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A molecular phylogeny of *Hebeloma* species from Europe

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ABSTRACT

In order to widen the scope of existing phylogenies of the ectomycorrhizal agaric genus *Hebeloma* a total of 53 new rDNA ITS sequences from that genus was generated, augmented by sequences retrieved from GenBank, and analysed using Bayesian, strict consensus and neighbour joining methods. The lignicolous *Hebelomina neerlandica*, *Gymnopilus penetrans*, and two species of *Galerina* served as outgroup taxa. *Anamika indica*, as well as representatives of the genera *Hymenogaster* and *Naucoria*, were included to test the monophyly of *Hebeloma*, which is confirmed by the results. *Hebeloma*, *Naucoria*, *Hymenogaster* and *Anamika indica* cluster in a strongly supported monophyletic hebelomatoid clade. All trees largely reflect the current infrageneric classification within *Hebeloma*, and divide the genus into mostly well-supported monophyletic groups surrounding *H. crustuliniforme*, *H. velutipes*, *H. sacchariolens*, *H. sinapizans*, and *H. radicosum*, with *H. sarcophyllum* being shown at an independent position; however this is not well supported. The section *Indusiata* divides with strong support into three groups, the position of the pleurocystidiate *Hebeloma cistophilum* suggests the possible existence of a third subsection within sect. *Indusiata*. Subsection *Sacchariolentia* is raised to the rank of section.

Introduction

The ectomycorrhizal basidiomycete genus *Hebeloma* (*Cortinariaceae*, *Agaricales*), with its main distribution in the temperate zones of the northern hemisphere, contains some 70–80 species in Central Europe. *Hebeloma* has been recognised as a group since Fries (1821) described it as a tribe of *Agaricus*. Kummer (1871) raised *Hebeloma* to the genus level, where it has since remained, with *H. fastibile* as type species (Singer 1961, 1986), the identity of which, however, is debatable (Kuyper & Vesterholt 1990; Vesterholt 2004). Singer (1986) recognised three subgenera within *Hebeloma*: *Porphyrospora*, *Myxocybe*, and *Hebeloma* and two sections within the subgenus *Hebeloma*: *Hebeloma* (species with a persisting cortina) and *Denudata* (cortina only present in the primordia). The genus *Hebeloma* belongs to the tribe *Hebelomateae* along with *Naucoria* and *Hebelomina* (Singer 1986). However, the infrageneric taxonomy remains controversial, as can be seen in the taxonomic history of the genus that is covered in detail, for example, by Aanen (1999); Boekhout (1982) and Kuyper and Vesterholt (1990). The most recent revision of the infrageneric classification is that of Vesterholt (2004).

As many *Hebeloma* species are frequently encountered in ecological, physiological, and biochemical studies, further knowledge of the existing species is desirable. Though carpophores of the genus are easily recognised in the field, species identification can be problematic, as most species look outwardly very much alike and comparatively few of the

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macroscopic characters are suitable for differentiation. It is therefore necessary in all cases to incorporate microscopic characters for species delimitation. However, morphological transitions and overlapping of differential characters such as, for example, degree of dextrinoidity of the spores, are frequently encountered. In addition, the studies of Aanen *et al.* (2000) revealed the existence of intercompatibility groups [icg, biological species *sensu* Boidin (1986)] within morphologically-defined species of *Hebeloma*.

Recent phylogenetic studies of *Hebeloma* (Aanen *et al.* 2000) using sequences of the rDNA ITS, show that this marker provides useful information for infrageneric classification. In order to extend the range of known sequences and to test the monophyly of *Hebeloma* shown, *e.g.* by Aanen *et al.* (2000) and Peintner *et al.* (2001) with a larger dataset, the ITS regions of further collections of *Hebeloma* were analysed. As Thomas *et al.* (2002) have shown the recently described genus *Anamika* to be close to *Hebeloma*, *A. indica* was included in the study to check the monophyly of *Hebeloma*, along with representatives of the *Hebeloma*/*Hymenogaster*/*Naucoria* clade of Peintner *et al.* (2001).

Materials and methods

Taxon sampling

Fresh material was collected and determined according to Bruchet (1970); Boekhout (1982); Vesterholt (1989, 1995, 2000), and Bon (2002). As many species of the investigated genera are rare or have a limited distribution, herbarium material was included to widen the range of species and the scope of geographic origin. All material was examined microscopically. Morphological characters will be covered in more detail in further papers as larger datasets are accumulated. The ITS dataset used contained a total of 107 sequences. Of these, 90 originated from *Hebeloma* spp., 53 of which were obtained in this study (Table 1), and 37 retrieved from GenBank (Table 2). Additionally, 13 sequences (six obtained in this study and seven retrieved from GenBank) belonged to further representatives of the *Cortinariaceae* that were included to test the monophyly of *Hebeloma*: *Anamika*, *Hymenogaster*, and *Naucoria*. *N. pseudoamarescens* (*Hebeloma funariophilum* M. Moser) was incorporated to test its affiliation to *Hebeloma*. *Hebelomina neerlandica*, a lignicolous species (Huijsman 1978) derived within *Gymnopilus* (Moncalvo *et al.* 2002) together with *Gymnopilus penetrans* and two sequences of *Galerina* were designated as outgroup taxa on the assumption of sufficient distance to the ectomycorrhizal genus *Hebeloma*.

Voucher specimens of our collections are preserved at the State Museum of Natural History, Görlitz, Germany (GLM). Others are located at the herbaria as indicated in Table 1.

The ITS sequences generated in this study are deposited in GenBank. Accession numbers are provided in Table 1.

DNA isolation

Nuclear DNA was isolated either from fresh carpophores or herbarium vouchers according to Štorchová *et al.* (2000) modified for fungi as follows: material was crushed in liquid nitrogen, 1.3 ml extraction buffer (0.35 M sorbitol, 0.1 M Tris–HCl pH 7.5, 5 mM EDTA, 0.1 % β-mercaptoethanol) was added directly to the powdered frozen tissue to form a suspension. The suspension was incubated for 10–20 min at room temperature and centrifuged at *ca* 4000 ***g*** for 5 min and the supernatant discarded. The pellet was resuspended in 0.3 ml extraction buffer and 0.3 ml lysis buffer (0.2 M Tris–HCl pH 7.5, 50 mM EDTA–Na_2, 2 M NaCl, 2 % CTAB). The samples were incubated for 15 min at 65 °C, then shaken with 0.6 ml chloroform: isoamylalcohol (24 : 1) and subsequently centrifuged for 10 min at *ca* 4000 ***g***. The supernatant was collected into a new microtube and 0.66 volume of cold isopropanol (−20 °C) was added, thoroughly mixed and the extracts stored at −20 °C for a minimum of 30 min. The tubes were then centrifuged at *ca* 14 000 ***g*** for 15 min, the supernatant discarded and the pellet washed in 1 ml 80 % ethanol at room temperature and centrifuged again at *ca* 14 000 ***g*** for 2 min. The supernatant was removed and the pellet briefly dried to remove the ethanol. Forty microlitres of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) were added, and the DNA rehydrated for a maximum of 1 h at 65 °C.

