate between hybridisation between divergent populations or an ancestral polymorphism retained in ICG 17. However, the lack of recombinants between the two types suggests that the two types have come together relatively recently.

We found both ITS types in one dikaryon in America as well as in Europe. This shows that this polymorphism is widespread. We do not know the cause of the divergence between the different ITS types. One of the possibilities is allopatric divergence. If allopatric divergence between type Ia and Ib has occurred between America and Europe, bilateral migration must be assumed to explain the current distribution of both types over both continents. A possibility for migration from North-America to Europe could be the migration as a symbiont of *Pseudotsuga menziesii*, which has been planted in Europe.

Interestingly, within a different clade of the *H. crustuliniforme* complex (Aanen *et al.*, 1999) many different ICGs have been found that are very closely related. However, the level of ITS divergence between these ICGs was substantially lower than the ITS divergence within ICG 17 of *H. velutipes*. This shows that there is no linear relationship between the level of ITS divergence and the level of incompatibility. However, within clade IIa of the *H. crustuliniforme* complex there was a fairly good correspondence between the level of incompatibility and the relative age of the last common ancestor. These results indicate that generalisations about the relationship between the level of incompatibility and genetic divergence have no firm basis.

A warning

These findings clearly show that caution must be taken when inferring organismal phylogenies from single sequences, especially from multicopy sequences such as ribosomal DNA (see also Buckler and Holtsford, 1996; O'Donnell and Cigelnik, 1997). Polymorphisms within the ITS region in the same individual have been reported in plants (reviewed in Baldwin *et al.*, 1995), beetles (Vogler and DeSalle, 1994) and nematodes (Zijlstra *et al.*, 1995). In fungi O'Donnell and Cigelnik (1997) have found a polymorphism in the ITS2 within single individuals. They showed that the incongruence between an organismal phylogeny of *Fusarium* species based on several gene sequences and one based on ITS2 sequences was due to nonorthologous ITS2 sequences. Within each species of *Fusarium* they studied, two ITS2 types were present, one in high copy number, and one in low. Direct sequencing gave only the sequence of the dominant type. Since copy numbers had shifted several times in the course of evolution, non-orthologous sequences could be obtained when doing direct sequencing.

This situation is different from the situation reported here, where different ITS types are present within single dikaryons. These ITS types represent different alleles at the same rDNA locus since they segregate in the monokaryons. Polymorphisms within biological species and even within single dikaryons, may be less exceptional than is generally assumed. Jonsson (1998) mentions heterogeneity in amplified ITS products from dikaryons as one of the explanations for the fact that the sum of lengths of the RFLP bands exceeds that of the PCR product. We know of several cases in which direct sequencing of ITS PCR products was not successful (D.K. Aanen, unpublished observations). This could be explained by a mixture of different ITS copies with minor length mutations in the PCR mixture.

Recently, within arbuscular mycorrhizal fungi, the Glomales, striking divergence in the ribosomal sequences of individuals has been found (Sanders, 1999; Hijri *et al.* 1999; Hosny *et al.*, 1999). Polymorphisms in the ribosomal DNA occurred inside individual spores. The Glomales are coenocytic and Hijri *et al.* (1999) found that the different rDNA sequences occurred in different nuclei. This is in agreement with our findings in ICG 17, where the different ITS types always resided in different nuclei. However, the Glomales are asexual, and the lack of recombination can -at least partially- explain the maintenance of

polymorphisms. *Hebeloma* species, on the other hand, are sexually outcrossing, and recombination would be expected to homogenise the ribosomal sequences.

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6

A comparison of the application of a biological and morphological species concept in the *Hebeloma crustuliniforme* complex within a phylogenetic framework.

Duur K. Aanen and Thomas W. Kuyper

Manuscript

Abstract

A method is presented to derive an operational species concept for the *Hebeloma crustuliniforme* complex that is based on (combinations of) biological species within an explicitly phylogenetic framework. Crucial in this analysis is a reliable estimate of the phylogeny of biological species in the *H. crustuliniforme* complex. Based on two nuclear sequences, we present a best estimate of the phylogeny of biological species within the *H. crustuliniforme* complex. Using this phylogeny, on the basis of (strict) monophyly only two species can be recognised among 22 biological species, viz. *H. velutipes* and *H. helodes*. An earlier phylogenetic analysis indicated that these two morphological species are not sister taxa. Relaxing the criterion of monophyly and allowing paraphyletic groupings of biological species as a morphospecies results in the recognition of three morphospecies, viz. *H. velutipes*, *H. incarnatulum* and *H. helodes*. A tree, with the five ICGs of the previously defined morphospecies *Hebeloma crustuliniforme* (1, 2, 3, 4 and 5) constrained as a monophyletic group cannot be rejected. This constrained tree, together with the relaxed criterion, allows the recognition of four species, viz. *H. helodes*, *H. crustuliniforme*, *H. velutipes* and *H. incarnatulum*. The limited ability to translate a biological species concept into an operational species concept is explained by the lack of qualitative characters and the plasticity of quantitative characters. Intercompatibility tests and an ITS based phylogeny indicated that most biological species are very closely related and hence provide support for the claim that a good correspondence between a biological species concept and a

morphological species concept in the *Hebeloma crustuliniforme* complex is not likely to be forthcoming.

Introduction

In this chapter we consider morphological variation, the criterion traditionally used to delimit species within this complex. In order to arrive at an operational taxonomy, we compare the biological species concept with morphological variation. This comparison is within the phylogenetic framework of chapters 3 and 4.

As a first step to arrive at an operational taxonomy, we considered the morphology of the biological species and tested if different biological species could be recognised by morphological criteria. If this was the case, such species were recognised. However, if they could not be recognised, the next important criterion that we used, was monophyly of morphologically recognisable entities. We tested if monophyletic groups of biological species could be delimited by morphological characters. These two criteria can conflict. For example, if one non-basal ICG can be separated from a monophyletic group of ICGs, the rest of this group automatically turns into a paraphyletic group.

The strict criterion of monophyly of species has been challenged (*e.g.* Sosef, 1997; Brummitt, 1997). We recognise that biological species can form paraphyletic groups (see Chapter 4, 5). De Queiroz and Donoghue (1988, 1990) have stressed that interbreeding units need not necessarily be monophyletic. As speciation can involve the splitting off of marginal and/or local populations (Levin, 1993), a consequence can be that after such a speciation event the parent species has become paraphyletic. This scenario could be an explanation for the phylogenetic relationships between ICGs 18 and 17 (Chapter 5). If one recognises the possibility of paraphyletic taxa at the biological-species level, one may wonder then why combinations of biological species could not form paraphyletic entities. We therefore also considered the consequences of relaxing the criterion of strict monophyly to recognising paraphyletic species as well. Alternatively, we could accept such paraphyletic taxa on infraspecific level. However, we considered polyphyletic entities unacceptable as morphospecies.

A crucial step in this approach is to have a reliable estimate of the ICG phylogeny. For the *H. crustuliniforme* complex as a whole, we have estimated phylogenetic

relationships based on ITS sequences (Chapter 3). In this ITS phylogeny the *H. crustuliniforme* complex consisted of two clades (clade I and II) that were not sister groups. For clade I, we only had the ITS sequence data to estimate the phylogeny. Within clade II, the organismal phylogeny of one subclade (IIa), consisting of nine ICGs belonging to different morphospecies such as *H. crustuliniforme*, *H. leucosarx*, and *H. pusillum*, was studied in more detail using other sequences (Chapter 4).

Different data sets that have the same evolutionary history, are expected to converge onto the true species phylogeny of the group under study, if analyzed using appropriate phylogenetic methods (Mes, 1995). In principle, such data sets can be combined. Kluge (1989) proposed that phylogenetic analysis should always be performed using all the available evidence (the 'total evidence' approach). In this approach, all of the independent characters available to the systematist should be combined and then analyzed using parsimony. However, others have argued against this approach (*e.g.* Lutzoni and Vilgalys, 1995). Miyamoto and Fitch (1995) argued that phylogenetic trees should be estimated separately from each data set and the different estimates should be compared using taxonomic congruence. Under this separate analysis approach, each partition represents an independent estimate of the tree, and these different estimates can be judged for congruence. It is often argued that congruence among different data partitions provides some of the strongest evidence that a particular phylogenetic estimate is accurate (Hillis et al., 1996). A compromise between the 'total evidence' approach and the 'separate analysis' approach is the 'conditional combination' approach (Huelsenbeck *et al.*, 1996) as advocated by Bull *et al.* (1993) and de Queiroz (1993). Under this approach, data sets are statistically tested for homogeneity. Heterogeneous data sets are those that result in significantly different estimates of phylogeny when analyzed separately and these data sets cannot be combined. If the test result is non-significant, *i.e.* the data sets do not result in significantly different estimates of the phylogeny, then these data sets should be combined (Huelsenbeck *et al.*, 1996). As an alternative to combining the data sets, the resulting trees can be combined (Mes, 1995, Sanderson *et al.*, 1998). A 'supertree' is an estimate of a phylogeny assembled from sets of smaller estimates (source trees) sharing at least some taxa (Sanderson *et al.*, 1998).

In order to get a best estimate of the phylogenetic relationships between the ICGs found sofar, we used the different estimates of phylogenetic relationships in the *Hebeloma*

crustuliniforme complex. We considered different possibilities to combine the data sets. Furthermore, we tested constraints with two traditionally recognised morphospecies, *H. crustuliniforme* and *H. pusillum*, as monophyletic groups.

To the morphological characters studied belong those traditionally used in the *Hebeloma* taxonomy (Bruchet, 1970, Boekhout, 1982, Vesterholt, 1995). Since many of the characters used are quantitative, we have not tried to reconstruct phylogenies based on these characters. Instead, we i) reconstructed organismal phylogenies based on molecular data and ii) tried to define morphologically recognisable monophyletic entities.

Using the best estimate of the phylogenetic relationships of ICGs within the *H. crustuliniforme* complex, we addressed the following questions:

i) how many morphological taxa, consisting of (strictly) monophyletic groups of ICGs can be recognised in this complex?

ii) how would relaxing the criterion of monophyly and allowing paraphyletic groupings of ICGs affect the number of morphospecies that can be recognised?

iii) how would trees which are constrained to produce monophyletic groupings of ICGs in previously recognised morphospecies, but which can not statistically be rejected against the most parsimonious trees, affect the number of morphospecies that can be recognised?

iv) what is the phylogenetic quality of previously recognised morphospecies such as *H. alpinum* or *H. pusillum*?

Materials and Methods

Material

Sexual intercompatibility was tested for 110 collections (Chapter 2). This analysis led to the recognition of at least 20 InterCompatibility Groups (ICGs). Two collections (9692 and 9694) were not compatible with any of the other collections. However, since these collections have neither shown compatibility in intracollection pairings nor in intercollection pairings, the possibility that these two collections were 'incompetent' could not be excluded (R. Petersen, pers. comm.). Therefore, it was not warranted to give a formal status as ICG to these two collections. However, assuming that the two collections are competent, they

represent two other ICGs. In this chapter we consider them as representants of two further ICGs: 13 (9692) and 22 (9694).

For the isolates that were used in the different phylogenetic studies we refer to chapters 3, 4 and 5. In this study also the IGS sequences of 9654 and 9509 were determined.

The macroscopical characters were determined for all 110 collections, the microscopical for 78 collections.

Morphological descriptions of ICGs and morphological characters used

Each ICG was described morphologically and the range of character states was described for each ICG. The morphological characters used are listed in Table 6-I. Many of these characters are quantitative.

Full descriptions of the ICGs are given in the Appendix.

Phylogenetic analysis and combining data sets

For clade I, we had only the ITS data to reconstruct an organismal phylogeny.

For clade IIa, we had different data sets. The first estimate of the phylogeny was based on ITS sequences (Chapter 3). In chapter 4, clade IIa, except for the two ICGs of *H. pusillum*, was studied in detail using different sequences: the nuclear IGS and a mitochondrial intron (Chapter 4). Here we include IGS sequences of the two ICGs of the morphospecies *H. pusillum*, ICG 7 and 9. We performed a new parsimony analysis with the inclusion of those two additional taxa. For the details of the parsimony analysis we refer to chapter 4. Gaps were coded according to Hibbett (1995) for all data sets. The reason that we used gap coding for the ITS data here and not in chapter 3 is that the analysis here is limited to a group of closely related taxa, the alignment of which was straightforward, whereas the alignment with the extended data set of chapter 3 was more ambiguous.

Sixteen taxa were common for the two nuclear data sets, the ITS and IGS sequences (ICG 1: 9503, 9618, 9621, 9673; ICG 2: 9570, 9627; ICG 3: 9680; ICG 4: 9602; ICG 5: 9581; ICG 7: 9654; ICG 8: 9538; ICG 9: 9509; ICG 14: 9566; ICG 15: 9624; ICG 20: 9688; ICG 21: 9650). Eleven taxa were common for all data sets (ICG 1: 9503, 9618,

9621, 9673; ICG 2: 9570, 9627; ICG 3: 9680; ICG 4: 9602; ICG 14: 9566; ICG 20: 9688; ICG 8: 9538). The Partition Homogeneity Test (Farris *et al.*, 1995, Huelsenbeck *et al.*,

Table 6-I. Morphological characters used to describe ICGs.

Macroscopical:	pileus:	diameter
		colour
		shape
		presence of hygrophanous spots
	lamellae:	number
		shape
		weeping
	stipe:	length
		width
		presence of bulb
		presence of pendent marrow strand
		covering
	general habit	
	smell	
Microscopical:	spores:	length
		width
		Q (ratio l/w)
		dextrinoidy (scale D0-D4, see Vesterholt, 1995)
		shape
		perispore loosening (scale P0-P3, see Vesterholt, 1995)
		ornamentation (scale O0-O4, see Vesterholt, 1995)
	cheilocystidia:	length
		width at median part
		width at apex
		Q (width apex/width median part)
		shape
		wall thickness
		presence of apical bifurcations
Host tree genera		

1996; implemented in PAUP*) was used (with 1000 replicates) to determine whether the different data sets were in conflict. In this test, the observed sites from all genes for each individual are pooled and resampled without replacement to give an artificial data set in which sites have been swapped randomly among loci. Many such artificial data sets are produced. MP trees are then made for each newly sampled partition in each artificial data set. If the data sets have the same evolutionary history, the sums of the lengths of the gene trees for the observed and resampled data should be similar, but if they have different evolutionary histories, the sums of the tree lengths should be longer than that for the actual data, because of extra homoplasy in the data (Geiser *et al.*, 1998). We discuss the different possibilities to estimate the phylogeny of clade II.

Furthermore, some alternative topologies were tested. Two of the species traditionally considered to be 'good' morphospecies are *H. crustuliniforme* sensu stricto and *H. pusillum*. We first did a parsimony analysis with the constraint that the biological species of which the morphospecies *H. crustuliniforme* consisted (ICGs 1, 2, 3, 4 and 5) formed a monophyletic group. Second, a constrained analysis was performed with the morphospecies *H. pusillum* as a monophyletic group (ICGs 6, 7, 8 and 12). The constrained trees found were compared with the unconstrained trees using the Kishino-Hasegawa test and Templeton's (1983) nonparametric test as implemented in PAUP*.

Strategy to arrive at an operational taxonomy

The first species concept that was tested as an operational species concept was the biological species concept. We considered the morphology of biological species and tested if different biological species could be recognised by morphological criteria. We recognised the possibility that biological species could represent paraphyletic taxa. For 8 ICGs we have included more than one strain in the phylogenetic analysis: ICGs 1, 2, 3, 4, 9, 11, 17 and 21. For those ICGs we tested the hypothesis that strains of a single ICG form a monophyletic group.

