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Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential*

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Within the basidiomycetes, the vast majority of known mycorrhizal species are homobasidiomycetes. It was therefore surprising when molecular and ultrastructural studies revealed a broad diversity of mycorrhizal associations involving members of the heterobasidiomycetous *Sebacinaceae*, fungi which, due to their inconspicuous basidiomes, have been often overlooked. To investigate the phylogenetic position of the *Sebacinaceae* within the basidiomycetes and to infer phylogenetic relationships within the *Sebacinaceae*, we made molecular phylogenetic analyses based on nuclear rDNA. We present a well-resolved phylogeny of the main lineages of basidiomycetes which suggests that the *Sebacinaceae* is the most basal group with known mycorrhizal members. Since more basal taxa of basidiomycetes consist of predominantly mycoparasitic and phytoparasitic fungi, it seems possible that a mycorrhizal life strategy, which was transformed into a saprotrophic strategy several times convergently, is an apomorphic character for the *Hymenomycetidae*. Mycorrhizal taxa of *Sebacinaceae*, including mycobionts of ectomycorrhizas, orchid mycorrhizas, ericoid mycorrhizas, and jungermannioid mycorrhizas, are distributed over two subgroups. One group contains species with macroscopically visible basidiomes, whereas members of the other group probably lack basidiomes. *Sebacina* appears to be polyphyletic; current species concepts in *Sebacinaceae* are questionable. *Sebacina vermifera sensu* Warcup & Talbot consists of a broad complex of species possibly including mycobionts of jungermannioid and ericoid mycorrhizas.

This wide spectrum of mycorrhizal types in one fungal family is unique. Extrapolating from the known rDNA sequences in *Sebacinaceae*, it is evident that there is a cosm of mycorrhizal biodiversity yet to be discovered in this group. Taxonomically, we recognise the *Sebacinaceae* as constituting a new order, the *Sebacinales*.

INTRODUCTION

Molecular phylogenetic studies (Weiß & Oberwinkler 2001) have revealed that the heterobasidiomycetous family *Sebacinaceae* does not belong to the *Auriculariales*, a group of wood-decaying fungi in which it had been placed mainly on the basis of ultrastructural and microscopical characters (Bandoni 1984). This was a surprise since species of the *Sebacinaceae* are morphologically very similar to members of the *Auriculariales* with which they share, for example, the longitudinally septate basidia. Subsequently a growing number of DNA sequences derived from plant roots were published that can be assigned to the *Sebacinaceae*; it became evident that members of this family are involved in a wide spectrum of mycorrhizal types: ectomycorrhizas (Glen *et al.* 2002, Selosse, Bauer & Moyersoen 2002, Tedersoo *et al.* 2003, Urban, Weiß & Bauer 2003), orchid mycorrhizas (McKendrick *et al.* 2002, Selosse *et al.* 2002, Taylor *et al.* 2003), ericoid mycorrhizas (Allen *et al.* 2003), and even in jungermannioid mycorrhizas, an only recently described association with liverworts (Kottke *et al.* 2003). Up to then in heterobasidiomycetes a mycorrhizal potential was only known from some taxa that occur as orchid symbionts (Rasmussen 2002). The broad diversity of mycorrhizal strategies present in *Sebacinaceae* is unique. This study presents the results of comprehensive molecular phylogenetic analyses using the nuclear gene for the ribosomal large subunit (nrLSU) that

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shed light on the ecology and evolution of a fascinating group of fungi whose striking biodiversity and ecological importance has only recently started to be recognised.