PCR conditions

The ITS regions (ITS 1 and ITS 2) and the 5.8 S rRNA gene were amplified in an Appligene Crocodile 3 Thermo Cycler (Appligene, Heidelberg, Germany), using the primer pairs ITS1 and ITS4 (White *et al.* 1990). PCR was performed in a total volume of 50 μl containing 2 U Taq DNA polymerase (Promega, Heidelberg, Germany), 5 μl of 10× *Taq* polymerase reaction buffer (Promega), 4 μl 25 mM magnesium chloride, 10 nmol of each dNTP (MBI-Fermentas, St Leon-Rot, Germany), 50 pmol of each of the two primers and 1 μl of the DNA extract. The reactions were performed with a hot-start PCR with 10 min initial denaturation at 94 °C before adding the *Taq* polymerase at 80 °C. The PCR programme was composed of 32 cycles (40 s at 94 °C, 30 s at 54 °C, 40 s at 72 °C). A final extension of 10 min at 72 °C followed the last cycle.

Cloning, sequencing and sequence analysis

PCR products were cloned into the pCR4-Topo Vector following the manufacturer's protocol of the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany) and transformed into TOP10 chemically competent *Escherichia coli*. Sequencing was performed on an LI-COR DNA Sequencer Long Reader 4200 using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, UK).

DNA sequences of the full ITS region were submitted to the European Molecular Biology Laboratory (EMBL) database under the accession numbers given in Table 1. Sequence length varied between 653 bp for *Hebeloma* sp. 'D' and 667 bp for *Naucoria geraniolens*. 107 ITS 1-, 5.8 S-, and ITS 2 rDNA sequences, 53 gathered in this study, were aligned manually with BioEdit version 5.0.9 (Hall 1999), resulting in an alignment including 603 putatively homologous sites.

Three methods of phylogenetic analysis were used to generate trees. The first tree (Fig 1) was generated using MrBAYES 3.0b4 (Huelsenbeck & Ronquist 2001) for Bayesian inference

Table 1 – Material used in this study

Species	Collection[a]	Collection site	Accompanied by	leg.; det.[b]	GenBank
Hebeloma					
H. aestivale	GLM 44051[d]	DK: Århus	*Quercus*	J. Vesterholt	AY308582
H. albocolossum	IB 95/57	N-Troms: Skibotndalen	*Betula pubescens*	M. Moser	AY308583
H. atrobrunneum	GLM 44050[e]	DK: Horsens NW, Lund	*Salix*	J. Vesterholt	AY308586
H. brunneifolium	L 0490421	NL-Overijssel: Olst-Wijhe	*Quercus*, *Salix*	G. & H. Piepenbroek; T. Boekhout	AY309959
H. bryogenes	C 21581	FIN-Varsinais-Suomi: Kustavi	*Pinus sylvestris*, *Picea abies*, *Betula*	J. Vauras; J. Vesterholt	AY309960
H. calyptrosporum	IB 93/53	A-Tirol: Hungerburg	*Picea*	M. Moser	AY309961
H. cistophilum	GLM 62249[f]	P-Minho:Esposende N	*Cistus salvifolius*	J. Vesterholt	DQ007992
H. cistophilum	GLM 62250[g]	I-Foggia: Mattinata, Tratturita	*Cistus monspeliensis*, *Quercus coccifera*	A. Hausknecht	DQ007993
H. collariatum	GLM 41874	D-Sachsen: Görlitz-Ludwigsdorf NW	*Quercus*	S. Hoeflich; H. Boyle & G. Zschieschang	AY309962
H. crustuliniforme sub H. alpinum	IB 97/775	A-Tirol: Innsbruck SW, Kalkkögel	*Dryas*, *Salix retusa*, *Loiseloiria procumbens*	U. Peintner; rev. J. Vesterholt	AY308584
H. cylindrosporum	GLM 34181	D-Sachsen: Kreba N	*Pinus*	R. Kießling; H. Boyle	AY309963
H. fragilipes	GLM 42703	D-Sachsen: Dahren	*Tilia*	S. Hoeflich; H. Boyle	AY309964
H. helodes f. *amoenolens*[c]	L 0490481	NL-Zuid Holland: Wassenaar	*Fagus*	T. Kops; T. Boekhout	AY311514
H. helodes v. *capitata*[c]	GLM 42707	D-Sachsen: Geheege S	*Quercus robur*	H. Boyle	AY311515
H. helodes v. *capitata*[c]	L 0490428	B-Namur: Vonêche NE	No record	T. Boekhout	AY311516
H. leucosarx	GLM 40669	D-Sachsen: Herrnhut	*Tilia*, *Carpinus*	G. Zschieschang	AY311517
H. malenconii	IB 94/419	I-Sardinia: Fontana Bona, Orgosolo	*Quercus ilex*	S. Tartarotti; M. Moser	AY311519
H. mesophaeum v. *crassipes*	GLM 43508	D-Sachsen: Görlitz-Südstadt	*Tilia*, *Betula*	S. Hoeflich; H. Boyle	AY311521
H. monticola	GLM 44052[h]	FIN-Pohjois-Karjala: Ilomantsi	No record	J. Heilman-Clausen; J. Vesterholt	AY311523
H. nigellum sub H. minus	IB 90/79	A-Tirol: Obergurgl SSE, Gaisbergtal	*Salix herbacea*	M. Moser; rev. H. Boyle, J. Vesterholt	AY311522
H. nigellum	GLM 44055[i]	DK-Grønland	No record	S. A. Elborne; J. Vesterholt	AY311524
H. oculatum	GLM 42741	D-Mecklenburg: Groß Pankow SSE	*Salix*, *Betula*	H. Boyle	AY311525
H. pallidoluctuosum	GLM 45575	D-Sachsen: Görlitz, Weinberg	*Tilia*, *Betula*	G. Zschieschang	AY311526
H. pallidum	JE	I-Sardinia: Cagliari E, Monte Cresia	*Pinus*	M. Contu	AY312976
H. polare	GLM 44054[j]	N-Svalbard: Longyearbyen	*Salix*	S. Huhtinen; J. Vesterholt	AY312977
H. populinum	GLM 41872	D-Sachsen: Charlottenhof	*Populus tremula*, *Betula*, *Pinus*, *Quercus*	S. Hoeflich; H. Boyle & G. Zschieschang	AY312978
H. populinum v. *tenuispora*[c]	L 0490451	NL-Oostelijke Flevoland, Handerbos	No record	H. v. d. Aa & J. Stalpers; T. Boekhout	AY312979
H. psammophilum	GLM 44441	D-Brandenburg: Niederlehme E	*Populus*, *Betula*, *Salix*	F. Gröger; rev. J. Vesterholt	AY308585
H. psammophilum	GLM 44053[k]	DK-NEJ: Hirtshals SW	No record	S. A. Elborne; J. Vesterholt	AY312980
H. pumilum	IB 1992/0061	A-Tirol: Matrei, Maria Waldrast	*Picea*, *Larix*	M. Moser	AY312981
H. pusillum	GLM 42941	D-Berlin: Berlin-Altglienicke	*Alnus*, *Salix*	F. Gröger	AY312982
H. remyi	IB 98/460	FIN-Lapland-N: Utsjoki, Kevosee Island	*Salix*, *Betula*	R. Pöder	AY312983
H. sacchariolens	GLM 43857	D-Thüringen: Greiz, Greizer Park	*Tilia cordata*	H. Boyle	AY312985
H. saliciphilum[c]	L 0490473	NL-Noord-Holland: Petten, Wilgendal	*Salix cinerea*	F. A. v.d.Bergh; C. Bas	AY312986
H. senescens	GLM 42680	D-Sachsen: Görlitz-Ludwigsdorf NW	*Tilia cordata*	S. Hoeflich; H. Boyle	AY312987
H. sinapizans	GLM 42554	D-Sachsen: Görlitz-Ludwigsdorf NW	*Tilia*	S. Hoeflich; H. Boyle	AY320380
H. sp. B	GLM 42698	D-Sachsen: Zentendorf NW	*Pinus*, *Quercus*, *Betula*	H. Boyle	AY320382
H. sp. D	GLM 42708	D-Sachsen: Geheege	*Betula*	H. Boyle	AY320384
H. sp. G	GLM 42711	D-Sachsen: Görlitz-Tauchritz S	*Betula*, *Quercus*	H. Boyle	AY320386
H. sp. I	GLM 43488	D-Sachsen: Görlitz	*Betula*	S. Hoeflich; H. Boyle	AY320387