The second species concept that was tested as an operational species concept was based on combinations of biological species within a phylogenetic framework. On the basis of an estimate of the phylogenetic relationships within *Hebeloma*, we tested for every sister group whether both sister taxa could be morphologically separated. The morphological

descriptions of the ICGs were used to do this. As an initial help, we used a set of 13 morphological characters, divided into discrete classes (Table 6-II). Sister taxa were separated if they showed no overlap in at least one of these characters. If both of them could indeed be unambiguously demarcated, they were (at least provisionally) accepted as valid morphospecies. The process was then repeated at the next higher level till all sister group relations had been dealt with. If sister taxa could not be recognised separately as morphospecies, both sister taxa were lumped and the morphological variability for the composite species was assessed. Again the process was repeated till all sister group relations have been dealt with. We introduced an additional criterion for recognition, viz. that morphological relationships between such provisional morphotaxa could be upheld across hierarchical levels. Essentially, in this approach the two sister groups A and B, even when sufficiently different to be kept apart by standard taxonomic practice, were lumped when clade C, the sister group of AB, could not be treated as separate from either group A or B. In cases where the consensus cladogram did not yield sister group relationships but showed unresolved polytomies, each taxon in a polytomy was compared with every other taxon. Inevitably, this could result in a complex pattern of relationships within the polytomy where some taxa could be unambiguously separated from each other whereas some other ones could not. Again, the criterion of consistency across levels was used.

A more relaxed version of this procedure was tested as well. In this version, paraphyletic taxa were recognised, viz. when the sister groups A and B could be separated, but clade C could only be separated from A, but not from B, we recognised the monophyletic A, and the paraphyletic (B,C).

Results

Phylogeny of ICGs

For the 16 taxa for which both ITS and IGS sequences were determined, we determined whether these data sets were in conflict using the partition homogeneity test. The actual summed tree length of 171 was equal to or longer than 65.6 % of the artificial

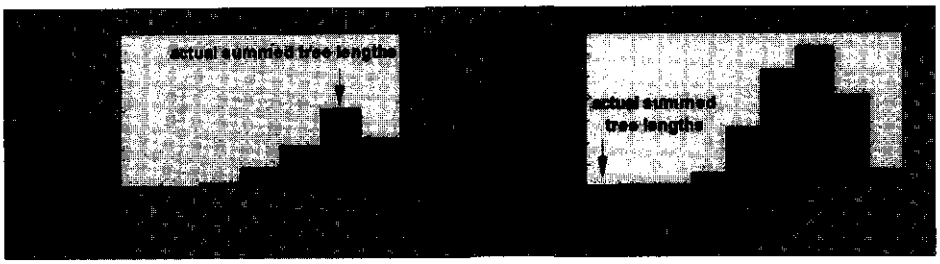

Fig. 6-1. Partition homogeneity test results.

data sets, indicating that the gene trees do not have significantly different topologies (Fig. 6-1a). Therefore, we combined the ITS and IGS data sets to reconstruct a nuclear phylogeny. The sister taxon of clade II, *H. sarcophyllum* was used as the outgroup. Doing a parsimony analysis with gaps coded according to Hibbett (1995) 4 trees were found of length 232 (c.i.=0.85; excluding uninformative characters, 1=113, c.i.=0.69), the strict consensus of which is depicted in Fig. 6-2.

For the 11 taxa for which all three sequences were determined, we also did the partition homogeneity test. The actual summed tree length of 134 was smaller than 99.9 % of the artificial data sets, indicating that the gene trees do have significantly different topologies (Fig. 6-1b). Therefore, we conclude that the mitochondrial and nuclear phylogenies cannot be combined.

In Chapter 3 we showed that the incongruence between the nuclear and mitochondrial tree was mainly due to ICG 1, which had a different position in both phylogenies. As a possible cause we proposed a hybridisation with different mitochondrial and nuclear contributions. Here we use the nuclear phylogeny, but we consider the consequences of other positions of ICG 1.

Monophyletic recognisable entities

Recognisability and monophyly of ICGs—In an appendix morphological descriptions are given of 20 ICGs and two putative ICGs (ICGs 13 and 22). *H. incarnatulum* is the single ICG that can be separated from all the other taxa of the *H. crustuliniforme* complex by the shape of its cylindrical to very narrowly clavate cheilocystidia. All other species of this complex have clavate to (sub)capitate cheilocystidia. Of the ICGs represented by more than

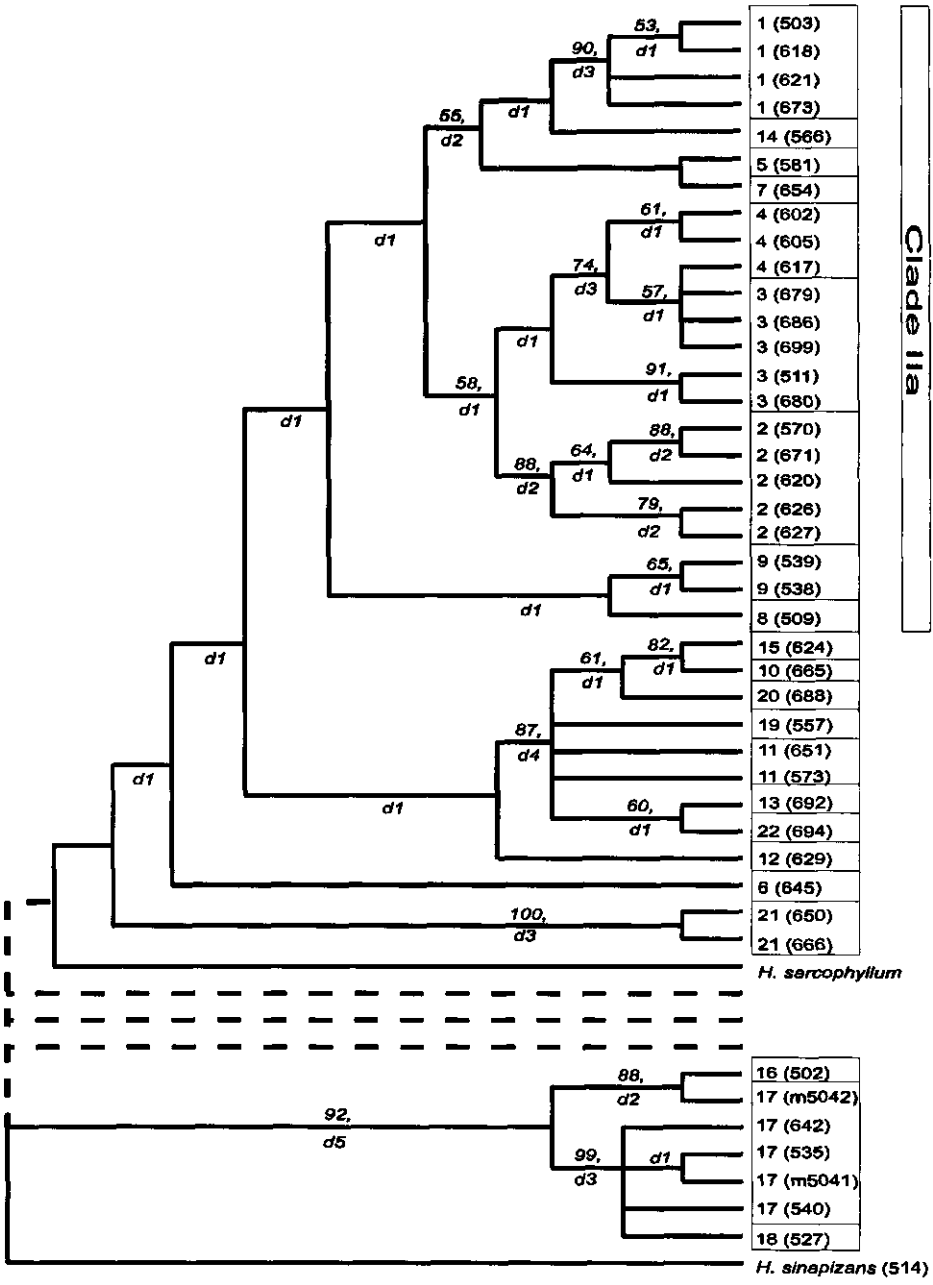

Fig. 6-2. Phylogenetic relationships in the *Hebeloma crustuliniforme* complex based on nuclear ribosomal ITS sequences. For clade IIa and strains 624, 688 and 650 IGS sequences were determined as well and these sequences were also used in this phylogenetic anallysis. The two clades I and II were analysed separately, but are placed in the same Figure. Indicated are bootstrap values and decay indices (preceded by d).

one collection, ICGs 1, 2, 9 and 21 were monophyletic, and the two strains of ICG 11 had identical ITS sequences but did not form a monophyletic group. The partially compatible ICGs 3 and 4 did not form monophyletic groups, together they constituted a monophyletic group, however. Strains of ICG 17 did not form a monophyletic group. Two ITS types were found within this ICG that belonged to two different clades (see Chapter 5).

Monophyletic recognisable entities—Since most of the ICGs could not be uniquely characterised, we tested if we could recognise monophyletic combinations of ICGs. For every sister group we tested whether both sister taxa could be morphologically separated. The consecutive steps for combining ICGs into monophyletic units are illustrated in Fig. 6-3 and described in Table 6-III. Some of the characters of the morphological descriptions of the ICGs and of combinations of sister groups are presented in Table 6-II.

To illustrate the procedure, we discuss some examples. At the lowest taxonomic level some sister groups could not be separated and were combined and others could be separated and were, at least provisionally, maintained (Fig. 6-3 and Table 6-III). ICG 1 and 14 could be separated on the basis of general habit and pileus colour and were therefore maintained at this point. The same was the case for ICG 5 and 7 (stipe-pileus ratio and pileus colour). However, in subsequent steps, those taxa could not be maintained any longer, because ICG 1 could not be separated from 5. ICGs 10 and 15 could be separated on the basis of general habit (stipe-pileus ratio). These taxa were therefore maintained at this point. However, the sister group of the pair [10,15], ICG 20 could not be separated from 10, although it could from 15. Therefore, these three taxa were lumped to g.

This analysis ultimately led to the recognition of 2 morphologically recognisable monophyletic groups, one consisting of three ICGs (clade I), and one consisting of 19 ICGs (clade II).

If paraphyletic species would be recognised, ICG 18 could be recognised as an additional monophyletic morphospecies, with the two ICGs of *H. velutipes* as a paraphyletic morphospecies. The acceptance of paraphyletic taxa does not have any influence on the final number of species recognised in clade II. Only the moment of combining ICGs is postponed in some cases if we accept paraphyletic entities.

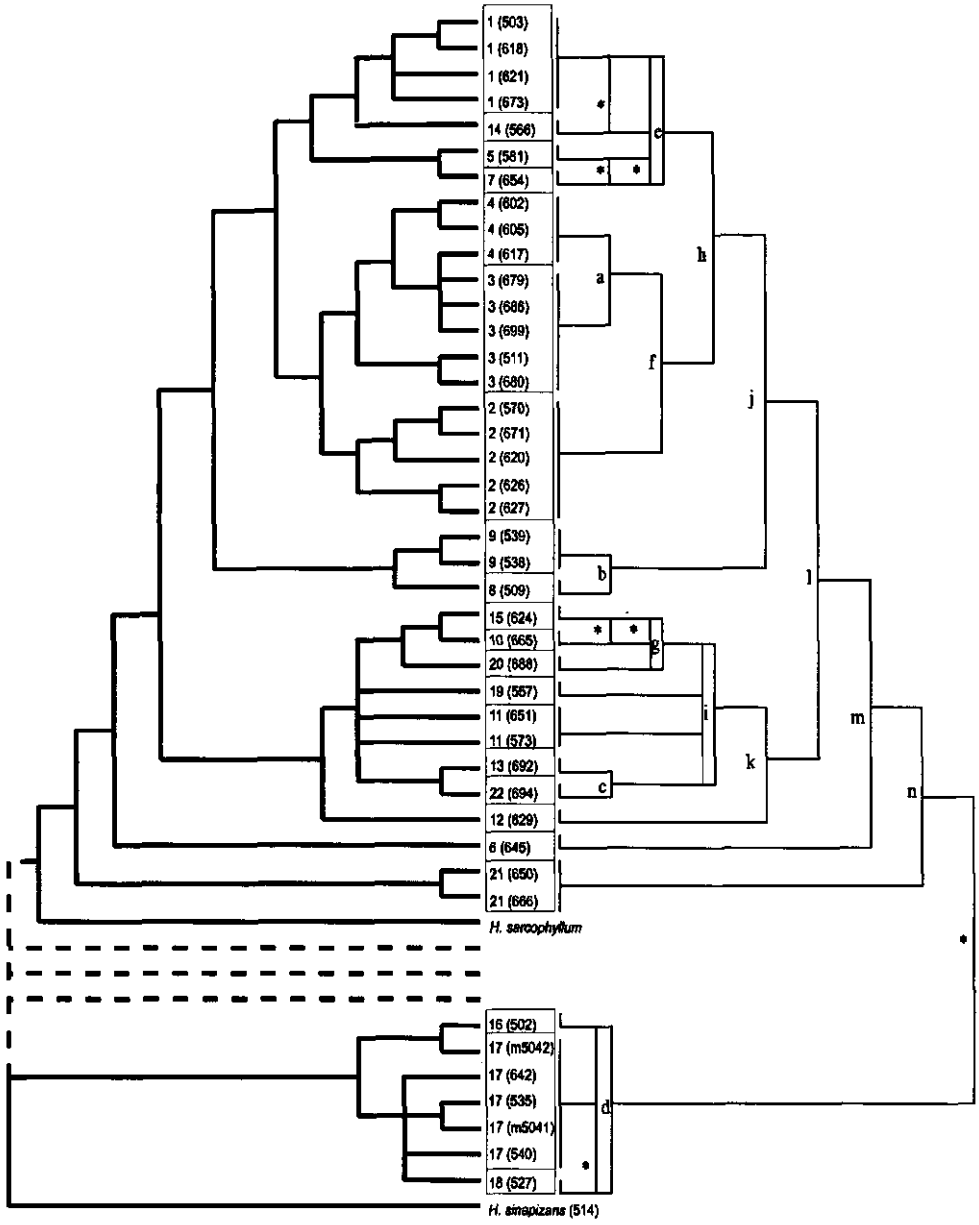

Fig. 6-3. Consecutive steps for combining ICGs into monophyletic units. If two sister groups can not be separated, this is indicated with *, if two sister groups can be separated, they are combined and a letter is given to the provisional taxon. These steps are repeated at the next level. This analysis ultimately leads to the seapration of 2 morphologically recognisable monophyletic groups, one consisting of three ICGs (clade I), and one consisting of 19 ICGs (clade II).

Table 6-III. Consecutive steps for combining ICGs into monophyletic units.

1st level

-compare 1, 14 – can be separated (general habit, stipe-pileus ratio, pileus colour) – maintain 1 and 14.

-compare 5, 7 – can be separated (general habit, stipe-pileus ratio) – maintain 5 and 7.

-compare 3, 4 – cannot be separated – combine to a.

-compare 8, 9 – cannot be separated – combine to b.

-compare 10, 15 – can be separated (general habit, stipe-pileus ratio) – maintain 10 and 15.

-compare 13, 22 – cannot be separated – combine to c.

-compare 16, 17, 18 in all combinations – 16 and 17 cannot be separated – combine to d.*

*ICG 18 can be separated from all ICGs because of the shape of its cheilocystidia. However, under the constraint of strict monophyly of recognisable groups, ICG 18 cannot be maintained, since 16 and 17 cannot be separated. Moreover, strains of ICG 17 itself form a paraphyletic group.

2nd level

-compare 1, 14, 5 and 7 in all combinations – 1 and 5 cannot be separated – combine to e.

-compare a, 2 – cannot be separated – combine to f.

-compare 10, 20 and 15, 20 – 10, 20 cannot be separated – combine 10, 15 and 20 to g.

3rd level

-compare e and f – cannot be separated – combine to h.

-compare g, 19, 11 and c in all combinations – none of them can be separated – combine to i

4th level

-compare h, b – cannot be separated – combine to j.

-compare i, 12 – cannot be separated – combine to k.