MATERIAL AND METHODS

Sample sources, DNA extraction, PCR and sequencing

DNA sequences that were determined for this study were obtained from fungal herbarium specimens, from mycorrhizas of different types, or from axenic fungal cultures. Extraction of genomic DNA, PCR amplification and sequencing of the nuclear coded D1/D2 region of the ribosomal large subunit was performed as described elsewhere: Weiß & Oberwinkler (2001) for dried reference collections and axenic fungal cultures, Selosse *et al*. (2002) for the mycorrhizas of the terrestrial orchids *Epipactis helleborine* and *E. microphylla*, and Selosse *et al*. (2002) and Urban, Weiß & Bauer (2003) for ectomycorrhizas. Provenance and plant hosts of the mycorrhizal samples are indicated in Fig. 2. We were pleased to be able to include in this study some of the original Warcup *Sebacina vermifera* strains, mostly isolated from Australian orchids (Warcup 1988); details of these strains are provided in Table 1.

Table 1. Strains of *Sebacina vermifera* included in this study. These strains were isolated from plant roots, grown in pure culture, and determined by induction of their basidial stages by J. H. Warcup (Warcup 1988).

Warcup isolate no.	GenBank accession no. (nrLSU)	Host plant
140	AY505548	*Eriochilus scaber* (*Orchidaceae*)
714	AY505549	*Eriochilus scaber* (*Orchidaceae*)
723	AF291366	*Cyrtostylis reniformis* (*Orchidaceae*)
750	AY505550	*Caladenia catenata* (*Orchidaceae*)
768	AY505551	*Glossodia minor* (*Orchidaceae*)
914	AY505552	*Phyllanthus calycinus* (*Euphorbiaceae*)
915	AY505553	*Caladenia catenata* (*Orchidaceae*)
963	AY505554	*Microtis uniflora* (*Orchidaceae*)
977	AY505555	*Microtis uniflora* (*Orchidaceae*)

Phylogenetic analysis

These sequences were analysed together with sequences already available in GenBank (http://www.ncbi.nlm.nih.gov/); accession nos are given in Figs 1 and 2. We analysed two data sets: (1) 65 sequences covering the major groups of basidiomycetes to estimate the phylogenetic placement of the *Sebacinaceae*; and (2) 107 sebacinoid sequences to elucidate phylogenetic relationships within the *Sebacinaceae*. Alignments were constructed using CLUSTAL X (Thompson *et al*. 1997) and manually edited with Se-Al (Rambaut 1996). Ambiguous alignment positions were excluded from the phylogenetic analyses.

To estimate phylogenetic relationships, alignments were analysed using a Bayesian approach based on Markov chain Monte Carlo (MCMC) as implemented in MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001). With this method it is possible to estimate *a posteriori* probabilities for the monophyly of given groups, i.e. the probability that a group is monophyletic given the DNA alignment.

For each alignment we ran four incrementally heated simultaneous Monte Carlo Markov chains over ten million generations using the general time-reversible model of DNA substitution, additionally assuming a percentage of invariable alignment sites with gamma-distributed substitution rates of the remaining sites (GTR + I + G; see Swofford *et al*. 1996), and random starting trees. Trees were sampled every 100 generations resulting in an overall sampling of 100 000 trees, from which the last 60 000 trees were used to compute a 50 % majority rule consensus tree to get estimates for the posterior probabilities. Stationarity of the chains was controlled using the Tracer software, version 1.0 (Rambaut & Drummond 2003). With the same software we calculated mean values for the parameters of the DNA substitution model that were sampled during the MCMC process (again using the last 60 000 samples). These mean values were then used to estimate branch lengths of the consensus trees with PAUP 4.0b10 (Swofford 2002) *via* maximum likelihood.

To avoid possible pitfalls of the MCMC approach (Huelsenbeck *et al*. 2002), we repeated the MCMC analysis for each alignment four times, always starting with random trees. We also performed neighbour-joining analyses (NJ; Saitou & Nei 1987) using Kimura 2-parameter distances (Kimura 1980), combined with non-parametric bootstrap analyses (Felsenstein 1985) in PAUP.