(*continued on next page*)

Table 1 – (*continued*)

Species	Collection[a]	Collection site	Accompanied by	leg.; det.[b]	GenBank
H. sp. K	GLM 43503	D-Sachsen: Niesky-See	*Quercus*, *Betula*	H. Boyle	AY320388
H. sp. M	GLM 46325	D-Sachsen: Oppach	*Picea*	H. Boyle	AY320390
H. sp. N	GLM 44136	D-Mecklenburg, Groß Pankow SSE	*Salix*	H. Boyle	AY320391
H. sp., sub *H. bruchetii*	IB 95/102	A-Tirol: Timmelsjoch	*Salix retusa*	M. Moser, rev. J. Vesterholt	AY309958
H. sp., sub *H. marginatulum*	IB 95/103	A-Tirol: Timmelsjoch	*Salix retusa*	M. Moser, rev. J. Vesterholt	AY311520
H. sp., sub *H. repandum*	IB 95/70	N-Troms: Ankerlia, Kofjordsdalen	*Salix herbacea*	R. Pöder & M. Moser; rev. J. Vesterholt	AY312984
H. sp., sub *H. subsaponaceum*	IB 96/717	I-Trentino: Val di Sella	*Abies*, *Fagus*, *Picea*	U. Peintner; rev. J. Vesterholt	AY320394
H. stenocystis	JE	D-Thüringen: Wiedersbach NE	*Picea*, *Pinus*	F. Gröger	AY320392
H. subconcolor	IB 98/462	N-Finnmark: Nesseby, Karlebotn	*Salix*	R. Pöder & B. Pernfuß; R. Pöder	AY320393
H. testaceum	L 0490468	NL-Flevoland: Dronten, Roggebotzand	*Quercus*	Tj. de Cock Buning	AY320395
H. vaccinum	GLM 43968	D-Brandenburg: Kotzen S	*Salix*, *Populus*	F. Gröger	AY320396
H. versipelle	L 0490475	NL-Overijssel: Delden	*Fagus*	C. Bas	AY320397
H. vinosophyllum	L 0490435	J-Honshu, Ôtsu, Ishizue	*Pinus*	T. Hongo	AY320398
Hebelomina					
H. neerlandica	L 0490460	NL-Overijssel: Rijssen S	On coniferous wood	W. Ligterink; C. Bas	AY320399
Naucoria					
N. alnetorum	GLM 43070	D-Berlin: Berlin-Karolinenhof	*Alnus*	F. Gröger	AY277276
N. amarescens	GLM 41994	D-Sachsen: Holtendorf N	*Alnus glutinosa*	S. Hoeflich; H. Boyle	AY303581
N. geraniolens	GLM 15686	D-Thüringen: Gotha	*Salix*	F. Gröger & G. Zschieschang	AY303582
N. pseudoamarescens sub H. funariophilum	L 0490429	D-Rh.-Pfalz: Gerolstein, Felsenhof	No record, on burnt place	Tj. de Cock Buning	AY351621
N. cf. *scolecina*	GLM 37718	D-Sachsen: Kleindehsa NE	*Alnus*	S. Hoeflich; H. Boyle	AY303583
N. tantilla	IB 88/79a	N-Spitzbergen: Kongsfjorden	*Salix polaris*	M. Moser	AY303584

a Herbarium acronyms according to Index Herbariorum (Holmgren *et al.* 1990): C, Copenhagen; GLM, Görlitz; IB, Innsbruck; JE, Jena; L, Leiden.
b If leg. & det. by the same person, the name is only listed once.
c *nom. prov.*
d Dupl. ex JV 87-502.
e Dupl. ex JV 00-612.
f Dupl. ex JV 00-677.
g Dupl. ex JV unnumbered.
h Dupl. ex JV 96-104.
i Dupl. ex SAE-86 135-GR.
j Dupl. ex TUR 071425.
k Dupl. ex SAE-1488.