5th level

-compare j, k – cannot be separated – combine to l.

6th level

-compare l, 6 – cannot be separated – combine to m.

7th level

-compare m, 21 – cannot be separated – combine to n.

8th level

-compare d, n – can be separated – maintain d and n.

individual characters that comprise a set, however, do not necessarily uniquely characterise morphospecies. Relatively broad spores, or slender, non-capitate cheilocystidia occur in clade II, but the combination of these characters has never been encountered in this clade. The last type of characters we recognise are statistical characters. For example, 74 % of the collections of clade II have been found with *Salicaceae* whereas only 7 % of the collections of clade I. Such characters can never be decisive themselves but can give additional support for doubtful collections.

It is obvious that the *H. crustuliniforme* complex consists of two divergent clades (Chapter 3) both of which consist of closely related ICGs. This close relationship of the ICGs within those two clades appears from molecular data (Chapter 4, 5) as well as from the uniformity in microscopical characters for the ICGs. Moreover, several cases of partial compatibility have been found between ICGs both within clades I as within clade II (Chapter 2). Some macroscopic characters on the other hand, such as general habit, pileus colour, slenderness and size are rather variable. This variability is evident at three levels: i) between ICGs. Some characters such as pileus colour, size and habit have evolved independently several times, according to the best estimate of the phylogeny; ii) within ICGs, although variation within ICGs is generally lower than between ICGs. Within ICG 9, we have found collections resembling *H. helodes* and a collection resembling *H. pusillum*; iii) within single collections. The high plasticity and variability in macroscopical characters might ultimately be the explanation for our failure to recognise more than four morphospecies with a minimal phylogenetic quality in this species-rich complex.

Provisional key to four recognised taxa

1a. Spores distinctly, often rather strongly dextrinoid, ellipsoid to oblong ($Q_{av} \leq 1.7$); cheilocystidia cylindrical to cylindrico-clavate, but sometimes with bifid apex; stipe usually distinctly bulbous, flocculose; often associated with *Pinaceae*, *Betulaceae*, *Carpinaceae* and *Fagaceae* → 2 [clade I; *H. velutipes*];

2a. Cheilocystidia cylindrico-clavate, in upper part on average more than 6.0 µm broad, and Q_{av} = 1.2-2.0 → *H. velutipes*

2b Cheilocystidia cylindrical, in upper part on average less than 6.0 µm broad, and Q_{av} = 1.1 → *H. incarnatulum* [making *H. velutipes* paraphyletic]

1b. Spores not to weakly dextrinoid, oblong to fusiform (Qav ≥ 1.7); cheilocystidia clavate to subcapitate, never with bifid apex; stipe usually cylindrical to clavate to subbulbous, often (coarsely) floccose; often associated with *Salicaceae* → 3 [clade II; *H. helodes*]

3a. Stipe usually less than 10 mm broad, (sub)floccose; pileus with straight margin when young, yellowish to red-brown, often distinctly paler towards margin and then ± bicolorous; cheilocystidia usually (sub)capitate → *H. helodes*

3b. Stipe usually more than 10 mm broad, coarsely floccose; pileus with involute margin when young, whitish to yellowish, ± unicolorous, cheilocystidia clavate to slightly subcapitate → *H. crustuliniforme* [if constrained to monophyly, making *H. helodes* paraphyletic]

Described morphological species

This chapter does not (and could not) aim at a full taxonomic revision of all taxa in this complex. It could not do so, because the biological species concept cannot be applied to type collections (unless there exist ex-type cultures). Therefore our nomenclator will be inevitably incomplete. We have only listed the major names that have been used recently and comment upon the biological (what is the relationship between biological species and morphological taxa?), phylogenetic (are morphospecies that are commonly mentioned, mono-, para- of polyphyletic?) and morphological (how well are different morphospecies in common use separated from each other?) quality of species names in common use.

To the morphospecies often mentioned in the literature about the *H. crustuliniforme* complex belong *H. crustuliniforme*, *H. alpinum*, *H. cavipes*, *H. fragilipes*, *H. helodes*, *H.*

leucosarx, *H. lutense*, *H. pusillum*, *H. longicaudum*, *H. incarnatulum*. These names are shortly discussed below.

H. crustuliniforme. *H. crustuliniforme* as presently conceived by Vesterholt (1995) can be maintained, if the criteria of monophyly and parsimony are relaxed to include paraphyletic groupings of ICGs and less parsimonious trees that cannot statistically be rejected against the most parsimonious trees.

H. alpinum. *H. alpinum* was originally described by Favre (1955) as a variety of *H. crustuliniforme*. Favre invoked a number of morphological characters to delimit this taxon but wondered whether these characters might just reflect adaptations to the microclimatic conditions in the alpine zone. However, as Favre did not observe specimens that were transitional between the alpine variant and the typical *H. crustuliniforme* (actually, he did not observe typical *H. crustuliniforme* at all in the upper subalpine zone), he considered these differences to be genetically fixed and hence worthy of recognition on varietal status. Bruchet (1970) elevated the taxon to species rank, placed it in another stirps than *H. crustuliniforme*, and gave an enlarged description of it, whereas Vesterholt (1995), who also accepted species status, used a partly different set of characters than Favre to keep this taxon apart. Our alpine collections from this group showed substantial variation in size. They did belong to three different ICGs, two of which contained both alpine and lowland collections (ICGs 1 and 2), and one of which (ICG 4) was partially compatible with a lowland ICG (ICG 3, Chapter 2). Partial intercompatibility between these four ICGs (Chapter 2 and 4) furthermore strongly suggest that *H. alpinum* cannot any longer be maintained. Moreover, in a phylogeny between populations of these ICGs (Chapter 4), the alpine populations did not form a distinct monophyletic group.

H. cavipes. *H. cavipes* was accepted by Vesterholt (1995) as a valid species, only known from the type collection. Vesterholt noted that in its microscopical characters it was very similar to both *H. crustuliniforme* and *H. helodes*, and these three taxa could only be kept apart on the basis of size and stipe covering. It is very likely, therefore, that finding additional collections of *H. cavipes* would either collapse the distinction with *H. crustuliniforme* (if somewhat larger specimens were found) or with *H. helodes* (if smaller specimens were found). Vesterholt further suggested that *H. cavipes* might be conspecific with *H. lutense* (q.v.), a species that is usually regarded as having a darker pileus.

H. fragilipes. *H. fragilipes* Romagn. was defined on the basis of the shape and median wall thickening of the cheilocystidia. The microscopical characters mentioned by Vesterholt

(1995) on the basis of a large number of collections from a number of European countries, suggests both elements of the *H. velutipes* clade (spores distinctly dextrinoid, cheilocystidia that are not much swollen apically) and the *H. helodes* clade (spores indextrinoid, oblong to fusiform). No collections have been made by us that exactly fit his description. However, we note that slightly thick-walled cheilocystidia have been observed in both ICG 16 and 17 (*H. velutipes* clade) and in various members of the *H. helodes* clade; such collections were completely interfertile with specimens with thin-walled cystidia, so some doubt exists whether this morphospecies could be maintained. Boekhout (1982) considered *H. fragilipes* to belong to the *H. helodes* clade.

H. helodes. *H. helodes* was described by Favre (1948) as a taxon very close to *H. pusillum* (same habitus, same size, and same colour). Subsequent authors have gradually enlarged this circumscription (*H. helodes* sensu Vesterholt is paler; *H. helodes* sensu Boekhout includes variants that are larger and/or with a more thick-set stipe, to 7.5 mm thick) or misinterpreted the name (*H. helodes* sensu Keizer and Arnolds (1995), which was said to include *H. velutipes*. In our cladogram *H. helodes* could be accepted as the name for a monophyletic clade (clade II) or as a paraphyletic grouping if *H. crustuliniforme* was considered a separate morphospecies. Species circumscription of the species would then still be quite large, which is consistent with the relatively high amount of molecular variation (compared to *H. crustuliniforme*). However, intermediates between *H. helodes* and *H. crustuliniforme* occur (cf *H. cavipes* above). Considering the wide circumscription of *H. helodes*, it becomes inevitable that *H. pusillum* and *H. lutense* have to be included; this conclusion is not surprising, considering the enlarged descriptions of *H. helodes* by Boekhout and Vesterholt.

H. leucosarx. *H. leucosarx* was described by Orton (1960) as a species with relatively slender spores, distinctly capitate cheilocystidia and associated with *Salix*. It has been considered a member of the *H. helodes* clade by Dutch mycologists. Vesterholt (1995), however, noted that the holotype had distinctly dextrinoid spores and non-capitate cheilocystidia, which makes this collection a member of the *H. velutipes* clade. Vesterholt also suggested that *H. velutipes* might be identical. *H. leucosarx* sensu auct. neerl. is almost certainly identical with *H. lutense*.

H. lutense. In the Netherlands *H. lutense* (= *H. leucosarx* sensu auct. neerl.) is recognised mainly by habit (relatively stout specimens, compared to *H. pusillum*) and habitat (usually associated with *S. repens* often in early successional, relatively open sites). The first character might reflect an adaptation to the relatively open structure (and hence dry microclimate) of *S.*

repens vegetation. The species also differs from *H. helodes* by darker colours. Collections that fit the description of *H. lutense* belong to two different ICGs. Morphological variability within some of these ICGs suggests that *H. lutense* can neither be separated from *H. helodes* nor from *H. pusillum*. Vesterholt (1995) suggested that *H. lutense* could be a synonym of *H. cavipes* (q.v.), another species that is doubtfully distinct from *H. helodes*.

H. pusillum. That name is used for small and slender specimens with a dark pileus, usually associated with willows. The species is somewhat variable in spore size. This species also got a slightly enlarged circumscription, e.g. by Bruchet (where it almost certainly includes *H. helodes*) and Phillips (which also seems to fit better into the concept of *H. helodes*). *H. pusillum* consists of four different ICGs. A tree, constrained to make *H. pusillum* a monophyletic entity, must statistically be rejected against the most parsimonious trees, indicating that the defining characters of *H. pusillum* (slender habit with small basidiocarps) have likely arisen repeatedly. This conclusion would also suggest that similar defining characters (stout habit of *H. lutense*) should also be considered with caution.

H. velutipes. The taxon is well recognised by relatively broad, dextrinoid spores and clavate, non-capitate cheilocystidia. Many collections also show a number of the following characters as well: pileus with hygrophanous spots; stipe distinctly bulbous (*H. bulbiferum* R. Maire); stipe hollow with pendent marrow strand; part of the cheilocystidia with bifid apex. The taxon can be accepted in a wide sense (a monophyletic taxon, similar to clade I) or in a more narrow sense (a paraphyletic grouping of ICG 16 and 17 -see Chapter 5- if *H. incarnatulum* is accepted as a valid morphospecies). *H. leucosarx* sensu Vesterholt and *H. longicaudum* sensu Keizer & Arnolds are identical (ITS-RFLP patterns of both taxa studied, D.K. Aanen, unpublished observations).

H. longicaudum. The taxon has originally been characterised by a pale pileus and a long stipe. However, the case of *H. pusillum* serves as a warning with regard to the taxonomic value of habit characters. Descriptions of several pale-coloured ICGs also indicate that habit can be very variable. *H. longicaudum* sensu Keizer and Arnolds is *H. velutipes*, *H. longicaudum* sensu Bruchet might probably also be best referred to *H. velutipes*. *H. longicaudum* sensu J. Lange seems to include both elements of *H. velutipes* and of *H. helodes*. Boekhout (1982) considered *H. longicaudum* to be the same taxon as *H. velutipes*, because he observed all intermediate forms between specimens with long stipes and short stipes.

H. incarnatulum. This morphospecies is characterised by very narrowly clavate to cylindrical cheilocystidia, and very small, rather broad, dextrinoid spores. The habitat appears to be restricted to *Picea* and *Pinus* among living *Sphagnum* moss (Smith, 1984). The single collection that was used in this study was also found under *Pinus* in a *Sphagnum* bog.

A consensus taxonomy of the Hebeloma crustuliniforme complex?

Implicit in the provisional key and in the subsequent discussion of names of previously recognised morphospecies, is the inevitable conclusion that there is no unique solution to the species problem in the *H. crustuliniforme* complex. Even under the assumptions (which not all mycologists would accept!) that i) the phylogeny estimate is sufficiently accurate and ii) the value of morphological characters has been exhaustively studied, the number of species ultimately depends on the rules of the game (acceptability of paraphyletic groupings, acceptability of less parsimonious trees that cannot be rejected in favour of the most parsimonious tree). It would therefore be necessary to seek consensus about these rules, so that the incompatible demands on taxonomy (species should really exist, be recognisable and have minimal phylogenetic quality) can be sorted out and an acceptable solution found for this complex.

Appendix

ICG 1

Pileus to 40-85 mm, convex to plano-convex, without umbo, margin involute, (slightly) viscid when moist, rather pale, in centre pale yellow to pale yellow-brown [10 YR 7-8/3; 2.5 Y 6-7/4], paler towards outer part, at margin whitish or white. Lamellae, L = 55-60, l = 1-3(-5), thin, crowded, to 6.5 mm, subventricose, narrowly adnate to emarginate, ochraceous [10 YR 7/3]; edge fimbriate, whitish; weeping. Stipe 35-50 x 7-14 mm, Q= 2.6-5.7 usually shorter than diameter of pileus, but sometimes equal to or slightly longer than diameter of pileus, at base somewhat clavate (to 16 mm) to almost equal, solid, but sometimes fistulose with age, white, coarsely floccose, especially in upper part. Context thick in pileus, firm, white. Smell raphanoid. Spores (9.5-)10.0-13.0(-14.0) x (5.5-)6.0-7.0 µm, on average 10.5-11.6 x 6.0-6.4 µm, Q = 1.7-2.1, Q_{av} = 1.74-1.92, not dextrinoid (D0-D1), regular to subamygdaliform, not or exceptionally tending to sublimoniform; perispore not (or very slightly) loosening (P0-P1); almost smooth to indistinctly verruculose (O1-O2(-O3)). Cheilocystidia (41-)44-70(-80) x 4-6 x (7-)8-12(-13) µm, on average 56.1-61.3 x 4.3-4.7 x 8.9-10.3 µm, Q = (1.4-)1.8-2.8(-3.0), Q_{av} = 2.0-2.2, (sub)cylindrico-(sub)clavate, gradually broadened towards apex, but only a (small) minority tending to subcapitate and then apical part more distinctly enlarged, thin-walled. Associated with *Salix* (4x), *Betula* (1x) or *Dryas* (1x).

ICG 2

Pileus to 36-170 mm, convex to applanate, without umbo or with indistinct umbo, margin (sub)involute, slightly viscid when moist, in centre yellowish [10 YR 7/3, 10 YR - 2.5 Y 7-8/4-6], but sometimes more brownish [10 YR 5/6-4/4], paler outwards, at margin whitish. Lamellae, L = 55-70, l = 3-5, thin, crowded, to 7 mm, subventricose, (very) narrowly adnate to emarginate, ochraceous brown [10 YR 6/4]; edge fimbriate, whitish; weeping. Stipe to 25-100 x 8-13 mm, Q=2.1-11.1 shorter to longer than diameter of pileus, slightly swollen to clavate (to 16 mm), but sometimes equal, solid or fistulose, white, coarsely floccose in upper part. Context thick, firm, white. Smell raphanoid. Spores (9.5-)10.0-12.0(-12.5) x (5.0-)5.5-6.5 µm, on average 10.3-11.2 x 5.8-6.1 µm, Q = 1.6-1.9(-2.0), Q_{av} = 1.76-1.87, not dextrinoid (D0-D1(-D2)), regular to subamygdaliform, not or very exceptionally tending to sublimoniform; perispore not loosening (P0); finely to distinctly verruculose (O1-O3).

