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British Mycological
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journal homepage: www.elsevier.com/locate/funbio



Phylogenetic relationships of the Gomphales based on nuc-25S-rDNA, mit-12S-rDNA, and mit-*atp6*-DNA combined sequences

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ARTICLE INFO

Article history:

Received 16 September 2009

Accepted 11 January 2010

Available online 28 January 2010

Corresponding Editor: G.M. Gadd

Keywords:

atp6

Gomphales

Homobasidiomycetes

rDNA

Systematics

ABSTRACT

Phylogenetic relationships among Geastrales, Gomphales, Hysterangiales, and Phallales were estimated via combined sequences: nuclear large subunit ribosomal DNA (nuc-25S-rDNA), mitochondrial small subunit ribosomal DNA (mit-12S-rDNA), and mitochondrial *atp6* DNA (mit-*atp6*-DNA). Eighty-one taxa comprising 19 genera and 58 species were investigated, including members of the Clathraceae, Gautieriaceae, Geastraceae, Gomphaceae, Hysterangiaceae, Phallaceae, Protophallaceae, and Sphaerobolaceae. Although some nodes deep in the tree could not be fully resolved, some well-supported lineages were recovered, and the interrelationships among *Gloeocantharellus*, *Gomphus*, *Phaeoclavulina*, and *Turbinellus*, and the placement of *Ramaria* are better understood. Both *Gomphus sensu lato* and *Ramaria sensu lato* comprise paraphyletic lineages within the Gomphaceae. Relationships of the subgenera of *Ramaria sensu lato* to each other and to other members of the Gomphales were clarified. Within *Gomphus sensu lato*, *Gomphus sensu stricto*, *Turbinellus*, *Gloeocantharellus* and *Phaeoclavulina* are separated by the presence/absence of clamp connections, spore ornamentation (echinulate, verrucose, subreticulate or reticulate), and basidiomal morphology (fan-shaped, funnel-shaped or ramarioid). *Gautieria*, a sequestrate genus in the Gautieriaceae, was recovered as monophyletic and nested with members of *Ramaria* subgenus *Ramaria*. This agrees with previous observations of traits shared by these two ectomycorrhizal taxa, such as the presence of fungal mats in the soil. *Clavariadelphus* was recovered as a sister group to *Beenakia*, *Kavinia*, and *Lentaria*. The results reaffirm relationships between the Geastrales, Gomphales, Hysterangiales, and the Phallales, suggesting extensive convergence in basidiomal morphology among members of these groups. A more extensive sampling that focuses on other loci (protein-coding genes have been shown to be phylogenetically informative) may be useful to answer questions about evolutionary relationships among these fungal groups.

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doi:10.1016/j.funbio.2010.01.002

Introduction

The gomphoid fungi occupy a unique position in the phylogeny of higher Basidiomycetes (Bruns *et al.* 1998; Pine *et al.* 1999; Hibbett & Thorn 2001; Hosaka *et al.* 2006). They are prominent in most forest ecosystems as saprotrophs and mutualists. The fungi in this group are also characterized by a wide range of basidiomal morphologies, from stalked ramarioid/clavarioid to cantharelloid-gomphoid, clavate, resupinate-odontoid, to sequestrate. Molecular studies reveal that gomphoid fungi are closely related to taxa in the Geastrales, Hysterangiales, and Phallales (Colgan *et al.* 1997; Hibbett *et al.* 1997; Pine *et al.* 1999; Humpert *et al.* 2001; Hosaka *et al.* 2006).

Taxonomy of the Gomphales has traditionally relied upon morphological characters now known to be subject to parallel evolution and phenotypic plasticity (Moncalvo *et al.* 2000). Consequently, many current genera and families are artificial, and taxonomic limits and identity of natural groups in the orders Geastrales, Gomphales, Hysterangiales, and Phallales are being re-examined.

Past workers (Maire 1902, 1914; Eriksson 1954; Heim 1954) recognized the relatedness of diverse morphologies within the Gomphales from microscopic and macrochemical characters, including cyanophilic spore ornamentation, chiasmic basidia, hyphal construction, and positive hymenial reaction to ferric sulfate (Eriksson 1954; Donk 1961, 1964; Petersen 1971a; Villegas *et al.* 1999). Donk (1961, 1964) proposed the family Gomphaceae to include the resupinate-odontoid genera *Kavinia* and *Ramaricium*, the stalked clavarioid-ramarioid genera *Lentaria* and *Ramaria*, the stalked hydroid genus *Beenakia*, the stipitate agaricoid genus *Gloeocantharellus*, and the pileate genera *Chloroneuron* and *Gomphus*. Corner (1970) proposed Ramariaceae to include *Delentaria*, *Kavinia*, *Lentaria*, and *Ramaria*. He excluded the pileate genera because no intermediate species linked the gomphoid and ramarioid morphologies. Petersen (1971a) suggested a gomphoid ancestral morphology for the family and later revised Donk's and Corner's familial classifications to include *Beenakia*, *Gomphus*, *Kavinia*, *Ramaricium*, *Ramariopsis*, and *Ramaria* (Petersen 1973, 1988).