Table 2 – Published sequences incorporated in this study

Species	GenBank
Hebeloma	
H. birrus	AF124693
H. bulbiferum	AF124673
H. cavipes	AF124670
H. crustuliniforme icg[a] 1 dkad[b] 621	AF124665
H. crustuliniforme icg 2 dkad 627	AF124696
H. crustuliniforme icg 3	AF124708
H. crustuliniforme icg 4	AF124694
H. crustuliniforme icg 5	AF124683
H. cylindrosporum	AF124695
H. danicum	AF124675
H. edurum/H. senescens	AF124698
H. helodes icg 9 dkad 538	AF124687
H. helodes icg 10	AF124674
H. helodes icg 11 dkad 651	AF124704
H. helodes icg 19	AF124690
H. helodes icg 20	AF124709
H. helodes icg 21 dkad 666	AF124707
H. helodes icg 21 dkad 650	AF124703
H. hiemale	AF124669
H. incarnatulum	AF124684
H. lutense icg 14	AF124678
H. lutense icg 15	AF124666
H. mesophaeum	AF124692
H. pusillum icg 12[c]	AF124697
H. pusillum icg 7	AF124706
H. pusillum icg 8	AF124681
H. pusillum icg 6	AF124702
H. radicosum	AF124700
H. sacchariolens	AF124689
H. sarcophyllum	AF124715
H. sinapizans	AF124682
H. tomentosum	AF124680
H. truncatum	AF124701
H. velutipes icg 16	AF124679
H. velutipes icg 17 dkad 535	AF124667
H. velutipes icg 17 dkad 540	AF124685
H. velutipes icg 17 dkad 642	AF124686
Anamika	
Anamika indica	AF407163
Galerina	
Galerina pruinatipes	AJ585510
Galerina pseudocamerina	AJ585508
Gymnopilus	
Gymnopilus penetrans	AF325663
Hymenogaster	
H. bulliardii	AF325641
H. glacialis	AF325634
Naucoria	
N. bohemica	AF124712
N. escharioides	AF124714
N. escharioides	AF325630
N. scolecina	AF325629

a icg, Intercompatibility group, see Aanen *et al.* (2000).
b dkad, Isolate number, see Aanen *et al.* (2000).
c Erroneously in GenBank as *H. helodes* icg 12 (Aanen, pers. comm. 2003).

of phylogeny. A MCMC run with four simultaneous chains and 5,000,000 generations was performed. The general time reversible model with invariable sites and gamma shape distributed substitution rates (GTR + I + G) was chosen as a substitution model. Every 500th generation the tree with the best likelihood score was saved, resulting in 10,000 trees. The first 1000 trees without reaching a stable likelihood score were deleted. Remaining trees were condensed in a majority rule consensus tree using PAUP* 4.0b10 (Swofford 2003). Branch supports were assigned as posterior probabilities on the consensus trees. Only high support values above 0.94 are shown. Following Larget and Simon (1999) branch supports less then 0.95 using Bayesian posterior probabilities are not significant.

Second, MP analyses were performed with PAUP* using the heuristic search mode with 10 addition sequence replicates, tree bisection–reconnection branch swapping, MULTrees option on and collapse zero-length branches off. All characters were treated as unordered and equally weighted. Strict consensus trees were calculated including all MP trees. The confidence of branching was assessed using 1000 bootstrap re-samplings.

The dataset used to reconstruct the tree (Fig 2) contained 603 characters of which 346 were constant, 80 parsimony-uninformative and 177 parsimony-informative.

The third tree (Fig 3) was based on a distance criterion. A NJ analysis was conducted based on the Kimura-2-Parameter model. The confidence of branching was assessed using 1000 bootstrap re-samplings.

Results

All three analysis methods show *Hebeloma* to be monophyletic with varying degrees of support (Bayesian with 0.98 BPP, strict consensus with less than 50 % support, and neighbour joining with a bootstrap value of 80 %), using *H. neerlandica*, *Gymnopilus penetrans* and representatives of *Galerina* as the outgroup taxa, *Naucoria*, *Anamika*, and *Hymenogaster* to test the monophyly of *Hebeloma*. *N. pseudoamarescens*, which was described by Moser (1970) as *H. funariophilum*, is placed in all analyses outside *Hebeloma* on a distinct lineage.

The 5.8 S rDNA region of *Hebeloma* was identical in all sequences generated by us, with the exception of *H. stenocystis*, which differed by one nucleotide. However, this may be an artefact of the PCR or sequencing process. The integrated published sequences (Aanen *et al.* 2000) also show virtually identical 5.8 S regions, most differences there were due to ambiguous nucleotides.

Sequencing several clones of one PCR product as done for *H. cylindrosporum*, *H. leucosarx*, *H. sinapizans*, and *Naucoria alnetorum* revealed no intrastrain polymorphism. Intraspecific variation can be found depending on the species concept applied. Especially when using a wider species concept certain intraspecific variation will be detected.

Tree topology

All three trees show a similar branch pattern within *Hebeloma*, corresponding largely to the infrageneric classification as