Cheilocystidia (36-)45-77(-83) x (3-)4-5(-6) x 7-10(-14) µm, on average 50.3-66.4 x 3.8-4.5 x 7.6-8.6 µm, Q = (1.5-)1.6-2.3(-2.8), Q_{av} = 1.7-2.0, (slenderly) cylindrico-(sub)clavate, very exceptionally tending to clavate-subcapitate with enlarged apical part, thin-walled, exceptionally slightly thick-walled in upper part, colourless. Associated with *Salix* (8x), *Dryas* + *Helianthemum* (1x) or *Corylus* (1x).

ICG 3

Pileus to 45-58 mm, plano-convex to applanate, without umbo to indistinctly umbonate, margin involute when young, slightly viscid when moist, pale ochraceous yellow [2.5 Y 7-8/2-4], uniformly coloured or paler outwards and at margin whitish. Lamellae, L = 55-65, l = 3-5, thin, crowded, to 8 mm, subventricose, narrowly adnate to emarginate, pale ochraceous [10 YR 7/3]; edge fimbriate, whitish; weeping. Stipe to 45-60 x 8-11 mm, Q=4.4-9.8 usually longer than diameter of pileus, equal to subclavate (13 mm), solid, with age becoming fistulose, white, coarsely floccose, especially in upper part. Context thick in pileus, firm, white. Smell raphanoid. Spores (10.5-)11.0-13.0 x 6.0-7.5 µm, on average 11.3-12.5 x 6.3-7.1 µm, Q = 1.6-1.9, Q_{av} = 1.68-1.81, not dextrinoid (D0-D1), regular to (sub)amygdaliform, not to partly tending to sublimoniform, perispore not to slightly loosening (P0-P1); finely to distinctly verruculose. Cheilocystidia (40-)43-74(-90) x 4-6 x 7-11 µm, on average 49.8-60.6 x 4.5-4.8 x 8.3-9.4 µm, Q = (1.3-)1.5-2.5, Q_{av} = 1.8-2.0, (sub)cylindrico-(sub)clavate to (sub)clavate, broadened towards apex, exceptionally to partly subclavate-subcapitate with enlarged apex, thin-walled or slightly thick-walled in upper part, colourless. Associated with *Populus* (4x), *Salix* (2x) and *Tilia* (1x).

ICG 4

Pileus to 42-70 mm plano-convex to applanate, without umbo, margin involute, slightly viscid when moist, pale yellow in centre [2.5 Y 7-8/2-4], but sometimes darker, to ochraceous or brownish ochraceous [10 YR 5-6/4-6, 10 YR 6/4], outwards paler and at margin whitish. Lamellae, L = 45-70, l = 3-5, thin, crowded, narrowly adnate; ochraceous; edge fimbriate, whitish; weeping. Stipe to 23-35 x 6-12 mm, Q=2.3-4.2 shorter than diameter of pileus, clavate (to 14 mm), white, coarsely floccose. Context thick, firm, white. Smell raphanoid, sometimes mixed with sweetish component. Spores (10.5-)11.0-13.0(-13.5) x 6.0-7.0(-7.5) µm, on average 11.3-12.4 x 6.4-7.0 µm, Q = (1.6-)1.7-1.9, Q_{av} = 1.72-1.83, not dextrinoid

(D0-D1), regular to subamygdaliform, not to partly tending to sublimoniform; perispore not loosening (P0); slightly verruculose to almost smooth (O1-O2(-O3)). Cheilocystidia (49-)50-70(-75) x 4-5 x (7-)8-11 μm, on average 56.4-65.3 x 4.5-4.6 x 8.0-9.6 μm, Q = 1.6-2.5, Q_{av} = 1.8-2.1, (sub)cylindrico-(sub)clavate, somewhat broadened towards apex, not or exceptionally tending to (sub)capitate, thin-walled. Associated with *Salix* (8x) and *Dryas* (3x).

ICG 5

Pileus to 45-60 mm, convex to applanate, without umbo or with an indistinct umbo, margin sometimes involute, viscid when moist, in centre pale yellowish [2.5 Y 7-8/2-4] to ochraceous [10 YR 5/4-6], outwards paler and at margin whitish to white. Lamellae, L = 80-100, thin, (very) crowded, to 6 mm broad, sometimes subventricose, narrowly adnate to slightly emarginate, ochraceous to pale grey-brown [10 YR 6/3]; edge fimbriate, whitish; weeping. Stipe to 40-115 x 10-11.5 mm, Q=3.6-11.5 shorter to longer than diameter of pileus, equal to subclavate (16 mm), solid to fistulose, white, coarsely floccose, especially in upper part. Context firm, white. Smell raphanoid. Spores (10.5-)11.0-12.5 x 6.0-7.0 μm, on average 11.5-11.9 x 6.1-6.4 μm, Q = 1.7-2.0, Q_{av} = 1.81-1.90, not to indistinctly dextrinoid (D0-D2), subamygdaliform, not tending to sublimoniform; perispore not loosening (P0), rather weakly to ± distinctly verruculose (O1-O3). Cheilocystidia 40-71(-89) x (3-)4-5 x (6-)7-10(-11) μm, on average 58.7-62.5 x 4.1-4.6 x 7.9-8.5 μm, Q = (1.2-)1.4-2.3(-2.5), Q_{av} = 1.7-2.1, cylindrico-clavate to subclavate, but sometimes almost cylindrical, a minority tending to clavate-subcapitate, but in one collection not swollen at apex at all, thin-walled, colourless or with slighly refringent wall. Associated with *Tilia* (2x) or *Quercus* (2x), sometimes mixed with *Corylus*.

ICG 6

Pileus to 13-23 mm, plano-convex to applanate, with or without umbo, margin sometimes subinvolute, viscid, subshiny, in centre reddish ochraceous to red-brown [5 YR 5/6, 7.5 YR 5/4], outwards slightly to distinctly paler, at margin slightly paler to whitish. Lamellae, L = 25-35, l = 1-3(-5), thin, normally crowded, to 2 mm broad, narrowly adnate, ochraceous brown; edge fimbriate, whitish; weeping. Stipe to 30-33 x 2.5-3 mm, Q=11-12 longer than diameter of pileus, equal, whitish above, somewhat darkening downwards, especially with age, slightly flocculose, especially in upper part. Context thin. Smell weak, raphanoid. Spores

12.0-14.5(-15.0) x 6.0-7.5 µm, on average 12.6-13.5 x 6.5-7.0 µm, Q = (1.7-)1.8-2.0(-2.1), Q_{av} = 1.92-1.93, nod dextrinoid (D0-D1), subamygdaliform, not tending to sublimoniform; perispore not loosening (P0); moderately weakly ornamented (O2). Cheilocystidia (50-)53-74(-75) x 4-5 x (8-)9-16(-17) µm, on average 60.2-64.0 x 4.1-4.4 x 9.3-13.3 µm, Q = 2.0-3.8(-4.0), Q_{av} = 2.3-3.0, cylindrico-subclavate to cylindrico-subspathuliform or cylindrico-subcapitate, sometimes rather conspicuously so, thin-walled or with slightly thickened yellowish wall in upper part, especially in subcapitate cheilocystidia. Associated with *Salix* (2x).

ICG 7

Pileus to 21-31 mm, plano-convex to applanate, with or without umbo, viscid, in centre (dark) brown [7.5 YR 4/4-5/6], towards margin paler. Lamellae, L = 30-40, l = 3, thin, normally crowded, emarginate, pale brown; edge fimbriate, whitish; weeping. Stipe to 35-40 x 3.5-4 mm, Q=10 equal to to longer than diameter of pileus, equal to slightly swollen, fistulose, ochraceous, flocculose. Context thin. Smell raphanoid. Spores 10.0-12.0(-12.5) x (5.5-)6.0-7.0 µm, on average 10.9-11.3 x 6.1-6.3 µm, Q = 1.6-1.9(-2.1), Q_{av} = 1.72-1.84, not to weakly dextrinoid (D0-D2), subamygdaliform, exceptionally tending to sublimoniform, perispore not or very slightly loosening (P0(-P1)); (sub)distinctly verruculose (O2-O3). Cheilocystidia (48-)49-78(-79) x 4-5 x (6-)7-12(-13) µm, on average 58.0-59.9 x 4.2-4.8 x 8.3-10.3 µm, Q = (1.4-)1.5-2.5(-2.6), Q_{av} = 2.0-2.1, subcylindrico-(sub)clavate, partly more tending to (sub)capitate, thin-walled or very slightly thick-walled in upper part. Associated with *Salix* (2x).

ICG 8

Pileus to 18-25 mm, plano-convex, usually (sub)umbonate, but sometimes without umbo, viscid, two-coloured, in centre red-brown [5 YR 3-4/4], outwards paler, at margin pale yellow-brown [10 YR 7-8/4] to whitish. Lamellae, L = 25-35, l = 1-3, thin, normally crowded, to 3 mm broad, rather narrow, narrowly adnate to emarginate, brownish ochraceous [10 YR 7/3]; edge fimbriate, whitish; weeping. Stipe to 25-58 x 2-3 mm, Q=10-25, equal to to much longer than diameter of pileus, equal to subclavate, soon fistulose, initially whitish, on damage discolouring to (yellow-)brown from base upwards, at apex (minutely) flocculose, downwards slightly fibrillose. Context thin, firm, whitish to pale brownish buff. Smell

raphanoid. Spores (10.5-)11.0-14.0(-15.0) x 5.5-7.0(-7.5) µm, on average 11.8-13.3 x 5.9-6.8 µm, Q = (1.8-)1.9-2.0(-2.1), Q_{av} = 1.93-1.99, not to weakly dextrinoid (D0-D2), subamygdaliform, none to a few tending to sublimoniform; perispore not or slightly loosening (P0-P1); weakly to distinctly verruculose (O1-O3). Cheilocystidia (36-)39-73(-80) x 4-5(-6) x (7-)8-15 µm, on average 44.1-60.9 x 4.2-4.5 x 9.3-12.2 µm, Q = (1.6-)2.0-3.5(-3.8), Q_{av} = 2.1-2.9, cylindrico-(sub)clavate, partly tending to subspathuliform or subcapitate, thin-walled or with slightly thickened yellowish wall in apical part. Associated with *Salix* (6x).

ICG 9

Pileus to 20-66 mm, plano-convex to applanate, usually not or hardly umbonate but sometimes more distinctly umbonate, viscid, sometimes only slightly so, in centre orange ochraceous to reddish brown [10 YR 6-7/6, 5/4-6], outwards paler, sometimes rather contrasting with centre of pileus and then ± bicoloured, at margin whitish to white. Lamellae, L = 30-45, thin, normally crowded, broadly to narrowly adnate or emarginate, to 6 mm, subventricose, ochraceous [10 YR 6/3]; edge fimbriate, whitish; (distinctly) weeping. Stipe to 26-90 x 3-7.5 mm, Q=6.4-14, longer than diameter of pileus, equal, not clavate or bulbous, solid but sometimes becoming fistulose, white, flocculose over whole length. Context thick, firm, white to brownish. Smell raphanoid. Spores (9.0-)10.0-12.0(-12.5) x 5.0-6.5 µm, on average 10.2-11.4 x 5.5-6.1 µm, Q = 1.7-2.0, Q_{av} = 1.79-1.88, not dextrinoid (D0-D1(-D2)), regular to subamygdaliform, a few tending to sublimoniform; perispore not or very slightly loosening (P0-P1), (moderately) distinctly verruculose ((O1-)O2-O3). Cheilocystidia (39-)40-74 x 3-5 x 6-12 µm, on average 50.5-58.6 x 3.7-4.1 x 6.7-9.1 µm, Q = 1.5-2.5(-3.0), Q_{av} = 1.8-2.2, often (conspicuously) flexuose but sometimes straight, cylindrico-subclavate, towards apex partly more (sub)spathuliform or subcapitate, but sometimes not or hardly broadened towards apex, thin-walled. Associated with *Salix* (5x).

ICG 10

Pileus to 50 mm, applanate to slightly depressed, without umbo, viscid, pale yellow [2.5 Y 8/2-4], outwards slightly paler. Lamellae, L = 63, l = 3, thin, (very) crowded, emarginate, ochraceous; edge fimbriate, whitish; weeping. Stipe to 75 x 8 mm, Q=9.3, longer than diameter of pileus, equal, fistulose, whitish, indistinctly flocculose. Context white. Smell raphanoid. Spores (10.0-)10.5-12.5(-14.5) x (5.5-)6.0-6.5(-7.0) µm, on average 10.9-12.0 x

6.2-6.4 µm, Q = 1.6-2.0(-2.1), Q_{av} = 1.72-1.94, not dextrinoid (D0-D1), subamygdaliform, not to partly tending to sublimoniform; perispore not loosening (P0); almost smooth, slightly to moderately verruculose (O1-O3). Cheilocystidia 36-50(-52) x 4-5 x (5-)6-7(-8) µm, on average 41.0-46.3 x 4.0-4.2 x 6.4-6.6 µm, Q = (1.3-)1.4-1.8, Q_{av} = 1.5-1.7, cylindrico-subclavate, only slightly swollen towards apex, not tending to subcapitate or subspathuliform, in general rather small and narrow, thin-walled. Associated with *Salix* (1x) and *Betula* (1x).

ICG 11

Pileus to 37-75 mm, plano-convex to applanate, without or with low broad umbo, (slightly) viscid when moist, in centre yellowish to pale yellow-brown [10 YR 7-8/3, 6-7/4], outwards paler, at margin whitish to white. Lamellae, L = 50-60, l = 3, thin, normally crowded, to 4 mm, not or hardly ventricose, narrowly adnate or emarginate, greyish ochraceous [10 YR 7/2]; edge fimbriate, whitish; weeping. Stipe to 50-53 x 5-10 mm, Q=5-10, shorter to longer than diameter of pileus, clavate to ± bulbous (10 mm), solid to subfistulose, white, flocculose. Context thick, firm, white to brownish. Smell raphanoid. Spores (9.5-)10.0-12.0(-12.5) x 5.5-7.0 µm, on average 10.7-11.1 x 5.9-6.4 µm, Q = (1.6-)1.7-1.9, Q_{av} = 1.74-1.82, not dextrinoid (D0-D1), subamygdaliform, not to exceptionally tending to sublimoniform; perispore not loosening (P0); rather distinctly verruculose (O2-O3). Cheilocystidia (39-)41-63(-76) x 4-5 x (5-)6-12 µm, on average 52.3-53.7 x 4.2-4.5 x 6.1-9.7 µm, Q = 1.2-2.5, Q_{av} = 1.4-2.2, cylindrical to (sub)clavate, not or only a minority tending to subspathuliform or subcapitate, thin-walled. Associated with *Salix* (2x) or at forest edge with various trees (*Fagus*, *Picea*).

ICG 12

Pileus to 25-38 mm, convex to applanate, without or with low umbo, margin sometimes involute, distinctly viscid, in centre pale yellow, ochraceous, (dark) yellow-brown to red-brown [10 YR 7/4-6, 5-6/6, 4-5/4, 7.5 YR 4/4], outwards paler, at margin whitish. Lamellae, L = 40-50, l = 3-5, thin, crowded, narrowly adnate to emarginate, pale ochraceous; edge fimbriate, whitish; weeping. Stipe to 53-65 x 3-7 mm, Q=9.3-17.7, shorter to longer than diameter of pileus, equal to slightly clavate, fistulose or solid, white, flocculose. Context white. Smell raphanoid. Spores 8.5-12.0 x 5.0-6.5(-7.0) µm, on average 9.0-11.2 x 5.0-5.8 µm, Q = (1.5-)1.6-2.0, Q_{av} = 1.74-1.96, not to weakly dextrinoid (D0-D2), regular to

subamygdaliform; not to partly tending to sublimoniform; perispore not loosening (P0); verruculose, sometimes rather coarsely so (O2-O3(-O4)). Cheilocystidia (37-)40-63(-68) x 4-5 x (7-)8-13 μm, on average 44.9-55.5 x 4.2-4.5 x 9.4-10.8 μm, Q = (1.4-)1.8-3.0(-3.3), Q_{av} = 2.1-2.6, (sub)clavate, usually (distinctly) swollen towards apex and sometimes tending to subcapitate, a minority remaining subcylindrico-subclavate, thin-walled or very slightly thick-walled, especially in apical part in ± subcapitate cheilocystidia, exceptionally slightly thick-walled throughout. Associated with *Salix* (5x) or *Populus* (1x).