RESULTS AND DISCUSSION

The results of the different runs of MCMC on the same alignment yielded very similar results, only differing slightly in posterior probability values. Stationarity of the Markov chains was obviously reached before 10 000 trees (alignment 1) and 20 000 trees (alignment 2) had been sampled. Figs 1 and 2 each present the consensus of one of the four MCMC analyses performed on each alignment. The results of the bootstrapped NJ analyses (data not shown) were widely consistent to the results of the MCMC analyses, i.e. groups supported by bootstrap values exceeding 50 % were generally not in conflict with groupings obtained by MCMC. An exception is the placement of *Urediniomycetes* and *Ustilaginomycetes* that appeared as sister groups in the NJ analysis. Bootstrap support was generally lower in the NJ analyses than the corresponding posterior probabilities inferred from MCMC analyses.

Basidiomycete phylogeny

Compared to other published molecular phylogenetic hypotheses concerning higher-level relationships in

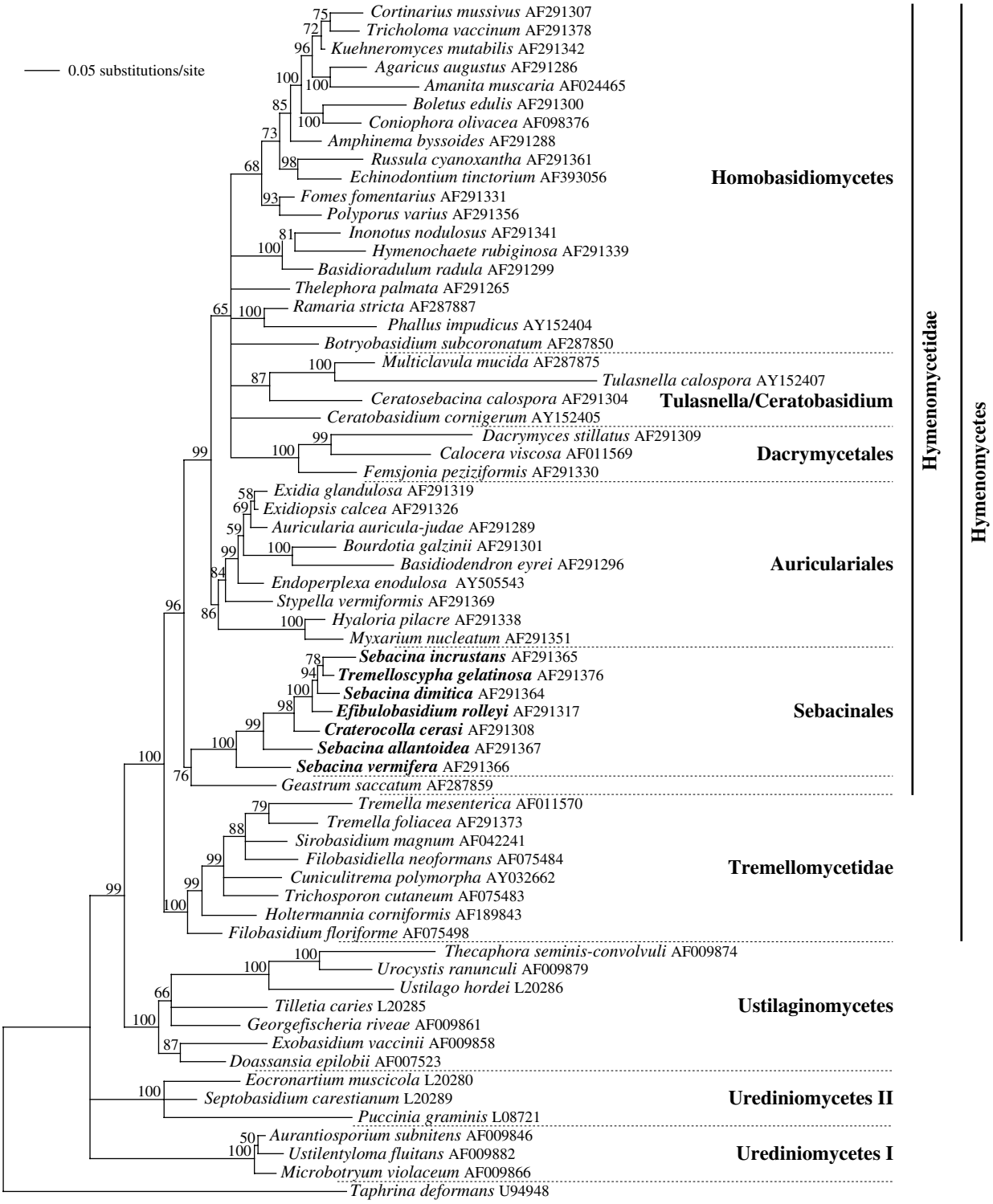

Fig. 1. Phylogenetic placement of *Sebacinales* within the basidiomycetes: Bayesian Markov chain Monte Carlo analysis of an alignment of nuclear DNA sequences from the D1/D2 region of the large ribosomal subunit. The topology was rooted with the ascomycete *Taphrina deformans*. Numbers on branches are estimates for *a posteriori* probabilities that the respective groups are monophyletic given the data. For more details see the text.

basidiomycetes (e.g. Swann & Taylor 1993, 1995, Gargas *et al*. 1995, Begerow, Bauer & Oberwinkler 1997) the present MCMC analysis of alignment 1 (Fig. 1) offers a high resolution, especially in the backbone of the phylogenetic tree. Thus, with a posterior probability of 99%, rust fungi and their relatives (*Urediniomycetes* I and II in Fig. 1) are basal in the basidiomycetes. Smut fungi (*Ustilaginomycetes*) and *Hymenomycetes* together form a monophyletic group, possibly with the type B secondary structure of the 5S rRNA as an apomorphy (Gottschalk & Blanz 1985).