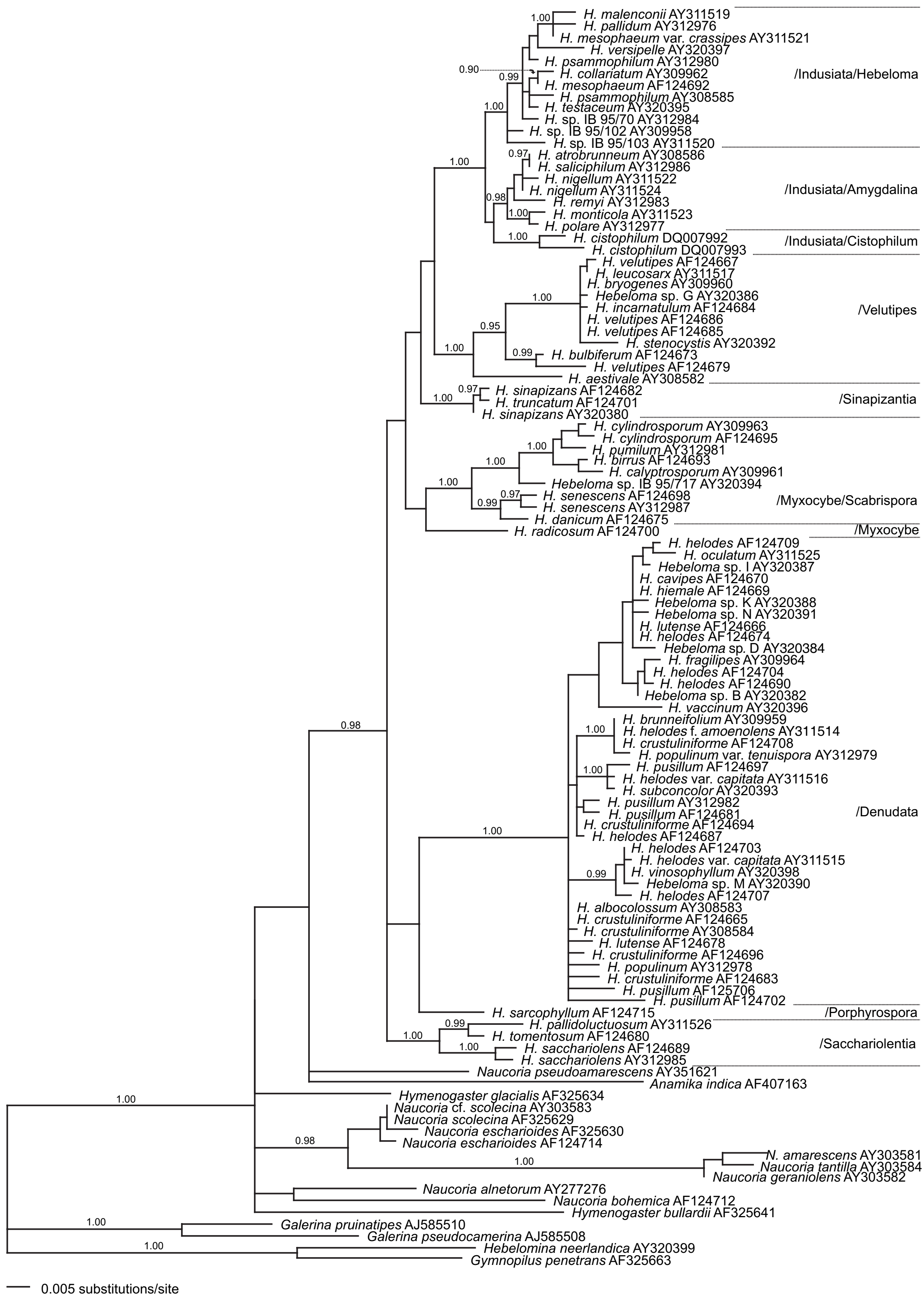

Fig 1 – Phylogenetic relationships in the genus *Hebeloma* based on ITS sequences using a Bayesian phylogenetic approach. Numbers above the branches refer to the Bayesian posterior probability of the node derived from 9000 MCMC sampled trees. *Hebelomina neerlandica*, *Gymnopilus penetrans* and two *Galerina* spp. were used as outgroup taxa; representatives of *Anamika indica*, *Hymenogaster*, and *Naucoria* were used to test the monophyly of *Hebeloma*.

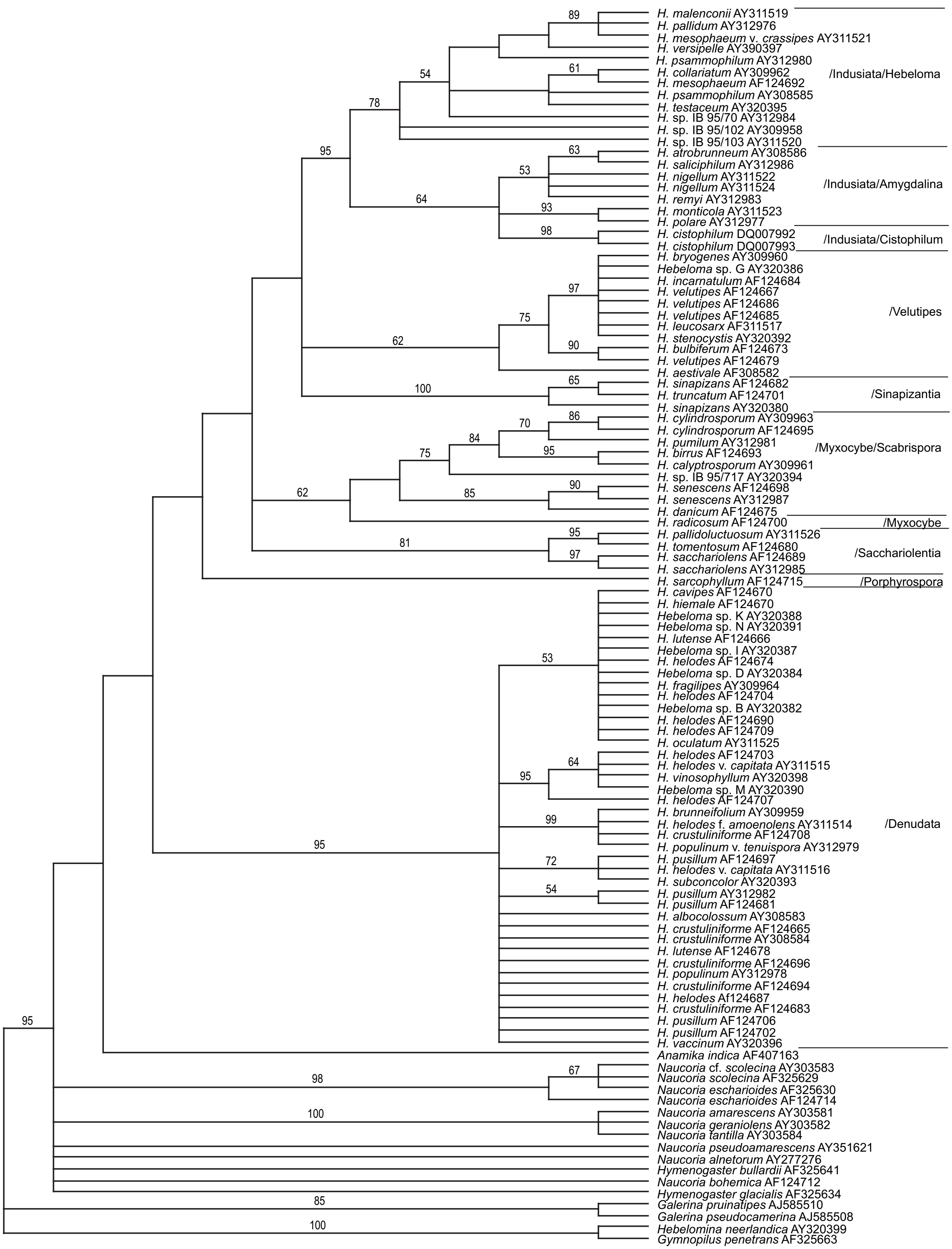

Fig 2 – Strict consensus tree of the genus *Hebeloma* based on ITS sequences. Bootstrap values (1000 resamplings) higher than 50 % are indicated above the branches. *Hebelomina neerlandica*, *Gymnopilus penetrans* and two *Galerina* spp. were used as outgroup taxa; representatives of *Anamika indica*, *Hymenogaster*, and *Naucoria* were used to test the monophyly of *Hebeloma*.