ICG 13

Pileus 25 to 37 mm, plano-convex to applanate, slightly umbonate, viscid, in centre pale yellow [2.5 Y 6-7/4], paler outwards, at margin white. Lamellae, L = 55, l = 3-5, thin, crowded, emarginate, pale ochraceous; edge fimbriate, whitish; weeping. Stipe to 45 x 6 mm, Q=7.5, slightly longer than diameter of pileus, subclavate, solid, white, (sub)floccose. Context white. Smell raphanoid. Spores (9.0-)9.5-10.0(-11.0) x (5.0-)5.5-6.0(-6.5) μm, on average 9.9 x 5.7 μm, Q = (1.6-)1.7-1.8(-1.9), Q_{av} = 1.74, not dextrinoid (D0-D1(-D2)), regular to subamygdaliform, not tending to sublimoniform; perispore not loosening (P0); verruculose (O2-O3). Cheilocystidia (38-)39-55(-56) x 4-5 x (5-)6(-7) μm, on average 45.0 x 4.3 x 5.9 μm, Q = 1.2-1.5(-1.8), Q_{av} = 1.4, cylindrical to somewhat subclavate, only slightly broadened apically, not tending to subspathuliform or subcapitate, sometimes even more subutriform and slightly broadened in lower part, thin-walled. Associated with *Populus* (1x).

ICG 14

Pileus to 22-35 mm, plano-convex to applanate, without umbo, not or hardly viscid, usually bicoloured, in centre (dark) red-brown to ochraceous brown [7.5 YR 4/2, 4-5/4, 10 YR 5-6/4], at margin paler, pale brown to whitish [10 YR 6-7/4, 8/3 or paler]. Lamellae, L = 30-40, l = 1-3, thin, normally crowded, to 4 mm, subventricose, almost free to narrowly adnate or emarginate, ochraceous brown [10 YR 5/4]; edge fimbriate, whitish; not distinctly weeping. Stipe to 18-32 x 4-7.5 mm, Q=2.6-8, usually shorter than but sometimes equal to diameter of pileus, equal or slightly bulbillose, solid to fistulose, white, discolouring to brown with age or on damage from base upwards, flocculose to subfloccose. Context thick, firm, white to brownish. Smell raphanoid. Spores (11.0-)11.5-14.0(-15.0) x 6.5-8.0(-8.5) μm, on average 12.0-13.1 x 6.9-7.2 μm, Q = 1.6-1.9(-2.0), Q_{av} = 1.72-1.84, not dextrinoid (D0-

D1(-D2)), regular to subamygdaliform, not to partly tending to sublimoniform; perispore not to very indistinctly loosening (P0(-P1)); moderately weakly to distinctly verruculose ((O1-)O2-O3). Cheilocystidia (39-)40-73(-75) x (3-)4-6 x (7-)8-12(-14) µm, on average 49.9-62.7 x 4.1-5.1 x 9.3-11.2 µm, Q = (1.3-)1.6-3.5(-3.7), Q_{av} = 2.1-2.6, usually clavate to (indistinctly) (sub)capitate or more (sub)spathuliform, a minority more cylindrico-(sub)clavate and hardly broadened towards apex, sometimes somewhat broadened in middle part and then cylindrico-subutriform, thin-walled or slightly thick-walled with brownish wall in apical part in subcapitate cheilocystidia. Associated with *Salix* (4x).

ICG 15

Pileus to 19-45 mm, plano-convex to applanate, without or with rather indistinct umbo, dry to slightly viscid, in centre dark red-brown to orange brown [2.5-5 YR 3/2, 5-7.5 YR 4-5/4-6, 10 YR 3-4/3], outwards almost concolorous to paler. Lamellae, L = 35-55, l = 1-3, thin, crowded, to 4.5 mm, subventricose, narrowly adnate to emarginate, brown, ochraceous brown to greyish brown [10 YR 4/4, 5-6/4, 6-7/3-4]; edge fimbriate, whitish; weeping, but sometimes not (distinctly) weeping. Stipe to 23-45 x 3-10.5 mm, Q=2.4-9, shorter than to equal to diameter of pileus (exceptionally somewhat longer than diameter of pileus), equal, solid to fistulose, white, discolouring to yellow-brown on ageing or damage from base upwards, at apex flocculose. Context thick, firm, white. Smell raphanoid. Spores 12.5-17.0 x (6.5-)7.0-8.0(-9.0), on average 13.7-14.9 x 6.9-7.6 µm, Q = (1.7-)1.8-2.1(-2.2), Q_{av} = 1.83-2.03, not to weakly dextrinoid (D0-D2), (sub)amygdaliform, partly tending to sublimoniform; perispore not or slightly loosening (P0-P1); slightly to distinctly verruculose (O1-O3). Cheilocystidia (34-)41-63(-65) x 4-6 x (6-)7-13(-14) µm, on average 50.4-52.0 x 4.4-4.9 x 8.3-10.7 µm, Q = 1.3-2.8(-3.0), Q_{av} = 1.9-2.3, subcylindrical to clavate, partly more tending to subcapitate, partly somewhat swollen in middle part and subcylindrical-subutriform, thin-walled or with a slightly thickened yellowish wall in apical part, especially in subcapitate cheilocystidia, in one collection with slightly thickened wall half-way. Associated with *Salix* (4x), *Populus* (1x) or *Pinus* (1x).

ICG 16

Pileus to 34-65 mm, convex to applanate, without or with rather distinct umbo, slightly viscid, in centre red-brown, yellow-brown to ochraceous [5 YR 4-5/3, 10 YR 4-6/4, 5-6/6],

uniformly coloured (especially in paler specimens) to ± distinctly paler outwards and at margin sometimes even whitish. Lamellae, L = 40-65, l = 3-7, thin, (very) crowded, rather broadly to narrowly adnate, to 6 mm, not ventricose to subventricose, ochraceous buff to brownish ochraceous [10 YR 7/2-3 to 6/3-4]; edge fimbriate, whitish; weeping (but sometimes not distinctly so). Stipe to 34-60 x 5-9 mm, Q=6.7-10.5, usually ± distinctly bulbous, sometimes (sub)clavate, fistulose, with pendent marrow strand, whitish, (sub)flocculose to subfloccose. Context thick, firm, white. Smell raphanoid. Spores (9.5-)10.0-12.5(-13.0) x 6.0-7.0 µm, on average 10.5-11.8 x 6.4-6.6 µm, Q = 1.5-1.8(-1.9), Q_{av} = 1.62-1.80, weakly to distinctly dextrinoid (D2-D4), regular to subamygdaliform, exceptionally sublimoniform; perispore not or very slightly loosening (P0-P1); slightly to rather distinctly verruculose (O2-O3). Cheilocystidia (40-)47-87(-106) x (4-)5-6(-8) x 6-9(-12) µm, on average 55.2-72.2 x 4.9-5.7 x 6.7-10.2 µm, Q = 1.2-1.8(-2.4), Q_{av} = 1.3-2.0, subcylindrical to subclavate, usually not distinctly enlarged apically, but exceptionally tending to subspathuliform, sometimes slightly swollen in basal part and then slenderly subutriform, thin-walled to very slightly thick-walled. Associated with various deciduous trees in mixed forest (*Betula*, *Fagus*, *Quercus*, *Carpinus*, *Corylus*).

ICG 17

Pileus to 32-78 mm, convex to applanate, without umbo to ± distinctly umbonate, very viscid to almost dry, sometimes seemingly hygrophanous with irregular spots, in centre usually varying between pale ochraceous yellow to pale yellow-brown [2.5 Y 7-8/2-4, 10 YR 7-8/4-6], sometimes more ochraceous brown [7.5-10 YR 5-6/4], uniformly coloured (especially in paler specimens) to distinctly paler outwards and then whitish at margin. Lamellae, L = 45-70, l = 1-3-7, thin, (very) crowded, to 8 mm, subventricose, rather broadly to narrowly adnate, ochraceous brownish [10 YR 6-7/3-4]; edge fimbriate, whitish; weeping. Stipe to 40-120 x 5-10 mm, Q=5.3-12, shorter to longer than diameter of pileus, usually distinctly bulbous (to 20 mm), but sometimes only subclavate or even equal, usually fistulose with pendent marrow strand but sometimes solid, white, discolouring to brownish on damage from base upwards, minutely flocculose to subfloccose, especially in upper part. Context thin, firm, white. Spores (9.5-)10.0-13.0 x 6.0-7.5 µm, on average 10.4-11.9 x 6.3-7.2 µm, Q = 1.5-1.7(-1.8), Q_{av} = 1.57-1.69, weakly to distinctly dextrinoid (D2-D4), regular to subamygdaliform, sometimes tending to sublimoniform; perispore not loosening (P0(-P1)); almost smooth

to distinctly verruculose (O1-O3). Cheilocystidia (36-)40-81(-83) x 4-7(-8) x 6-13 µm, on average 45.5-66.0 x 4.5-6.3 x 6.2-9.3 µm, Q = (1.0-)1.2-2.2(-2.8), Q_{av} = 1.2-2.0, straight to flexuose, usually subcylindrico-subclavate, only slightly broadened towards apex (but in two collections more distinctly broadened and even tending to subspathuliform or subcapitate), a few more subcylindrical and hardly swollen towards apex, sometimes slightly swollen in basal part and then slenderly subutriform, thin-walled to slightly thick-walled, sometimes bifid in apical part in varying frequency (absent to fairly common, and then apex to 19 µm broad). Smell raphanoid. Associated with various conifers (*Pinus*, 6x; *Picea*, 4x) and deciduous (*Betula*, 5x, *Quercus*, 2x, *Fagus*, 1x, *Carpinus*, 2x) trees; in 2 collections vicinity of *Salix* also noted.

ICG 18

Pileus to 60 mm, convex to almost applanate, with a low broad umbo, very viscid, uniformly yellow-brown [10 YR 7-8/4-6]. Lamellae, L = 55, l = 1-3, thin, normally crowded, to 5 mm, not ventricose, broadly adnate, ochraceous [10 YR 7/2-3]; edge fimbriate, whitish; weeping. Stipe to 110 x 7 mm, Q=15.7, longer than diameter of pileus, bulbous (to 20 mm), fistulose with pendent marrow strand, white, finely flocculose. Context thin, firm, white. Smell raphanoid. Spores (10.0-)10.5-11.5(-12.0) x (6.0-)6.5-7.0 µm, on average 10.9 x 6.5 µm, Q = 1.6-1.7(-1.8), Q_{av} = 1.67, distinctly dextrinoid (D3-D4), regular to subamygdaliform, not sublimoniform; perispore not loosening (P0); distinctly verruculose (O2-O3). Cheilocystidia (45-)46-59(-72) x (4-)5-6 x 5-6(-7) µm, on average 54.5 x 5.0 x 5.6 µm, Q = 1.0-1.2(-1.3), Q_{av} = 1.1, cylindrical, partly somewhat inflated in basal part and then subventricose-slenderly utriform, near apex not or hardly inflated, not clavate, thin-walled. Associated with *Pinus* among living *Sphagnum*.

ICG 19

Pileus 35 to 49 mm, applanate, only indistinctly umbonate, viscid, ochraceous yellow-brown [10 YR 6/6] in centre, outwards paler. Lamellae, L = 55, l = 3, thin, normally crowded, to 5.5 mm, subventricose, narrowly adnate, ochraceous brown [10 YR 6/4]; edge fimbriate, whitish; weeping. Stipe 42-75 x 4-7 mm, Q=10-12.5, longer than diameter of pileus, equal, not bulbous, white, flocculose in upper part. Context thick, firm, white. Smell raphanoid. Spores 10.5-11.0 x 6.0-6.5 µm, on average 10.7 x 6.2 µm, Q = (1.6-)1.7-1.8, Q_{av} = 1.74,

not dextrinoid (D0-D1), subamygdaliform, not tending to sublimoniform; perispore not or hardly loosening (P0(-P1)); moderately coarsely verruculose (O2-O3). Cheilocystida (38-)39-55(-57) x 4-5 x (5-)6-7(-8) μm, on average 46.2 x 4.6 x 6.4 μm, Q = (1.2-)1.6(-1.8), Q_{av} = 1.4, subcylindrical-subclavate, exceptionally more distinctly clavate, partly somewhat swollen below middle part and then tending to slenderly subutriform, thin-walled. Associated with *Quercus* (1x).

ICG 20

Pileus to 48-60 mm, plano-convex to applanate, with or without umbo, viscid, pale brownish yellow [10 YR - 2.5Y 6-8/4], outwards paler, at margin whitish. Lamellae, L = 60-70, l = 1-7, thin, normally crowded, to 5 mm, not ventricose, narrowly adnate to emarginate, pale ochraceous grey; edge fimbriate, whitish; probably weeping. Stipe to 60-62 x 8-9 mm, Q=6.7-7.8, equal to diameter of pileus, equal to slightly clavate, whitish, solid, flocculose in upper part. Context thick, firm, white. Smell raphanoid. Spores (10.5-)11.0-12.0(-12.5) x 6.0-7.0(-7.5) μm, on average 11.3-11.4 x 6.5-6.8 μm, Q = 1.6-1.8, Q_{av} = 1.64-1.76, not to weakly dextrinoid (D0-D2), subamygdaliform, not sublimoniform; perispore not loosening (P0); very slightly to distinctly verruculose (O1-O3). Cheilocystidia (44-)45-72(-74) x 4-5 x (6-)7-9 μm, on average 55.0-59.7 x 4.3-4.5 x 7.4-7.5 μm, Q = 1.4-2.0(-2.3), Q_{av} = 1.7, subcylindrico-subclavate to somewhat more distinctly clavate, partly even tending to somewhat subcapitate, but partly somewhat swollen in lower part and then tending to slenderly utriform, thin-walled or sometimes distinctly thick-walled in upper part, especially in subcapitate cheilocystidia. Associated with *Quercus* (2x).

ICG 21

Pileus to 46-50 mm, plano-convex to applanate, slighty umbonate, slightly viscid, in centre brownish yellow [10 YR 6-7/4-6], outwards paler, at margin whitish. Lamellae, L = 40-50, l = 3, thin, normally crowded, not ventricose, emarginate, ochraceous; edge fimbriate, whitish; weeping. Stipe to 70-75 x 6.5-9 mm, Q=8.3-10.8, longer than diameter of pileus, equal to slighty swollen, fistulose, whitish, flocculose. Context thick, firm, white. Smell raphanoid. Spores 9.5-12.5 x 5.5-6.5(-7.0) μm, on average 10.4-11.6 x 5.8-6.3 μm, Q = 1.7-2.0, Q_{av} = 1.75-1.88, not dextrinoid (D0-D1), subamygdaliform, not to weakly sublimoniform; perispore not loosening (P0); verruculose (O2-O3). Cheilocystidia (42-)45-68(-73) x

4-5 x 6-11(-15) µm, on average 48.9-57.3 x 4.3-4.6 x 7.3-9.8 µm, Q = (1.2-)1.4-2.5(-3.0), Q_{av} = 1.6-2.3, cylindrico-subclavate, usually only slightly broadened apically to more distinctly subspathuliform or subcapitate, a minority tending to subcylindrical-subclavate, thin-walled, but sometimes with slightly thickened wall in middle part. Associated with *Betula* (1x) and *Tilia* (1x).