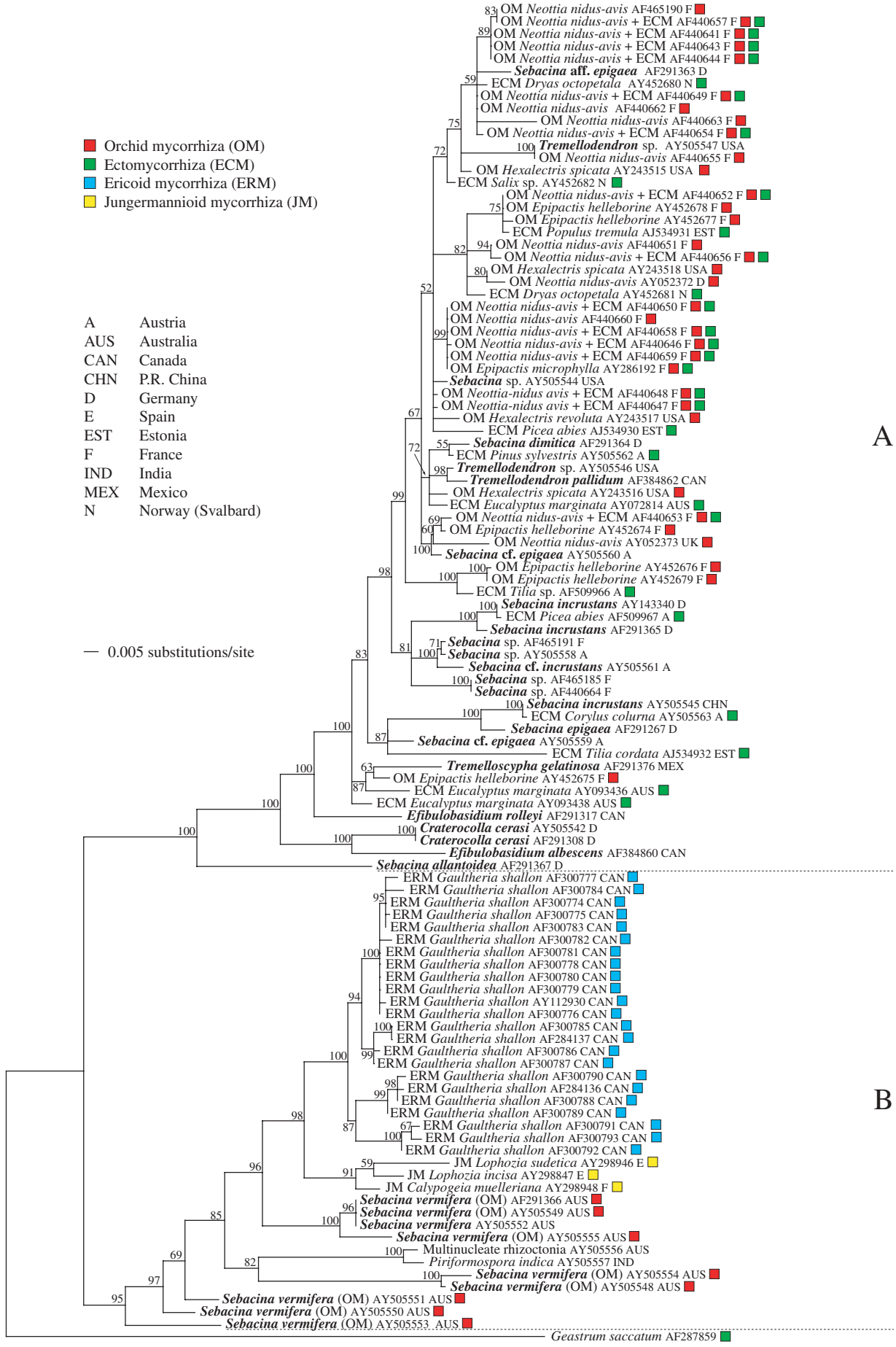

Fig. 2. For legend see opposite page.

Our study confirms that it is difficult to separate homobasidiomycetes, as currently defined, from other hymenomycetous taxa such as *Tulasnellales* and *Ceratobasidiales* (Hibbett & Thorn 2001, Weiß, Bauer & Begerow 2004). In our analysis, *Tulasnella calospora* groups as highly supported with the lichen-forming homobasidiomycete *Multiclavula mucida*, representing the cantharelloid clade *sensu* Hibbett & Thorn (2001). This position is consistent with other molecular phylogenetic analyses (e.g. Bruns *et al*. 1998, Hibbett, Gilbert & Donoghue 2000, Bidartondo *et al*. 2003). Also with respect to other homobasidiomycetous clades, our results are consistent with clades recognised in Hibbett & Thorn (2001), with one exception: *Geastrum* was separated from the gomphoid-phalloid clade, which in our analysis is represented by species of *Ramaria* and *Phallus*, and appears as a sister taxon of the *Sebacinaceae* (see the discussion below).

Phylogenetic position of the Sebacinaceae

With a high posterior probability, the *Sebacinaceae* occupy a basal position within the *Hymenomycetidae*, with *Geastrum* as a sister group (Fig. 1). A close phylogenetic relationship between the *Sebacinaceae* and *Geastrum* has recently also been found in another molecular phylogenetic analysis of nrLSU D1/D2 sequences (Taylor *et al*. 2003). Considering that *Geastrum* is also capable of forming mycorrhizas (Agerer & Beenken 1998), and the more basal groups in basidiomycetes mainly include mycoparasitic and plant parasitic fungi (Weiß, Bauer & Begerow 2004), we hypothesise that the common ancestor of this *Geastrum*/*Sebacinaceae* clade, or even the common ancestor of the whole group of the *Hymenomycetidae*, was ectomycorrhizal. If this assumption of an apomorphic mycorrhizal status in *Hymenomycetidae* holds, then the distribution of mycorrhizal taxa within the homobasidiomycetes could be explained by multiple independent origins of saprotrophism rather than by convergent evolution of mycorrhizas, in contrast to current hypotheses on the evolution of ectomycorrhizas in basidiomycetes (e.g. Hibbett, Gilbert & Donoghue 2000).