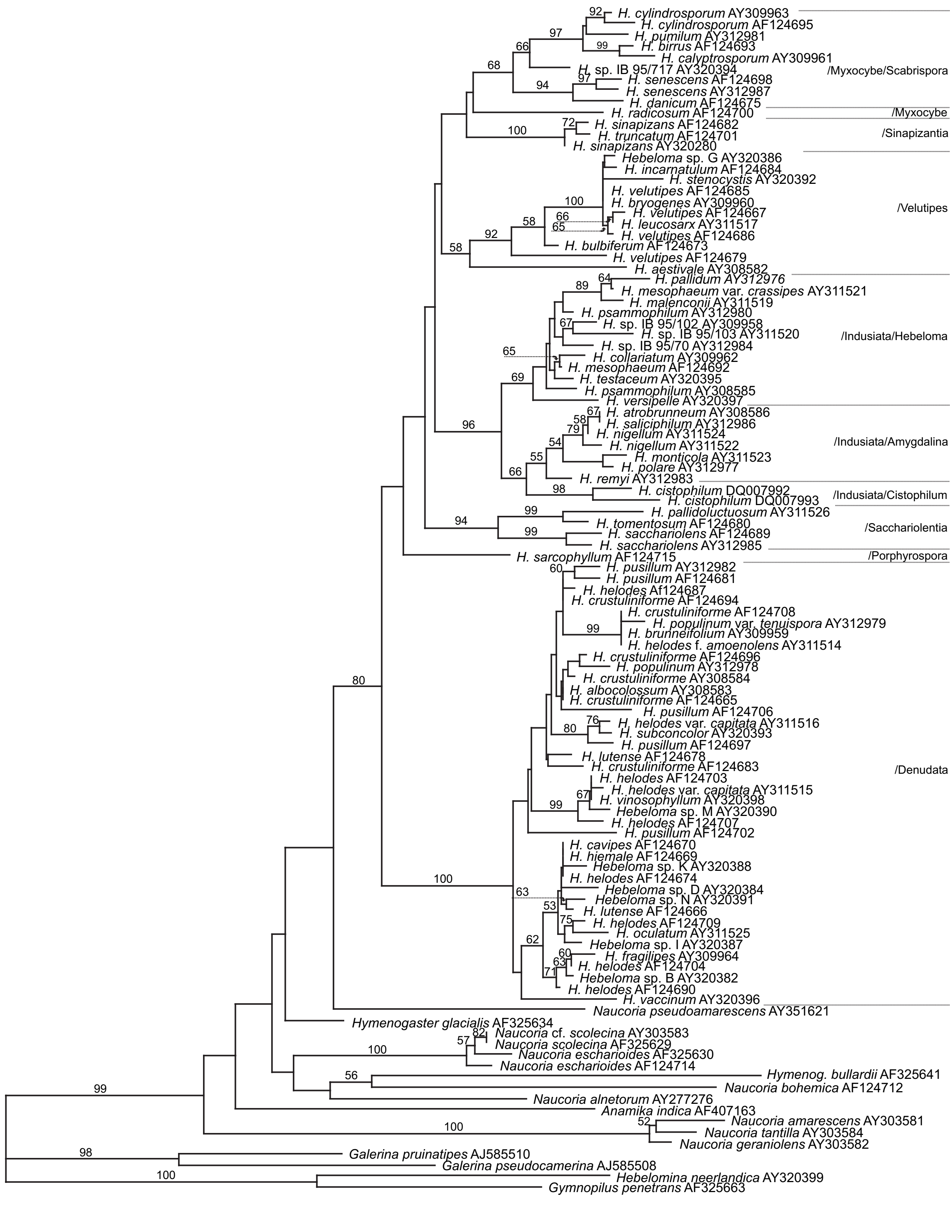

Fig 3 – Phylogenetic relationships in the genus *Hebeloma*, based on sequences of the whole ITS region. The NJ tree was obtained based on the Kimura-2 genetic distance correction. Bootstrap values (1000 resamplings) higher than 50 % are indicated above the branches. *Hebelomina neerlandica*, *Gymnopilus penetrans* and two *Galerina* spp. were used as outgroup taxa; representatives of *Anamika indica*, *Hymenogaster*, and *Naucoria* were used to test the monophyly of *Hebeloma*.

described by Vesterholt (2004), but only to a much lesser degree to that of Bruchet (1970) or Bon (1986). The major clades are therefore designated following Vesterholt (2004). The methods of analyses are abbreviated in the following as: BA = Bayesian (Fig 1), SC = strict consensus (Fig 2), and NJ (Fig 3).

The /*Indusiata* clades (support: BA 1.00 BPP, SC 95 %, NJ 96 %) consist of the veiled species and subdivide, also well supported, into three distinct clades, *viz.* /*Hebeloma* (BA 0.99 BPP, SC 78 %, NJ 69 %), /*Amygdalina* (BA 0.98 BPP, SC 64 %, NJ 66 %), and /*Cistophilum* (BA 1.00 BPP, SC 98 %, NJ 98 %). The /*Denudata* clades (support: BA 1.00 BPP, SC 95 %, NJ 100 %) consist of the *H. crustuliniforme* complex. The /*Sinapizantia* clades consist of *H. sinapizans* and *H. truncatum*, in all three analyses with 100 % support. The /*Velutipes* clades (support: BA 1.00 BPP, SC 62 %, NJ 58 %) embrace the *H. velutipes* complex. The /*Myxocybe* clades (support: BA < 0.95 BPP, SC 62 %, NJ 68 %) contain species that are known at least occasionally to form pseudorhizas (subsect. *Scabrispora*). *H. radicosum* is at a separate position within these clades (support: BA < 0.95 BPP, SC 62 %, NJ < 50%). The /*Sacchariolentia* clades (support: BA 1.00 BPP, SC 81 %, NJ 94 %) encompass the *H. sacchariolens* group. The /*Porphyrospora* clades contain only *H. sarcophyllum*. This single lineage was placed at different positions within the phylogenetic trees without any support.

Outgroup and other taxa

All three analysis methods depict *Naucoria* and *Hymenogaster* with differing degrees of resolution as paraphyletic genera. However, together with *Hebeloma*, they form a highly supported monophyletic clade (BA 1.00 BPP, SC 95 %, NJ 99 %) as similarly shown by Peintner *et al.* (2001). Thomas *et al.* (2002) showed a monophyletic clade containing *Hebeloma*, *Naucoria*, and *Anamika indica*. Our results are consistent with this and reflect the proximity of all four genera in a hebelomatoid clade.

Bayesian analysis places *A. indica* and *N. pseudoamarescens* at independent positions on a branch adjacent to *Hebeloma*, though without support. The strict consensus tree places *A. indica* proximate to *Hebeloma*, while *N. pseudoamarescens* is at an unresolved position within *Naucoria*. Neither of these positions attains bootstrap support. NJ places *N. pseudoamarescens* proximate to *Hebeloma* and *A. indica* at an independent position within *Naucoria*, these relationships also without support.

Discussion

The 5.8 S region is highly conserved within the genus *Hebeloma*. However, the flanking ITS regions show a certain degree of variability, though a high resolution at the species level was not always apparent. Close sequence similarity of many morphologically defined species within our clades indicates that the assertion of Aanen *et al.* (2000) that the *Hebeloma crustuliniforme* complex, *i.e.*, those species belonging to the section *Denudata*, subsection A of Bruchet (1970), is at present undergoing speciation can be generally considered applicable to the rest of the genus as well. Aanen and Kuyper (2004) demonstrated the difficulty of finding a serviceable correspondence between a morphological and a biological species concept within the *H. crustuliniforme* complex.