ICG 22

Pileus 35 to 70 mm, convex, without umbo, viscid, pale ochraceous yellow[2.5 Y 7/8], more or less uniformly coloured, only at margin somewhat paler. Lamellae, L = 55, l = 3-7, thin, normally crowded, emarginate, ochraceous; edge fimbriate, whitish; weeping. Stipe 20-45 x 3.5-10 mm, Q=4.5-7.5, shorter than diameter of pileus, at base slightly swollen, white, subflocculose. Context thick, firm, white. Smell raphanoid. Spores (11.0-)11.5-12.0(-12.5) x 6.0-6.5 µm, on average 11.7 x 6.2 µm, Q = 1.8-2.0, Q_{av} = 1.88, not dextrinoid (D0-D1), (sub)amygdaliform, partly tending to sublimoniform; perispore not or very slightly loosening (P0); almost smooth to slightly verruculose (O1-02). Cheilocystidia (43-)47-67(-70) x (3-)4(-5) x 6-8(-9) µm, on average 57.5 x 3.9 x 7.2 µm, Q = (1.5-)1.6-2.0(-2.3), Q_{av} = 1.8, cylindrico-(sub)clavate, at apex slightly to distinctly broadened but not or hardly tending to (sub)capitate or (sub)spathuliform, thin-walled, colourless. Associated with *Betula* (1x).

7

General dicussion

Species concepts applied to the *Hebeloma crustuliniforme* complex

The basal species concept applied to the *Hebeloma crustuliniforme* complex is the biological species concept. For ectomycorrhizal fungi, the use of the biological species concept has been restricted to a few genera (*Laccaria*, Mueller and Gardes 1991; Fries and Mueller 1984; *Suillus*, Fries and Neumann 1990; *Paxillus*, Fries 1985). The reason for this limited impact of the biological species concept as applied to ectomycorrhizal fungi is that in vitro spore germination is problematic for most ectomycorrhizal genera. This spore germination for ectomycorrhizal species has been stimulated in different ways, for example by co-inocculation of certain micro-organisms, such as *Rhodotorula*, growth with living tree roots or dikaryotic mycelium of the same fungal species (see the different studies of Fries and coworkers). Germination of *Hebeloma* spores had previously been shown to be stimulated by living plant roots (Fries and Birraux, 1980). This way of stimulating spore germination was also tested in this thesis but only incidentally good results were obtained. Spore germination of fresh spores, without additional stimulation, was found to be more successful. Therefore, for most collections monokaryons were obtained using spores collected directly from fresh basidiocarps on agar plates.

Although in vitro spore germination had been obtained before in the genus *Hebeloma*, no intercompatibility tests had been performed. Here at least 20 InterCompatibility Groups (ICGs, biological species) were found (Chapter 2). A large number of ICGs consisted of only few collections, especially in the *H. helodes* clade (clade II, Chapter 3, 6). Since only 110 collections were tested, expanding the sample size will therefore probably increase the number of ICGs, especially in clade II.

A low level of partial intercompatibility between ICGs was observed between some ICGs (1-2, 3-4, 2-3/4 and 16-17). One strain (605) was compatible with all strains of the two ICGs 3 and 4. This phenomenon is similar to that found by Petersen and Ridley (1995)

with a New Zealand strain of *Pleurotus*. In all other cases, however, assignment of isolates to a single ICG was unambiguous. Individual compatible combinations between members of the partially compatible ICG 1 and 2 and between members of the partially compatible ICG 16 and 17 showed signs of reduced compatibility. This was reflected by: (i) no or unidirectional nuclear migration, (ii) reduced growth rate of dikaryon and (iii) aberrant morphology of hyphae. These observations show that these dikaryons are less vigorous than the combinations within ICGs. These observations also support Boidin's (1986) claim that intercompatibility should not automatically be equated with interfertility. On the other hand, most of the individual compatible combinations between the partially compatible ICG 3 and 4 did not show any sign of reduced compatibility and the single compatible combination between ICG 2 and 3/4 (strain 605) did not either, suggesting that these combinations have a higher persistence.

For eight ICGs more than one strain was included in at least one of these phylogenetic analyses. Strains of five of these eight formed monophyletic groups, and three ICGs formed paraphyletic groups in a nuclear phylogeny: ICGs 3, 4 (both in the IGS phylogeny, Chapter 4) and 17 (ITS phylogeny, Chapter 3, 5). However, the partially compatible ICGs 3 and 4 formed monophyletic groups in a mitochondrial phylogeny and together they constituted a monophyletic group in the nuclear phylogeny (Chapter 4). ICG 17 formed a clade with the partially compatible ICG16 and with the fully incompatible ICG 18. Overall, a fairly good correspondence between the biological species concept and the relative age of the most recent common ancestor has been found in this group of species. This is consistent with findings in other mushroom genera (*Pleurotus*, Vilgalys *et al.*, 1996, Vilgalys and Sun, 1994; *Armillaria*, Piercy-Normore, 1998). However, within the genus *Pleurotus* some recently evolved ICGs were paraphyletic (Vilgalys *et al.*, 1996, Vilgalys and Sun, 1994). Usually, populations of an ICG within a continent formed monophyletic groups but the populations within an ICG from different continents sometimes formed a paraphyletic group. Future studies in the *H. crustuliniforme* complex should therefore include strains from a broader geographic area, to consider the possibility of other paraphyletic ICGs.

Paraphyly of ICGs shows that intercompatibility must sometimes be considered as a plesiomorphic character state. The ultimate criterion that is used to delimit species, either intercompatibility or monophyly, is arbitrary. However, more detailed comparisons about phylogenetic relationships (preferentially based on more than one gene sequence) within

ICGs and between partially compatible ICGs are necessary to estimate the frequency of paraphyletic ICGs. If monophyly of ICGs is the norm, there is a good argument to use reproductive isolation as a criterion. However, if paraphyly of ICGs is frequently observed, this can be used as an argument to use monophyly as a criterion, although one could still argue that compatibility gives at least theoretically the possibility for genetic exchange. Overall, species problems can best be approached by studying both sexual compatibility and phylogenetic relationships. Such studies are also most informative on the origin of reproductive isolation (here defined as speciation) in Basidiomycetes (see also the discussion below).

Using a morphological species concept with a minimal phylogenetic quality, two or at the most four species could be delimited (Chapter 6). This indicates that most biological species within the *H. crustuliniforme* complex are cryptic species (sibling species, biological species that can not be separated morphologically). In other genera, mating studies have shown the existence of such sibling species as well. Sibling species have been found for instance within *Xeromphalina campanella* (Johnson, 1997), *Lentinula boryana* (Petersen et al., 1998), the *Corticiaceae* (Hallenberg, 1991) and the genera *Paxillus* (Fries, 1985) and *Laccaria* (Mueller, 1991, 1991), although in some cases morphological differences could be found between ICGs. In the genus *Armillaria* many species had been described, but over time most mushroom taxonomists agreed that there was only one species of *Armillaria*, *A. mellea* (Petersen and Hughes, 1999). However, mating studies indicated the existence of many biological species (Ullrich and Anderson, 1978). Having this knowledge, mycologists re-examined *Armillaria* morphology and ecology, and the majority of biological species were found to represent morphological species that had previously been named but were historically overlooked (but see Termorshuizen and Arnolds, 1997).

Three different hypotheses could explain why most biological species do not represent morphological species in the two clades of the *H. crustuliniforme* complex:

i. Recent origin of ICGs;
ii. Morphological stasis;
iii. Morphological plasticity, resulting in high intraspecies variability, combined with a very limited number of (qualitative) characters.

In the *H. crustuliniforme* complex i and iii are probably the most important factors to explain the high number of cryptic species. A close relationship of the ICGs within the two

clades appears from molecular data (Chapter 3, 4) as well as from the uniformity in microscopical characters for the ICGs. Moreover, several cases of partial compatibility have been found between ICGs both within clade I as within clade II (Chapter 2). Some macroscopic characters on the other hand, such as general habit, pileus colour, slenderness and size are rather variable. This variability is evident at three levels: i) between ICGs. Some characters such as pileus colour, size and habit have evolved independently several times, according to the best estimate of the phylogeny; ii) within ICGs, although variation within ICGs is generally lower than between ICGs and iii) within single collections. The high plasticity and variability in macroscopical characters might ultimately be the explanation for our failure to recognise more than four morphospecies with a minimal phylogenetic quality in this species-rich complex.

Strong evidence was found that some of the traditionally recognized species are not monophyletic. Among these are *H. pusillum*, *H. lutense* (=*H. leucosarx* sensu auct. neerl.) and *H. alpinum*. The criteria used to define these species, are macroscopical characters such as general habit and geographic/ecological characters. However, we showed that these macroscopical characters are rather plastic (at different levels), and in these cases this plasticity may either be convergent evolution due to the same microclimatic conditions (*H. pusillum*, slender basidiocarps in humid *Salix* bushes, *H. lutense*, stout basidiocarps usually associated with *S. repens* often in early successional, relatively open sites) or phenotypic plasticity (*H. alpinum* in the alpine zone, *H. crustuliniforme* in non-alpine areas). Therefore, it is not surprising that these species which are defined on the basis of macroscopical characters in combination with geographic/ecological characters do not represent monophyletic entities.

Phylogenetic relationships within the genus *Hebeloma*.

In Chapter 3 phylogenetic relationships were determined between the ICGs within the *H. crustuliniforme* complex and between them and the other groups within the genus *Hebeloma* using ITS 1 and 2 sequences. Several unexpected results were obtained. The proposed sister group of *Hebeloma*, *Alnicola* appeared to form a paraphyletic group. Although trees with *Alnicola* constrained as a monophyletic group are 7 steps longer than the most parsimonious trees, these could not be rejected by both Templeton's and the Kishino-

Hasegawa test. Clearly, for a better understanding of the evolutionary relationships between *Alnicola* and *Hebeloma* and within *Alnicola* a larger sample of the latter taxon needs to be studied in future studies. If *Alnicola* indeed turns out to be paraphyletic, at least two possibilities exist to define monophyletic entities: i) *Alnicola* can be split into monophyletic entities; ii) *Hebeloma* and *Alnicola* can be fused to a single monophyletic genus. The latter solution has been proposed by Kühner (1980).

Another unexpected result was that the 20 ICGs of the *H. crustuliniforme* complex did not form a monophyletic group, but instead formed two distinct clades. One of these clades (clade II) consisted of 17 ICGs that belong to morphospecies as *H. crustuliniforme*, *H. alpinum*, *H. pusillum*, *H. lutense* and *H. helodes*. The other (clade I) consisted of three ICGs that belong to the morphospecies *H. velutipes* and *H. incarnatulum*. Within clade II, many of the phylogenetic relationships were unresolved, because of low sequence divergence. Within clade I, two well supported clades were found, one consisting of ICG 16 and a strain of ICG 17, and the other consisting of strains of ICG 17 and ICG 18.

Several other well supported clades could be recognized, such as the two species of the *H. sacchariolens* complex (*H. tomentosum* and *H. sacchariolens*) the two veiled species (*H. collariatum* and *H. mesophaeum*) and *H. sinapizans* and *H. truncatum*. However, the basal relationships between the major monophyletic groups were not well resolved.

To change taxonomic divisions on the basis of new insights in phylogenetic relationships, one should test whether new phylogenetic hypotheses are significantly better than existing. Several methods now exist to test a particular phylogenetic hypothesis using sequence data (*e.g.* Templeton, 1983, Kishino and Hasegawa, 1989, Huelsenbeck and *et al.* 1996). Many of these tests are not very powerful, *i.e.* a null hypothesis is rarely rejected. The likelihood ratio test has proven to be more powerful (Huelsenbeck and *et al.* 1996). However, the evolutionary model should be realistic, else this test rejects a null-hypothesis too often. In this study alternative topologies with other clades as basal in the genus could not be rejected. It was therefore not possible to translate this cladogram into a newly proposed infrageneric classification for *Hebeloma* and only few of the existing subdivisions of the genus *Hebeloma* (Introduction, Table I) which are all contradictory to some extent, could be rejected. Many of the characters upon which these divisions are based are quantitative, with diffuse transitions. This makes it very difficult to objectively assign character states in many cases. More

importantly, some of the character states that have been used for infrageneric classification represent plesiomorphic states, as was shown by a most parsimonious reconstruction of several character states on the best tree (Chapter 3). Dividing the genus on the basis of such character states inevitably leads to paraphyletic entities. These two factors, the difficulty in assigning character states and the plesiomorphic nature of important character states, have probably contributed to the contradictory opinions on *Hebeloma* infrageneric divisions.

One could wonder whether a phylogenetic analysis of other sequences than the ITS 1 and 2 could give a better resolution of the phylogenetic relationships between the main clades of the genus *Hebeloma*. The low resolution of phylogenetic relationships between the basal taxa was not caused by a lack of variation of the used sequence data (ribosomal ITS 1 and 2), but instead by a lack of phylogenetically informative sites for resolution at this level. Therefore, major improvements cannot be expected from other sequence data. More variable sites are most likely to be saturated, less variable sites probably have no phylogenetic information at all. I hypothesize that the major groups within the genus *Hebeloma* have diverged within a relatively short period.

Future studies should include representants of the other described morphological species as well, preferentially type specimens.

Host specificity.

In Chapter 1 it was shown that ICGs did fall in two host preference groups. One clade of ICGs preferentially grows with *Salicaceae* (74 %) and the other clade of ICGs has a broad host range, but is rarely found with *Salicaceae* (7 %). *Hebeloma* species have generally been considered to have broad host ranges (Molina *et al*, 1992; Smith and Read, 1997), but the uncertainty surrounding species recognition made evaluation of this claim difficult. In this thesis it was clearly shown that host specificity of *Hebeloma* species is stronger than is generally assumed, especially at the level of ICGs.

Other ectomycorrhizal genera have restricted host ranges as well. For example, *Suilloideae* are almost exclusively associated with *Pinaceae* (Singer, 1986; Kretzer *et al.*, 1996). Martin *et al.* (1998) have recently found that *Pisolithus* species also show strong host preference. On the other hand, some ectomycorrhizal fungi have been thought to have broad host ranges such as *Laccaria*. However, detailed studies in this genus (Mueller, 1992) have

shown that many species in fact have more restricted host ranges than had been assumed. It was found that many species of the genus *Laccaria* are either found with *Pinaceae* or *Fagaceae*.

This may be a more general phenomenon: although a lack of host specificity is found at a certain taxonomic level, for example at the genus level or the morpho-species level, there may be host specificity at a lower taxonomic level, for instance at the biological-species level. Therefore, in order to make statements about host specificity, one should clearly specify the taxonomic level for which the statement is made.

Within the other groups of *Hebeloma*, *Salicaceae* preference is also found in the *H. mesophaeum* group and the *H. sacchariolens* group. We can only speculate about the cause of this difference in host preference: *Salicaceae* preferring and non-*Salicaceae* preferring. It has been shown that salicylic acid plays an important role as a signaling molecule in disease resistance in plants (for an overview, see Durner et al 1997). A hypothesis to explain the distinction between species that can grow with *Salicaceae* and species that can not, is that some species are able to break the resistance associated with salicylic acid, whereas others are not.

A most parsimonious reconstruction of host preference, coded as a character with two states, *Salicaceae* preferring and non-*Salicaceae* preferring, indicated that a preference for *Salicaceae* has arisen at least three times independently within *Hebeloma* (Fig. 4, Chapter 3). Strong evidence was found for a basal position of ICG 21 in clade II. This ICG has not been found with *Salicaceae*. The *Salicaceae* preferring clade consists of 16 ICGs, its non-*Salicaceae* preferring sister group of only one ICG. Interestingly, the two other groups in the genus *Hebeloma* that have *Salicaceae* preference (*H. mesophaeum* group and *H. sacchariolens* group), belong to the most species rich clades within the genus, judging from morphological studies (Vesterholt, 1989; Gröger and Zschieschang, 1981). These findings suggest that the host tree switch to *Salicaceae* has been followed by extensive and rapid speciation.

We can only speculate if there is a causal relationship between the switch to *Salicaceae* and this speciation, and if so what explanations there are. *Salicaceae* belong to the monophyletic order Malpighiales (Bremer *et al.* 1998; Doyle, 1998). All the other families that belong to this clade do not form ectomycorrhiza. The *Fagales* and *Pinaceae*, the other tree groups with which *Hebeloma* species frorm ectomycorrhiza, belong to

different clades. This means that the most parsimonious explanation for this pattern of host tree colonization is that *Salicaceae* have been colonized by ectomycorrhizal fungi separately and probably recently. Assuming that only a limited number of fungi had the ability to grow with *Salicaceae*, the colonisation of this relatively new, empty niche may be causally related to the evolution of the high number of relatively closely related ICGs.