Regarding the phylogenetic position of the *Sebacinaceae* within the basidiomycetes (Fig. 1), which is corroborated by the ecological data at hand, it is appropriate to establish a new basidiomycetous order:

Sebacinales M. Weiß, Selosse, Rexer, A. Urb. & Oberw., **ordo nov.**

Fungi Hymenomycetum. Basidia longitudinaliter septata. Septa doliporis parenthesomatibus imperforatis, efibulata. Cystidia nulla.

Typus ordinis: *Sebacinaceae* Oberw. & K. Wells 1982 (*in* Wells & Oberwinkler 1982: 329).

This new order can be morphologically separated from species of *Auriculariales*, in which the *Sebacinaceae* has been placed up to now (Bandoni 1984), by a combination of longitudinally septate basidia, imperforate parenthesomes at the septal pores, and a lack of both clamp connections and cystidia. The lack of cystidia has to be included in this diagnosis since *Endoperplexa enodulosa*, a species which according to our molecular phylogenetic analyses does not belong to the *Sebacinales* (Fig. 1), differs from our description of *Sebacinales* only in the presence of cystidia (Roberts 1993).

Consequently, the order *Auriculariales* has to be emended to include saprotrophic hymenomycetes with septate basidia and septa with imperforate parenthesomes, where cystidia are present in species that lack clamp connections. Unfortunately, this emendation is not perfectly congruent with our molecular phylogenetic analysis. It is, in the literal meaning of the word, an improvement in the exclusion of *Sebacinales*, but not the ultimate solution, as there are taxa such as *Ceratosebacina calospora* or *Exidiopsis gloeophora* that fit the emended concept of *Auriculariales*, but obviously should be excluded from that group according to molecular phylogenetic results (Fig. 1; Weiß & Oberwinkler 2001). At the moment, we see no way to solve this problem. Hopefully other characters will be detected in the future that will allow a more elegant morphological circumscription of both *Auriculariales* and *Sebacinales*.

Phylogenetic relationships within Sebacinales

The MCMC hypothesis of phylogenetic relationships in the *Sebacinales* shows a division into two subgroups referred to here A and B (Fig. 2). Group A contains all the sequences obtained from basidiomes, from ectomycorrhizas, and sebacinoid mycobionts of the orchids *Neottia nidus-avis*, *Epipactis* and *Hexalectris* (i.e. of at least partly heterotrophic orchids; Taylor *et al*. 2003, Selosse *et al*. 2004). *Sebacina* and *Tremellodendron* appear to be polyphyletic.

Subgroup B contains in basal positions various sequences of *Sebacina vermifera* that were obtained from axenic fungal cultures, mostly originating from the roots of green, autotrophic Australian orchids (Warcup 1988; Table 1). The three sebacinoid sequences from liverwort rhizoids included in this study (Kottke *et al*. 2003) appear as a monophyletic group, which according to our MCMC analysis (Fig. 2) represents the sister group to the one containing the sebacinoid rDNA sequences from ericoid mycorrhizas

Fig. 2. Phylogenetic relationships within *Sebacinales*: Bayesian Markov chain Monte Carlo analysis of an alignment of nuclear DNA sequences from the D1/D2 region of the large ribosomal subunit. The topology was rooted with *Geastrum saccatum*. Numbers on branches are estimates for *a posteriori* probabilities that the respective groups are monophyletic given the data. The two main subgroups of *Sebacinales* discussed in the text are designated A and B. Sequences from teleomorphic specimens are printed in bold.

of *Gaultheria shallon* (Allen *et al*. 2003). All teleomorphs known for species in group B were observed only in axenic culture and morphologically assigned to *Sebacina vermifera* (Warcup 1988).