Monophyly of Hebeloma

The monophyly of *Hebeloma* shown here is in accordance with earlier findings based on ITS (*i.e.* Aanen *et al.* 2000; Peintner *et al.* 2001). The placement of the genera *Hebeloma*, *Naucoria*, *Hymenogaster*, and *Anamika* in a large monophyletic clade suggests that *Anamika*, along with *Hymenogaster*, should be included in the tribe *Hebelomateae*. Conversely, *Hebelomina*, at least *Hebelomina neerlandica* by virtue of its derivation within *Gymnopilus* (Moncalvo *et al.* 2002), should be excluded from the tribe *Hebelomateae*. *Naucoria* appears in all three analyses to be paraphyletic, indicating a necessity for further studies on the status of that genus. Although Peintner *et al.* (2001) detected only two paraphyletic clades within *Naucoria* our study indicates at least four clades/lineages containing sequences of *Naucoria*. However, all three analyses depict it close to *Hebeloma*. Nevertheless, the sequences differed sufficiently from those of *Hebeloma* to suggest that *Naucoria* should still probably better be treated as a separate genus, rather than fusing it with *Hebeloma* as proposed by Kühner (1980). This separation is also morphologically supported by differences in the pileipellis structure between *Hebeloma* and *Naucoria* (*e.g.* Singer 1986).

Monophyletic groups within Hebeloma

The /Indusiata *clade*

The /*Indusiata* clade largely reflects the division by Vesterholt (1989) of the section *Indusiata* into the subsections *Hebeloma* (with non-dextrinoid, ovoid to ellipsoid spores) and *Amygdalina* (with ± dextrinoid, ovoid to amygdaloid spores). One exception within the clade /*Amygdalina* is the species pair *H. monticola*/*H. polare*. However, it seems that this is a further example showing that separating characters such as spore shape have probably developed several times independently, or perhaps have been partially lost as suggested by Aanen *et al.* (2000) for the *H. crustuliniforme* complex. The position of *H. cistophilum*, a pleurocystidiate species (Heykoop & Esteve-Raventós 1997), may indicate the existence of a third subsection within *Indusiata*.

H. saliciphium, a provisionally named collection made in 1969 by C. Bas, exhibits an identical sequence to that of *H. atrobrunneum*, which was first described 20 years later. We consider the *H. saliciphilum* collection to represent *H. atrobrunneum*, as it also corresponds morphologically to the original description of that species by Vesterholt (1989). Both of these are shown to be quite close to *H. nigellum*. *H. monticola* forms a subclade with *H. polare* while *H. remyi*, probably synonymous with *H. monticola* (Vesterholt 1989), appears fairly distant from the latter differing by a total of 10 nucleotides. It cannot be excluded that the voucher labelled *H. remyi* may represent some other closely related *Hebeloma*, as several of the species in sect. *Amygdalina* differ only slightly in their microscopic characters and differentiation is mainly based on macroscopic features of fresh fruit bodies (Vesterholt 1989).

Hebeloma sp. IB 95/103 *sub H. marginatulum* and *H.* sp. IB 95/102 *sub H. bruchetii* appear as sister species in the NJ tree, but this relationship is not well resolved in the BA and SC trees. *Hebeloma* sp. *sub H. repandum* differs from *H.* sp. *sub H. bruchetii* by a total of eight nucleotides, and therefore, the voucher examined here may indeed represent a separate species having slightly smaller spores than the *H.* sp. *sub H. bruchetii* collection.

The distinction shown between the *H. psammophilum* collection of Vesterholt and that of Gröger *sub H. ammophilum* (Bon, *non* Bohus) was quite unexpected, as both exsiccates were microscopically virtually indistinguishable. This is interpreted as further evidence of the existence of cryptic species within the *Indusiata*.

The epithet *versipelle* is regarded by Vesterholt (1989) as a *nomen confusum* and we agree with this view. The specimen examined here appears to represent a distinct entity, though it is morphologically hardly separable from *H. mesophaeum*, with which *H. versipelle sensu* Romagnesi is considered to be synonymous. We have not yet been able to investigate *H. subcaespitosum*, which is considered synonymous with *H. versipelle sensu* Konrad & Maublanc and has also been listed as a synonym for *H. collariatum* by Vesterholt (1989). The epithet *testaceum* is illegitimate (Vesterholt 1989) and is only used here as the studied voucher, which represents an entity close to *H. mesophaeum*, was thus labelled.

H. mesophaeum var. *crassipes* appears clearly distant to *H. mesophaeum* var. *mesophaeum*, and its position indicates that it may represent a distinct species close to *H. malenconii*, though it is microscopically indistinguishable from *H. mesophaeum*. Prior to its renaming by Vesterholt (1989) the var. *crassipes* was treated at the species level and known as *H. fastibile*, the type species of *Hebeloma*. *H. pallidum*, synonymous with *H. mesophaeum* var. *lacteum*, may also represent a distinct species.

The /Myxocybe *clade*

The pseudorhiza-forming species group together in all three trees, with *Hebeloma radicosum* at a basal position in those clades. This gives support to Vesterholt (1989, 2004) who assigned the 'rooting' species to the section *Myxocybe*, which had been originally erected by Fayod (1889) as a monotypic subgenus for *Agaricus radicosus* (*H. radicosum*).

All trees infer that *H. pumilum* and *H. birrus* are probably not conspecific as considered by Gröger (1987) on the basis of close morphological similarities. *H. birrus* is depicted as a sister species to *H. calyptrosporum*. *H. danicum* also clusters separately from these as a sister species to *H. senescens*, with strong support. These results reflect the existence of a number of entities in this group in spite of the difficulties encountered in morphological differentiation.

The /Sinapizantia *clade*

The strong support and the independent position of the clade containing *Hebeloma sinapizans* and *H. truncatum* substantiate their classification as a group in their own right as, *e.g.*, proposed by Vesterholt (2004), who transferred this group to section level.

The /Porphyrospora *clade*

The separate position of *H. sarcophyllum* appears not to support Vesterholt's conclusion that this species should, along with the pseudorhiza-forming species, also belong to sect. *Myxocybe* and that the monotypic section *Porphyrospora* is synonymous with *Myxocybe* (Vesterholt 1989). It rather agrees with the classification by Bruchet (1970), who placed *H. sarcophyllum* in sect. *Porphyrospora*, the most conspicuous characteristic of which is the reddish spore colour. We propose that this section be maintained.

The /Sacchariolentia *clade*

The *H. sacchariolens* group, which is characterised by the saccharine-like odour of the carpophores, is shown in all three trees as a strongly supported clade of its own. Bruchet's (1970) classification as *Denudata* Subsection B, placing these species together with *H. sinapizans*, *H. truncatum*, and others appears artificial. In view of the distinct phylogenetic position of this group, we propose to raise the subsection *Sacchariolentia* to section level.