In future studies it will be interesting to study the specificity for *Salicaceae* of the *Salicaceae* specific ICGs in the lab. Comparisons can be made of the growth of trees in combinations between *Salicaceae* specific ICGs, and *Salicaceae* and other trees, as well as between non-*Salicaceae* specific ICGs, and *Salicaceae* and other trees.

Intercompatibility and molecular variation: clues for the origin of intercompatibility?

The interface between population genetics and systematics is most informative for studies of speciation (Templeton, 1998; Schilthuizen, 1999; Coyne and Orr, 1999). The more distant from the actual speciation event, the greater the expected genetic differences among the species, but the more difficult it becomes to infer which genetic differences were causally involved in speciation versus which were consequences of speciation (Templeton, 1998). More importantly, at this interface between population biology and systematics examples exist where incompatibility is not qualitative ('all-or-nothing') but quantitative with different levels of partial incompatibility (*Armillaria*, Anderson et al. 1980; *Heterobasidion annosum*, Chase and Ullrich 1990a+b; Garbelotto *et al.*, 1998; *Hebeloma*, this thesis, Chapter 2). This provides the opportunity to compare different levels of compatibility with phylogenetic history of strains.

In Chapter 4 phylogenetic relationships were determined between individuals belonging to ICGs of clade IIa of the *H. crustuliniforme* complex. In this clade 9 ICGs were found, 4 of which were partially intercompatible. This partial intercompatibility was organized hierarchically with an intermediate level of intercompatibility between ICGs 3 and 4 and very limited between 2 and 3/4, and between ICGs 1 and 2. This partial intercompatibility provided the opportunity to compare different levels of intercompatibility with phylogenetic history. A mitochondrial and a nuclear phylogeny were reconstructed. For ICGs 2, 3 and 4 a positive correlation was found between the level of intercompatibility and the relative age of the most recent common ancestor. ICGs generally formed

monophyletic groups, ICG 3 and 4 (15% partial intercompatibility) were sister groups and the sister group of (3,4) was ICG 2 (0.4% intercompatible with (3,4)). This is consistent with a gradual origin of sexual incompatibility ('divergence-first'). ICG 1 had a different position in the nuclear and mitochondrial phylogeny. In the nuclear phylogeny it was the sister taxon of ICG 5, and in the mitochondrial the sister group of ICG 2. A possible explanation is that ICG 1 has a hybrid origin, with the ancestor of ICG 5 as the nuclear donor and the ancestor of 2 as the mitochondrial donor. In future studies, phylogenies based on other molecular markers, both nuclear and mitochondrial, could be used to confirm the proposed hybrid origin of ICG 1.

In Chapter 5 a polymorphism in the ITS of ICG 17 was described, with some dikaryotic individuals having two different ITS types differing at 17 nucleotide positions. One of these types was highly similar to the single type found in ICG 16. It was shown that the different ITS types within ICG 17 represented different alleles at homologous rDNA loci, since monokaryons always had a single type. The polymorphism was widespread with both types occurring both in Europe and in America. However, no recombinants between the two types were found, suggesting that the two types have come together relatively recently in evolutionary time. Several lines of evidence were described why the polymorphism within ICG 17 was not the result of actual hybridisation between the partially compatible ICGs 16 and 17.

Future studies should address the question if other molecular markers are also polymorphic within ICG 17. If this is the case, recent hybridisation between divergent populations must be assumed. The inclusion of samples from a larger geographic area in such a study potentially could shed light on the geographic origin of the divergent populations. Alternatively, the polymorphism is unique for the ITS region. In that case, either introgression of one ITS type has to be assumed, or some other mechanism to explain the divergence and the maintenance of the two different ITS types within an ICG. However, I do not know of such a mechanism other than allopatric divergence and recent genetic exchange.

It will be very interesting to perform intercompatibility tests between strains of *H. velutipes* sampled in a large area. The outcome of intercompatibility tests between North American populations and European populations of the *H. velutipes* clade can shed light on the relationship between the level of partial compatibility found in this species complex and

molecular variation. If allopatric populations are found, compatible with both ICGs 16 and 17, this could be evidence for reinforcement of reproductive isolation between ICGs 16 and 17 in sympatry.

The results described in this thesis show that in two different species complexes, two different patterns were observed. In clade II of the *H. crustuliniforme* complex, genetic divergence was very low (Chapter 3, 4) and many ICGs were found. In clade I on the other hand, a low number of ICGs was found although substantial genetic divergence was observed within this clade and even within a single ICG (Chapter 5). This shows that there is no linear relationship between the level of ITS divergence and the level of incompatibility. However, within clade IIa of the *H. crustuliniforme* complex there was a fairly good correspondence between the level of incompatibility and the relative age of the last common ancestor. These results indicate that generalisations about the relationship between the level of incompatibility and genetic divergence have no firm basis.

Sexual incompatibility and genetic exchange.

The results described in this thesis raise further questions about the relationship between phylogenetic relationships, sexual intercompatibility and genetic exchange. Speciation in this thesis was defined as the origin of reproductive isolation. The study on speciation was addressed here by comparing the level of intercompatibility with the relative age of the most recent common ancestor. In this approach, initially the possibility of genetic exchange between divergent populations was not explicitly considered. Although generally a fairly good correspondence was found between phylogenies based on different nuclear as well as mitochondrial sequences, for one ICG strong discordance was found between its position in a nuclear and that in a mitochondrial phylogeny. Among other possible causes, hybridisation between ancestors of different ICGs was proposed as an explanation. Moreover, within ICG 17 two highly divergent ITS types were found. These findings suggest that genetic exchange between divergent populations may be more frequent than is generally assumed. Even between ICGs genetic exchange may be more frequent than is generally assumed on the basis of outcomes of mating tests. Intersterility barriers between most species are generally considered to be prezygotic and nearly always absolute, and natural hybridisation is considered to be rare or absent (Boidin, 1986; Vilgalys and Sun,

1994; Vilgalys *et al.*, 1996). However, evidence for rare mating events between divergent populations may not be obtained by outcomes of mating tests. Recently, in several Ascomycete species in which never a sexual state had been observed, recombination was found by comparing phylogenies based on single gene sequences (Burt *et al.*, 1996; Geiser *et al.*, 1998). This approach, comparing phylogenies based on single gene sequences, could be used to detect rare genetic exchange between ICGs in Basidiomycetes. By comparing these nuclear phylogenies with phylogenies based on mitochondrial sequences, cytoplasmic exchange between ICGs could be discovered.

Until recently, in Agaricales molecular phylogenies have especially been used to infer phylogenies at a taxonomic level higher than the (biological) species level. However, there is still much uncertainty surrounding species circumscription. More research is necessary at or below the species level, to study the relationship between divergence, incompatibility and genetic exchange. The approach of comparing closely related partially or fully incompatible ICGs seems very promising. In this approach first ICGs and cases of partial incompatibility are defined within a morpho-species (complex). Having found these ICGs, phylogenetic relationships between them are defined at a relatively high taxonomic level. Finally, in this approach the study focuses on closely related fully or partially incompatible ICGs. Especially at this taxonomic level, the relationship can be studied between divergence, incompatibility and genetic exchange, by comparing multiple gene trees of populations belonging to these ICGs.

If genetic exchange between ICGs is more general than is generally assumed, this raises new questions about the genetic basis of sexual incompatibility.

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Summary

This thesis deals with species delimitation and speciation in the ectomycorrhizal *Hebeloma crustuliniforme* complex. The species concept traditionally used in this complex is based on morphology of basidiocarps. However, species delimitation has been controversial. One of the best known names is *H. crustuliniforme* (Bull.) Quél., a name regularly encountered in the mycorrhizal literature. However, this name has been used for a species complex of taxa with relatively pale (but sometimes more brown-tinged), veilless carpophores with weeping lamellae. In this thesis the basal criterion to delimit species within the *Hebeloma crustuliniforme* complex is sexual intercompatibility. Therefore, speciation is here defined as the origin of sexual interincompatibility. The strategy that has been used in this thesis to address the questions of species delimitation and speciation in the *Hebeloma crustuliniforme* complex can be summarized as follows:

1) Define InterCompatibility Groups (ICGs) and look for examples of partial incompatibility (Chapter 2);
2) Determine phylogenetic relationships between those ICGs (Chapter 3);
3) Focus on closely related, and preferentially partially incompatible, ICGs to study speciation (Chapters 4, 5);
4) Derive an operational species concept for the *Hebeloma crustuliniforme* complex that is based on morphological recognition of (combinations of) biological species within an explicitly phylogenetic framework (Chapter 6).

In Chapter 2 the results are described of intercompatibility tests in this species complex. In a sample of 110 collections 20 InterCompatibility Groups (ICGs) were found. Partial compatibility was found between ICGs 3 and 4, between 2 and 3/4, between 1 and 2 and between 16 and 17. One strain (605) was compatible with all strains of ICGs 3 and 4. In all other cases, however, assignment of isolates to a single ICG was unambiguous. Individual compatible combinations between members of the partially compatible ICG 1 and 2 and between members of the partially compatible ICG 16 and 17 showed signs of reduced compatibility. This was reflected by: (i) no or unidirectional nuclear migration, (ii) reduced growth rate of the dikaryon and (iii) aberrant morphology of hyphae.

In Chapter 3 phylogenetic relationships were studied between the ICGs of the *H. crustuliniforme* complex and between them and the other main groups in the genus *Hebeloma* based on nuclear ribosomal ITS sequences, using cladistic methods. The 20 ICGs of the *H. crustuliniforme* complex do not form a monophyletic group, but instead form two distinct clades, one consisting of three ICGs (clade I) and the other of 17 ICGs (clade II). Most of the ICGs in the latter clade were very closely related as suggested by a low sequence divergence. The majority of ICGs of this clade showed a preference for *Salicaceae*, but the basal ICG (ICG 21) did not. The host tree switch to *Salicaceae* has probably been followed by extensive and rapid speciation.
Several other well supported clades were found in the genus *Hebeloma*, but the basal relationships between them were not well resolved. It is therefore impossible to propose a new infrageneric division for *Hebelama*.

In Chapter 4 a subclade of clade II (subclade IIa) was studied in detail. In this subclade nine ICGs were found, four of which were partially compatible. This partial intercompatibility was organised hierarchically with an intermediate level of compatibility between ICGs 3 and 4 and very limited compatibility between 2 and 3/4 and between 1 and 2. The single strain (605) that was intercompatible with all strains of ICGs 3 and 4 was a member of subclade IIa as well. A mitochondrial and a nuclear phylogeny of strains belonging to these partially compatible populations were reconstructed. For ICGs 2, 3 and 4 a positive correlation was found between the level of interincompatibility and the relative age of the most recent common ancestor. ICGs generally formed monophyletic groups, ICG 3 and 4 (15% partial intercompatibility) together formed a monophyletic group and the sister group of (3,4) was ICG 2 (0.4% intercompatible with (3,4)). This is consistent with a gradual origin of sexual incompatibility (divergence-first). ICG 1 had a different position in the nuclear and mitochondrial phylogeny. In the nuclear phylogeny it was the sister taxon of ICG 5, and in the mitochondrial the sister group of ICG 2. A possible explanation is that ICG 1 has a hybrid origin, with the ancestor of ICG 5 as the nuclear donor and the ancestor of 2 as the mitochondrial donor.

The subject of Chapter 5 is a polymorphism in the ribosomal Internal Transcribed Spacer of ICG 17. Within this ICG of the morphospecies *Hebeloma velutipes* a dikaryotic strain (d504) was found with two divergent types ITS. These two types segregated in monokaryotic progeny of the same strain, showing that the different ITS types represent

different alleles at homologous rDNA loci. RFLP analysis of more strains of ICG 17 showed that the polymorphism is widespread, with both types occurring in Europe as well as in America. Cladistic analyses of the two ITS sequences showed that they did not form a monophyletic group. One of the types belonged to a clade together with the single ITS type found in the partially compatible ICG 16 and the other to a clade together with the single ITS type found in the fully incompatible ICG 18. RFLP analysis of the mitochondrial ribosomal SSU showed that there were fixed differences between the mitochondria of ICG 16 and 17. Several lines of evidence were described that the ITS polymorphism in ICG 17 is not the result of actual hybridisation between 16 and 17. The polymorphism within ICG 17 must therefore be of a different origin. The lack of recombinants, neither within the rDNA locus nor between ITS 1 and 2, suggests that the two types have come together relatively recently. The ITS polymorphism described in this Chapter clearly showed the potential danger of using single ribosomal sequences for reconstructing species phylogenies and the potential problems for molecular identification of species.

In Chapter 6 a method is presented to derive an operational species concept for the *Hebeloma crustuliniforme* complex that is based on (combinations of) biological species within an explicitly phylogenetic framework. Crucial in this analysis is a reliable estimate of the phylogeny of biological species in the *H. crustuliniforme* complex. Based on two nuclear sequences, we presented a best estimate of the phylogeny of biological species within the *H. crustuliniforme* complex. Using this phylogeny, on the basis of (strict) monophyly only two species could be recognised among 20 biological species, viz. *H. velutipes* and *H. helodes*. An earlier phylogenetic analysis indicated that these two morphological species are not sister taxa. Relaxing the criterion of monophyly and allowing paraphyletic groupings of biological species as a morphospecies resulted in the recognition of three morphospecies, viz. *H. velutipes*, *H. incarnatulum* and *H. helodes*. A tree, with the five ICGs of the previously defined morphospecies *Hebeloma crustuliniforme* (1, 2, 3, 4 and 5) constrained as a monophyletic group could not be rejected. This constrained tree, together with the relaxed criterion, allowed the recognition of four species, viz. *H. helodes*, *H. crustuliniforme*, *H. velutipes* and *H. incarnatulum*. The limited ability to translate a biological species concept into an operational species concept was explained by the lack of qualitative characters and the plasticity of quantitative characters. Based on the close relationship between the ICGs in the two clades of the *H. crustuliniforme* complex, it was

shown that a good correspondence between a biological species concept and a morphological species concept is not likely to be forthcoming.

In the final Chapter the results found in this study were integrated and discussed in a broader context and directions for future research were suggested. Future phylogenetic studies should consider the possibility of genetic exchange between divergent populations more explicitly.

Samenvatting

Dit proefschrift gaat over een onderzoek aan het *Hebeloma crustuliniforme*-complex (radijsvaalhoed-complex), een groep nauw verwante plaatjeszwammen (Agaricales, Basidiomycota) die ectomycorrhiza's vormen. Ectomycorrhiza is een wederzijds voordelige symbiose tussen een schimmel en een plant, meestal een boom. De omgrenzing van soorten in dit soortencomplex was tot nu toe gebaseerd op morfologische kenmerken van de vruchtlichamen (de paddestoelen). Op die manier was echter geen ondubbelzinnige omgrenzing van de soorten gevonden: verschillende onderzoekers onderscheidden verschillende aantallen soorten.

In dit onderzoek stonden de volgende vragen centraal

i) Hoe kunnen we soorten omgrenzen en herkennen in dit complex?

ii) Hoe zijn de soorten ontstaan?