Generic and species concepts in Sebacinales

Our results indicate that basidiome gross morphology is not useful for the definition of monophyletic groups in *Sebacinales*, a statement which obviously can be generalised for wide parts of a natural systematic concept in basidiomycetes (e.g. Oberwinkler 1977, Bandoni 1984, Hibbett & Thorn 2001). Thus, *Sebacina*, defined for resupinate forms (Tulasne & Tulasne 1871), is polyphyletic according to the present analysis. A morphological transition from certain species of *Sebacina* to *Tremellodendron*, a genus name introduced for clavarioid species, has been described (McGuire 1941). Our analysis supports this point of view. *Efibulobasidium*, a genus based on pustulate basidiomes (Wells 1975), is, according to our results, another example of a probably polyphyletic group circumscribed by basidiome morphology.

Not only the generic concept, but also the species concepts in *Sebacina* appear to be questionable. The molecular analysis shows that diverse species might be included in the present circumscription of *Sebacina incrustans*, the type of *Sebacina*; the same situation holds for *S. epigaea*. The problem for an accurate delimitation of species is the lack of useful macro- and microscopical characters. Obviously, biodiversity in this group is much higher than hitherto assumed.

This is corroborated by the molecular diversity detected in mycobionts of ectomycorrhizas or orchid mycorrhizas, from which up to now no data about sexual stages are available. Judging from our molecular phylogenetic hypotheses, most of the mycorrhizal mycobionts contained in subgroup A should morphologically be classified in *Sebacina* or *Tremellodendron*. There is a particularly high probability that one of the *Neottia* mycobionts (AF440655), sequenced from roots of a French specimen of *N. nidus-avis* (Selosse *et al*. 2002), is a *Tremellodendron* species, since its D1/D2 sequence is identical with that obtained from a *Tremellodendron* sample from North America. If they are conspecific, this would be the first molecular evidence for a wide geographical distribution of a single species of the *Sebacinales*.

It is, however, premature to speculate about geographical distribution patterns of sebacinoid species, since the present sampling of DNA sequences of *Sebacinales* is strongly biased on collections from Europe, Australia (*Sebacina vermifera* and mycobionts of *Eucalyptus*) and North America (*Gaultheria* mycobionts). From the limited molecular data, however, we can infer that both *Sebacinales* subgroups A and B are distributed over Europe, Australia, and North America. In our analysis we were able to also include a Chinese specimen of the *S. incrustans* complex. Judging from herbarium material of *S. incrustans* (Lowy 1971; Peter Roberts, pers. comm.), we suggest that the *Sebacinales* has a wide distribution.

Mycorrhizal diversity

In the field, ectomycorrhizas involving *Sebacinales* mycobionts have only been well documented for group A (Glen *et al*. 2002, Selosse, Bauer & Moyersoen 2002, Urban, Weiß & Bauer 2003). The potential to form ectomycorrhizas *in vitro* has been demonstrated for species of the *Sebacina vermifera* complex (Warcup 1988), but it is not clear whether such associations also occur in the field. On the other hand, orchid mycorrhizal species of *Sebacinales* occur in both subgroups A and B (Warcup 1988, McKendrick *et al*. 2002, Selosse *et al*. 2002, Taylor *et al*. 2003), but they may not be homologous. In subgroup A, sebacinoid mycobionts colonise achlorophyllous species, such as *Neottia nidus-avis* and *Hexalectris spicata* (Selosse *et al*. 2002, Taylor *et al*. 2003), or green *Epipactis* species for which achlorophyllous individuals exist and that are likely to be partially heterotrophic (Selosse *et al*. 2004). These orchid mycobionts also form ectomycorrhizas with diverse surrounding trees (Selosse, Bauer & Moyersoen 2002, Selosse *et al*. 2002, 2004), suggesting a tripartite association, where the orchid derives resources from the tree *via* the sebacinoid mycobiont, as described for other achlorophyllous orchids (e.g. Taylor & Bruns 1997, McKendrick, Leake & Read 2000). It is therefore possible that all of the sebacinoid orchid mycobionts of subgroup A have ectomycorrhizal potential. Species of the *Sebacina incrustans* complex cannot be grown in pure culture (F.O., unpubl.), which may be indicative of a strictly ectomycorrhizal life strategy for species belonging to group A. On the other hand, orchid symbionts of group B have been isolated and grown in pure culture (Warcup & Talbot 1967, Warcup 1988). Only these species, and not the orchid symbionts found in group A, may belong to the highly polyphyletic form genus *Rhizoctonia* (Milligan & Williams 1987, Taylor *et al*. 2003).