The following new combination is proposed:

Hebeloma sect. Sacchariolentia (J. E. Lange ex M. Bon) H. Boyle, **comb. nov.**

Basionym: *Hebeloma* subsect. *Sacchariolentia* J. E. Lange ex M. Bon, *Docums mycol*. **17** (65): 52, 1986. Type species: *Hebeloma sacchariolens* Quél. 1880 (1879).

The /Denudata *clade*

There is no high resolution within the /*Denudata* clade, though it is possible to recognise differences between most morphologically defined entities. This clade basically corresponds to clade II (a–d) of Aanen *et al.* (2000), augmented by the addition of *ca* 20 further species.

Hebeloma cavipes and *H. hiemale* show identical sequences. *H. helodes* icg 10 [biological species *sensu* Aanen *et al.* (2000); Aanen and Kuyper (2004)] and *H. leucosarx sensu auct. neerl.* (Syn. *H. lutense*) icg 15 differ from these only by two ambiguous bases each. The virtually identical ITS sequences of *H. cavipes* and *H. lutense* reinforce the argument of Vesterholt (1995) that these species are probably synonymous. *H. hiemale* is a poorly understood and rarely collected species. Boekhout (1982) studied five historical collections from the herbarium Bresadola and found them to be very close to *H. helodes* in their microscopic characters. The sequences are a further indication of this proximity.

The identical sequences of the microscopically inseparable *H. albocolossum* and *H. crustuliniforme* (icg 1) suggest that these are probably conspecific. *H. brunneifolium*, *H. helodes* f. *amoenolens* and one collection of *H. crustuliniforme* (icg 3) also display identical sequences, which, particularly in the case of *H. brunneifolium*, was unexpected. This species has, in contrast to the latter two, strongly ornamented, calyptrate and dextrinoid spores. The dextrinoid-spored *H. vaccinum* also appears in the /*Denudata* clade but at a distinct position in BA and NJ and unresolved with SC. This was, as with *H. brunneifolium*, not anticipated.

The scattered distribution of *H. helodes* icgs within the trees was attributed by Aanen and Kuyper (2004) to the rather wide circumscription of this species. We agree with this observation and find further confirmation in that the majority of our (as yet) unnamed collections also cluster here, though none of these appear to be a 'classical' *H. helodes*.

Both collections of *H. helodes* var. *capitata* fit morphologically to the original description by Boekhout (1982), however, they cluster at different positions. Boekhout (1982) questioned whether this variety could be identical with *H. leucosarx sensu auct. neerl*. (syn. *H. lutense*) and subsequently confirmed this after studying the type of that species (Boekhout, pers. comm., 2000). The two icgs of *H. lutense* from Aanen *et al.* (2000) cluster, however, clearly at different positions to *H. helodes* var. *capitata*. This also should probably be interpreted as a consequence of the wide morphological circumscription within this entire group.

H. vinosophyllum is depicted by BA and NJ to be identical to *H. helodes* icg 21, the ITS sequence of which only differs from that of *H. vinosophyllum* by two ambiguous bases. As *H. vinosophyllum* is an ammonium fungus (*e.g.* Sagara 1995, Tibbett & Carter 2003), it could be of interest to test the physiological ecology of *H. helodes* icg 21 and other species within that group.

The separation of the two *H. pusillum* groups and the comparatively large difference between the icgs 6 and 7 (18 bp) signify that the status of *H. pusillum* should be further investigated.

*The /*Velutipes *clade*

There is no high resolution within the /*Velutipes* clade, though, as with the /*Denudata* clade, it is possible to recognise differences between most morphoentities. These subclavate-cystidiate, dextrinoid-spored species cluster together but distinctly separate from the section *Denudata* in the classical sense. This supports the classification at section level by Vesterholt (2004).

Hebeloma aestivale, a recently described and certainly distinctive species forms the basal lineage of this entire group in all three trees. Three specimens have identical sequences, *viz*. *H. bryogenes* and two collections by Aanen of *H. velutipes* icg 17 numbered DKAd 540 and 642. The latter differs in having three ambiguous nucleotides, here only reflected in the NJ tree. *H. incarnatulum* differs from *H. bryogenes* merely by one nucleotide in ITS 1 and is considered by Aanen (pers. comm., Aug. 2002) to be synonymous with that species. Both are, in contrast to *H. velutipes*, thus far known to be restricted to conifers.

The relationships within *H. velutipes* were discussed by Aanen *et al.* (2000) and Aanen and Kuyper (2004). Aanen *et al.* (2000) reported polymorphisms in the ITS of various *H. velutipes* strains. Taking this into consideration in view of the similarity of the sequences, it may be appropriate to interpret this very common species in a broad sense, incorporating the species concepts of Boekhout (1982) and Vesterholt (1995, 2000), and perhaps even including *H. bryogenes*.

The holotype of *H. leucosarx* P. D. Orton 1960 must be studied in order to resolve the difference between *H. leucosarx sensu auct. neerl*. (syn. *H. lutense*) and *H. leucosarx sensu* Vesterholt (1995, 2000), which our results indicate to represent *H. velutipes*.

H. stenocystis, *H. bulbiferum* and *H. velutipes* icg 16 differ most from the rest of this group. *H. stenocystis* is rarely found and thus poorly investigated. It is considered by Boekhout & Kuyper (1999) to probably belong to the *H. velutipes* complex. The voucher we used, collected under conifers, closely resembles *H. bryogenes* both outwardly and microscopically and should also perhaps be included in the abovementioned broad species concept of *H. velutipes*. *H. bulbiferum* and *H. velutipes* icg 16 are depicted as intermediate between *H. aestivale* and the rest of the group in all three trees. In spite of close general morphological similarity within this group (with the exception of *H. aestivale*) considerable ITS polymorphism is evident.

Conclusions

The ITS sequences, especially within the /*Denudata* and /*Velutipes* clades differ in a number of cases only by few nucleotides. Considering the potential variability of the ITS region, this situation can lead the conclusion that the ITS within *Hebeloma* is, due to the recent and current process of speciation, not yet divergent enough to reflect the morphological specific differences. The biological species found by Aanen *et al.* (2000) are indicative of this.

Aanen *et al.* (2000) laid a solid foundation for molecular studies in *Hebeloma*. We have been able to add a considerable number of species to this framework and to show that there are well-defined distinct groups within the genus. We agree with and support the statement of Aanen *et al.* (2000) that inference of phylogenies on the basis of single markers (single gene trees) should be taken with caution, especially in areas where strong support is lacking. Future studies will have to incorporate a number of further markers in multigene analyses to elucidate the interspecific relationships within *Hebeloma*. It would also be desirable in that case to sample a large number of collections of the respective species in order to generate a basis for comparison of polymorphisms within the DNA regions used.

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