In dit proefschrift is het **biologisch-soortconcept** als het basale soortconcept gehanteerd: individuen behoren tot één soort als ze samen vruchtbare nakomelingen kunnen produceren. Bij sexuele Basidiomyceten is een monokaryon het mycelium dat ontstaat uit een gekiemde spore; alle cellen van een monokaryon hebben één kern. Een dikaryon ontstaat uit fusie van twee monokaryons. Alle cellen in een dikaryon hebben twee afzonderlijke kernen, één van ieder monokaryon. Dikaryons kunnen microscopisch gemakkelijk van monokaryons onderscheiden worden. Tweekernige mycelia kunnen vruchtlichamen (paddestoelen) vormen, waarin de kernversmelting plaats vindt, gevolgd door meiose en sporenvorming (zie Fig. 1). Biologische soorten worden bij sexuele Basidiomyceten meestal gedefinieerd door het testen van sexuele intercompatibiliteit: kunnen monokaryons van verschillende individuen met elkaar fuseren en een stabiel dikaryon vormen. Om deze reden wordt een biologische soort in dit proefschrift meestal aangeduid met de term InterCompatibiliteits-Groep (ICG).

De vraag hoe soorten zijn ontstaan (speciatie) hangt natuurlijk af van hoe we soorten definiëren. Aangezien hier het biologisch-soortbegrip gehanteerd is, kunnen we het ontstaan van soorten gelijkstellen met het ontstaan van reproductieve isolatie, waarvan sexuele incompatibiliteit bij sexuele Basidiomyceten de belangrijkste vorm is.

De werkwijze die gebruikt is om de verschillende onderzoeksvragen te beantwoorden is als volgt:

1) Het bepalen van ICG's (biologische soorten) en het vinden van voorbeelden van gedeeltelijke compatibiliteit (hoofdstuk 2);
2) Het reconstrueren van evolutionaire relaties tussen ICG's (hoofdstuk 3);
3) Het nauwkeuriger onderzoeken van nauw verwante ICG's, bij voorkeur slechts gedeeltelijk reproductief geïsoleerde ICG's, om het proces van het ontstaan van reproductieve isolatie te kunnen bestuderen/reconstrueren (hoofdstukken 4, 5);
4) Het afleiden van een gemakkelijker te hanteren soortbegrip voor het *Hebeloma crustuliniforme*-complex, gebaseerd op morfologische herkenning van (combinaties) van biologische soorten die een unieke gemeenschappelijke voorouder hebben (hoofdstuk 6).

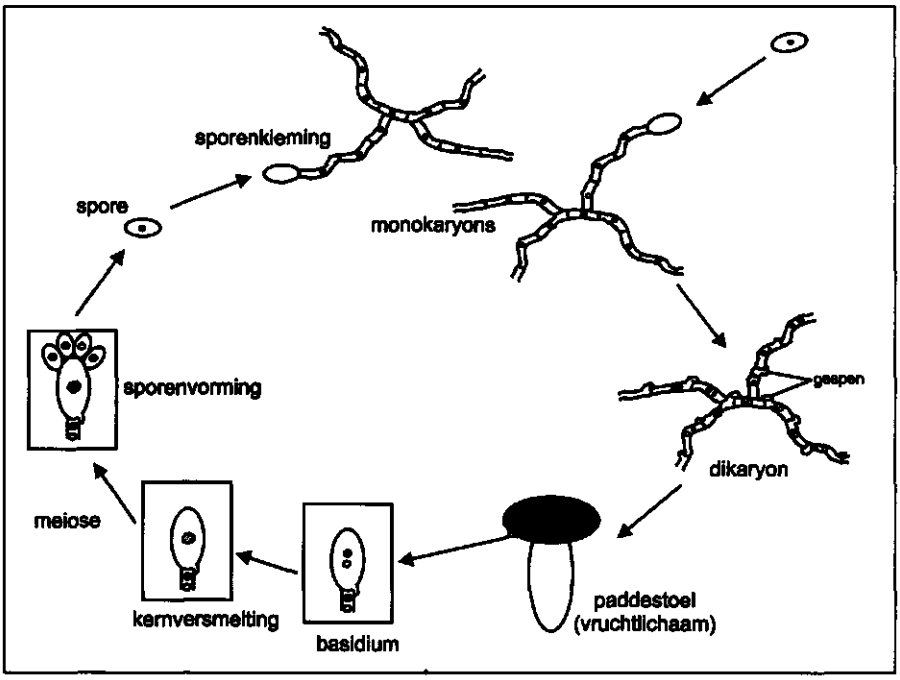

Fig. 1. De vereenvoudigde levenscyclus van een sexuele heterothallische Basidiomyceet.

In hoofdstuk 2 zijn de resultaten beschreven van intercompatibiliteits-testen in dit soorten-complex. In een verzameling van 110 collecties (dikaryotische individuen, meestal

bestaande uit meerdere vruchtlichamen), die door mij gedurende drie jaren in noordwest-Europa was aangelegd, werden maar liefst 20 biologische soorten (ICG's) gevonden. Er werd ook gedeeltelijke compatibiliteit gevonden tussen een aantal ICG's: tussen ICG 3 en 4, tussen 2 en 3/4, tussen 1 en 2 en tussen 16 en 17. Eén collectie (605) was compatibel met alle collecties van zowel ICG 3 als 4. In alle overige gevallen konden individuele collecties ondubbelzinnig tot een enkele ICG gerekend worden. Individuele combinaties tussen collecties van de partieel compatibele ICG 1 en 2 en tussen collecties van de partieel compatibele ICG 16 en 17 vertoonden tekenen van verminderde compatibiliteit. Dit kwam tot uiting in: i) geen of eenzijdige kernmigratie, ii) verminderde groei van het dikaryon en iii) een afwijkende morfologie van het dikaryon.

In hoofdstuk 3 werden evolutionaire verwantschappen bestudeerd tussen de gevonden ICG's en tussen deze en de overige groepen binnen het geslacht *Hebeloma*. Deze verwantschappen werden afgeleid door een cladistische analyse te doen op de basenparen van een stukje DNA, de ITS, van een groot aantal collecties. Uit deze analyse bleek dat de 20 gevonden ICG's geen unieke gemeenschappelijke voorouder hebben, maar twee groepen vormen die ieder een andere unieke laatste gemeenschappelijke voorouder hebben. Een van deze twee groepen bestond uit drie ICG's (clade I, *H. velutipes/H. incarnatulum*), de andere uit 17 ICG's (clade II, *H. crustuliniforme/H. pusillum/H. helodes*). De meeste ICG's binnen deze laatste clade waren zeer nauw verwant, wat bleek uit de geringe sequentie-divergentie van de soorten in deze clade. Het grootste deel van de ICG's binnen deze clade toonde een voorkeur voor *Salicaceae* (wilg en populier) als gastheer. Maar liefst 74 % van de collecties uit deze clade waren bij deze bomen verzameld. De ICG die basaal is binnen deze clade, ICG 21, was echter niet bij *Salicaceae* gevonden. Dit wijst erop dat de wisseling van gastheervoorkeur is gevolgd door uitgebreide en snelle soortvorming. In clade I was slechts 7 % van de collecties verzameld bij *Salicaceae*.

Verschillende andere goed ondersteunde monofyletische groepen werden gevonden binnen het geslacht *Hebeloma*, maar de verwantschappen *tussen* deze groepen konden niet met grote zekerheid gereconstrueerd worden. Daarom was het onmogelijk een nieuwe indeling binnen het geslacht *Hebeloma* voor te stellen.

In hoofdstuk 4 werd een monofyletische groep binnen clade II (subclade IIa) in detail bestudeerd. In deze subclade waren negen ICG's gevonden, waarvan er vier slechts gedeeltelijk incompatibel waren. Deze vier ICG's behoorden samen met een volledig

incompatibele ICG tot de door sommige taxonomen onderscheiden soorten *H. crustuliniforme* en *H. alpinum*. De partiële incompatibiliteit was hiërarchisch georganiseerd met een redelijk niveau van intercompatibiliteit tussen ICG 3 en 4 en zeer beperkte intercompatibiliteit tussen ICG 2 en 3/4 en tussen 1 en 2. De ene collectie die compatibel was met alle collecties van zowel 3 als 4 (collectie 605) behoorde ook tot subclade IIa. Er werden een mitochondriële en een nucleaire fylogenie gereconstrueerd van collecties die tot deze gedeeltelijk compatibele ICG's behoorden. De mate van incompatibiliteit tussen de verschillende ICG's werd vergeleken met de relatieve ouderdom van de meest recente gemeenschappelijke voorouder. Voor de drie ICG's 2, 3 en 4 werd er in het algemeen een positieve correlatie gevonden tussen de mate van incompatibiliteit en deze relatieve ouderdom. De leden van afzonderlijke ICG's vormden in het algemeen monofyletische groepen, ICG 3 en 4 vormden samen op hun beurt ook een monofyletische groep en de zustergroep van (3,4) was ICG 2. Dit was in overeenstemming met de voorspellingen van de hypothese dat sexuele incompatibiliteit geleidelijk ontstaat bij een toename van de genetische verschillen (divergentie-eerst). ICG 1 had in de mitochondriële fylogenie een andere positie dan in de nucleaire. In de mitochondriële fylogenie was ICG 1 de zustergroep van ICG 2, en in de nucleaire de zustergroep van ICG 5. Een mogelijke verklaring is dat ICG 1 een hybride oorsprong heeft, waarbij de voorouder van ICG 5 de nucleaire donor van 1 is, en de voorouder van ICG 2 de mitochondriële donor.

Het onderwerp van hoofdstuk 5 is een polymorfisme in de ITS van ICG 17 (clade I). Binnen ICG 17 die tot de morfologische soort *Hebeloma velutipes* behoorde was een dikaryon (d504) gevonden met twee sterk verschillende ITS-types. Monokaryotische nakomelingen van dit dikaryon hadden slechts één ITS-type, wat aantoont dat de twee ITS-types verschillende allelen zijn van homologe rDNA-loci. Een restrictie-fragment-lengte-polymorfisme-analyse (RFLP-analyse) toonde aan dat het polymorfisme wijdverspreid is. Beide types komen zowel in Amerika als Europa voor. Een cladistische analyse van de twee ITS-sequenties van ICG 17 toonde aan dat ze samen geen monofyletische groep vormen. Een van de twee ITS-types vormt een clade samen met het unieke type dat gevonden was in de partieel compatibele ICG 16 en het andere ITS-type van ICG 17 vormt een clade samen met het unieke ITS-type dat in ICG 18 gevonden was. RFLP-analyse van een mitochondrieel stukje DNA toonde aan dat er permanente verschillen zijn tussen de mitochondriën van ICG 16 en 17. Verschillende aanwijzingen duidden erop dat het ITS-

polymorfisme binnen ICG 17 niet het resultaat is van recente hybridisatie tussen de partieel compatibele ICG 16 en 17. Het polymorfisme moet daarom een andere oorsprong hebben. Het ontbreken van recombinanten, zowel binnen het rDNA-locus als tussen ITS 1 en 2, suggereerde dat de twee types pas recent zijn samengekomen in ICG 17. Het ITS-polymorfisme dat in hoofdstuk 5 werd besproken toonde duidelijk het mogelijke gevaar aan van het gebruik van een enkele ribosomale sequentie voor het reconstrueren van een soortenfylogenie. Daarnaast wees het beschreven polymorfisme duidelijk op de mogelijke problemen voor moleculaire identificatie van soorten.

In hoofdstuk 6 wordt een methode gepresenteerd om te komen tot een gemakkelijk hanteerbaar soortbegrip voor het *Hebeloma crustuliniforme*-complex. Dit soortbegrip was gebaseerd op morfologische herkenning van (combinaties van) biologische soorten met een unieke gemeenschappelijk voorouder. Cruciaal voor het toepassen van zo'n soortbegrip, is een betrouwbare reconstructie van de fylogenie van de biologische soorten. Er werd een cladistische analyse toegepast op twee verschillende nucleaire sequenties en een beste schatting van de fylogenie werd gepresenteerd. Op basis van deze fylogenie, konden slechts twee monofyletische, morfologisch herkenbare soorten binnen de 20 biologische soorten erkend worden, namelijk *H. velutipes* (clade I) en *H. helodes* (clade II). Als we het criterium van strikte monofylie verruimden tot het eveneens erkennen van parafyletische soorten (soorten die geen *unieke* gemeenschappelijke voorouder hebben), konden drie soorten erkend worden: *H. incarnatulum*, *H. velutipes* en *H. helodes*. Een stamboom waarin de vijf ICG's van de vroeger beschreven *H. crustuliniforme* (ICG's 1, 2, 3, 4 en 5) geforceerd een monofyletische groep vormden, kon statistisch niet worden verworpen. Op basis van deze 'gedwongen' boom konden met behulp van het verruimde criterium vier soorten erkend worden: *H. incarnatulum*, *H. velutipes*, *H. crustuliniforme* en *H. helodes*. Het beperkte vermogen om een biologisch-soortbegrip te vertalen naar een gemakkelijk hanteerbaar soortbegrip werd verklaard door een gebrek aan kwalitatieve morfologische kenmerken en de plasticiteit van kwantitatieve kenmerken. Op basis van de nauwe verwantschap tussen de ICG's in de twee claden van het *H. crustuliniforme*-complex, werd aannemelijk gemaakt dat het vinden van een goede overeenstemming tussen een biologisch-soortbegrip en een morfologisch-soortbegrip niet waarschijnlijk is.

In het laatste hoofdstuk werden de resultaten die in dit proefschrift beschreven staan geïntegreerd en in een breder kader geplaatst. Richtingen voor vervolgonderzoek werden

gesuggereerd. Een belangrijke aanbeveling was dat nieuwe fylogenetische studies nadrukkelijker rekening dienen te houden met de mogelijkheid van genetische uitwisseling tussen genetisch gedivergeerde populaties.

Dankwoord

Het onderzoek dat tot dit proefschrift heeft geleid heeft voor een deel plaats gevonden op het voormalige biologisch station in Wijster en voor een deel bij de vakgroep genetica in Wageningen. Hoewel beide plaatsen een geheel eigen sfeer hadden, heb ik het op beide plaatsen goed naar mijn zin gehad. Terugkijkend herinner je je vooral leuke momenten. Maar er zijn natuurlijk ook momenten geweest waarop het minder ging en waarop ik me afvroeg of het wel zou lukken. Gelukkig heb ik veel hulp en steun gevonden op verschillende terreinen, op verschillende momenten bij vele mensen.

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Last but not least I mention Birgith. Birgith, I want to thank you for your love and friendship. Jeg elsker dig.

Curriculum Vitae

Duur Aanen werd geboren op 19 maart 1971 te Hoornaar (Zuid-Holland). In 1989 werd door hem het diploma aan het gemeentelijk Gymnasium Camphusianum te Gorinchem behaald. In datzelfde jaar begon hij met de studie biologie te Utrecht. In de specialisatiefase deed hij onderzoek aan de fylogenie van soorten in het plantengeslacht *Aeonium* gebaseerd op chloroplast-DNA onder begeleiding van Ted Mes. Daarnaast werd een afstudeervak bewerkt aan de Universiteit van Amsterdam over de evolutionaire betekenis van dagelijkse verticale migratie van *Daphnia*-soorten, onder begeleiding van Prof. J. Ringelberg en Dr. B. Flik. Tijdens zijn studie schreef hij ook een scriptie over de populatiegenetische consequenties van kleine-populatiegrootte onder begeleiding van Dr. G. De Jong. Tijdens zijn studie werden twee studentassistentschappen bij de (toenmalige) projectgroep 'Genoomevolutie' vervuld. In mei 1994 studeerde hij af en begon in juli van dat jaar als Onderzoeker in Opleiding aan het onderzoeksproject 'Species and speciation in the *Hebeloma crustuliniforme* complex' dat tot dit proefschrift heeft geleid. Dit onderzoeksproject was een samenwerking tussen het toenmalige Biologisch Station Wijster (Thom Kuyper) en de vakgroep Erfelijkheidsleer van de Landbouwuniversiteit Wageningen (Rolf Hoekstra). Een deel van het onderzoek werd uitgevoerd in Wijster en een deel in Wageningen.

Na zijn aanstelling als o.i.o. heeft hij twee maanden gewerkt bij de Open Universiteit waarbij examenvragen voor de cursus Evolutiebiologie werden gemaakt. Daarnaast is hij vier maanden in dienst geweest als toegevoegd docent bij de vakgroep Erfelijkheidsleer.