The Sebacina vermifera *complex*

The present molecular phylogenetic study includes fungal isolates that have been assigned to *Sebacina vermifera* (Warcup & Talbot 1967, Warcup 1988). These isolates were obtained from roots of Australian orchids and it was shown that some isolates were able to stimulate the germination of orchid seeds and to form ectomycorrhizas with myrtaceous species *in vitro* (Warcup 1988). Concerning his isolates of *S. vermifera*, which varied in microscopical measurements as well as in growth parameters of axenic cultures, Warcup (1988) states that 'without further data it is difficult to decide whether *S. vermifera* is a variable species or a complex of closely allied species.' Our analyses strongly suggest that *S. vermifera* is indeed a broad complex of species

(Fig. 2). It is even possible that all the species that can currently be assigned to *Sebacinales* subgroup B belong to this morphologically defined complex, since the liverwort mycobionts (Kottke *et al*. 2003) and those of *Gaultheria shallon* (Berch, Allen & Berbee 2002, Allen *et al*. 2003), which are also included in subgroup B, have up to now only been detected by molecular or ultrastructural means, and nothing is known about their sexual stages.

We also included in the present study two isolates obtained from arbuscular mycorrhizas (AM); the multinucleate rhizoctonia DAR 29830 was isolated from a vesicle of *Glomus fasciculatum* (Williams 1985, Milligan & Williams 1987) and the anamorphic *Piriformospora indica* isolated from a spore of *Glomus mosseae* (Verma *et al*. 1998). Both isolates are closely related according to our molecular phylogenetic analysis, which is consistent with their morphological characters (Milligan & Williams 1987, Varma *et al*. 2001), and positioned among isolates of the *Sebacina vermifera* complex (Fig. 2). Similar isolates were frequently obtained in Australia from pot cultures of AM, but also from diverse host plants in the field (Milligan & Williams 1987). So far nothing is known about the specific diversity of these organisms, nor do we have data about their interaction with AM fungi, but it was shown that the isolate designated as *Piriformospora indica* was able to benefit plant growth and increase resistance against pathogens in a broad range of host plants (Varma *et al*. 2001).

This phylogenetic analysis shows a broad mycorrhizal capacity in *Sebacinales*, including ectomycorrhizas (Warcup 1988, Glen *et al*. 2002, Selosse, Bauer & Moyersoen 2002, Selosse *et al*. 2002), orchid mycorrhizas (Warcup 1988, McKendrick *et al*. 2002, Selosse *et al*. 2002), ericoid mycorrhizas (Allen *et al*. 2003), and also the recently recognised jungermannioid mycorrhizas (Kottke *et al*. 2003). Despite the tremendous increase of data in recent years, sampling of *Sebacinales*, both geographically and with respect to the host plants, is still erratic. The present data are but the tip of an iceberg, as corroborated by a recent quantitative study (Avis *et al*. 2003), in which 5% of the ectomycorrhizas in a temperate oak savanna were ascribed to *Sebacinales*. Future studies will bring more detailed insight into the ecology and phylogeny of this fascinating fungal order, which may have a future economically important plant-beneficial potential, which has hitherto been overlooked.

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