Morphological and recent molecular data (mitochondrial and nuclear rDNA) have been used to infer inter- and intra-specific relationships among genera in this group of fungi. According to Pine *et al.* (1999), Villegas *et al.* (1999), Humpert *et al.* (2001), and Hosaka *et al.* (2006), the Gomphales includes the genera *Beenakia*, *Clavariadelphus*, *Gautieria*, *Gloeocantharellus*, *Gomphus*, *Kavinia*, *Lentaria*, *Phaeoclavulina*, *Ramaria*, *Ramaricium*, and *Turbinellus*. Hosaka *et al.* (2006) demonstrated the Gomphales to be a sister group to the Phallales, represented by the families Clathraceae (*sensu* Chevallier), Phallaceae (*sensu* Corda), Lysuraceae, Protophallaceae (*sensu* Zeller), Claustulaceae, and Trappeaceae. Both Gomphales and Phallales are closely related to the Hysterangiales (*sensu* Hosaka & Castellano) and the Geastrales.

Villegas *et al.* (1999), using morphological traits, proposed the Gomphales to be monophyletic and delimited by the presence of mycelial cords or rhizomorphs. It included the families Beenakiaceae (*Beenakia*, *Kavinia*, and *Ramaricium*), Gomphaceae (*Gomphus* and *Gloeocantharellus*), Lentariaceae (*Lentaria*), and Ramariaceae (*Ramaria*). According to Singer (1949), Heim

(1954), Heinemann (1958), Donk (1964), Giachini (2004) and Hosaka *et al.* (2006), *Clavariadelphus* is a member of the Gomphales. The results of Villegas *et al.* (1999), however, disagree with the premises that *Clavariadelphus*, *Gomphus*, and *Ramaria* are members of the same order. According to those authors *Clavariadelphus* is not grouped within but rather a sister group to the Gomphales.

Pine *et al.* (1999) and Humpert *et al.* (2001), on the other hand, using sequences of both mitochondrial (mit-12S-rDNA) and nuclear (nuc-25S-rDNA) loci, showed that a gomphoid-phalloid clade including *Clavariadelphus*, *Geastrum*, *Gloeocantharellus*, *Gomphus* (monophyletic), *Lentaria*, *Ramaria* (paraphyletic), *Pseudocolus*, and *Sphaerobolus* was recovered in all analyses performed. Pine *et al.* (1999) showed that *Gomphus sensu lato* represented a terminal monophyletic group in the gomphoid-phalloid clade (although just two taxa were sampled), having *Ramaria* as sister group (Figs 1–3 in Pine *et al.* 1999). Based on morphological as well as molecular characters, Giachini (2004) revisited the generic concepts in the family Gomphaceae and recombined the species of *Gomphus sensu lato* into *Gloeocantharellus*, *Gomphus sensu stricto*, and the resurrected genera *Phaeoclavulina* and *Turbinellus*.

Cantharelloid/gomphoid and clavarioid fungi have historically been prominent in hypotheses about the origin of fleshy basidiomycetes (Singer 1947, 1986; Heim 1954; Corner 1966; Harrison 1971; Petersen 1971a; Corner 1972; Jülich 1981; Miller & Watling 1987). Their fruiting forms can be arranged in a transformation series, from clavate at one end, cantharelloid/gomphoid intermediately, and agaricoid at the other extreme. Corner (1972) proposed the “*Clavaria* theory” of basidiomycete evolution in which cantharelloid and clavarioid fungi were to be regarded as ancestral, and from which all other Homobasidiomycetes have been derived. He suggested that simple clavate morphologies (e.g. *Clavaria*) with smooth hymenia gave rise to intermediate cantharelloid species (e.g. *Cantharellus*, *Craterellus*), and from those were derived the wrinkled or folded hymenial gomphoid species (e.g. *Gomphus*, *Turbinellus*). Other authors agree on transformations among ramarioid, cantharelloid, and agaricoid forms but propose the opposite polarity, suggesting that lineages containing cantharelloid, ramarioid, and club-like fungi have been derived from agaricoid ancestors (Fiasson *et al.* 1970; Arpin & Fiasson 1971; Petersen 1971a; Singer 1986).

In this paper we analyze phylogenetic relationships among major evolutionary lineages of gomphoid fungi using combined sequence data from nuclear (nuc-25S-rDNA) and mitochondrial-encoded ribosomal and non-ribosomal RNA genes (mit-12S-rDNA, mit-*atp6*-DNA). Our taxonomic sampling focused on the Gomphales *sensu* Jülich (1981). Major questions tested in this study were:

- 1) Is *Gomphus sensu lato* monophyletic?
- 2) Are genera within *Gomphus sensu lato* monophyletic?
- 3) Are the Gomphales, Hysterangiales, Phallales, and Geastrales closely related?
- 4) How have basidiomatal morphology, presence or absence of clamp connections, and substrate affinity evolved within the Gomphales?

Materials and methods

Taxonomic sampling

The sampling of Geastrales, Gomphales, Hysterangiales, and Phallales included 19 genera and 58 species (total of 81 taxa) as listed along with GenBank accession numbers (Table 1). One species each of *Bondarzewia* and *Russula* plus *Cortinarius iodes* were included as outgroups. Holotypes and representative specimens were examined and sampled when available. Dried specimens were obtained from the following herbaria: BPI, BR, DSH, FH, K, MICH, NYS, O, OSA, OSC, PERTH, PDD, SFSU, SUC, TENN, UC, and UPS (<http://www.nybg.org/bsci/ih/ih.html>). Two or more fresh collections of each species or variety were included when available.

Species identification and nomenclature were based on holotype and paratype specimens and taxonomic keys and species descriptions (Corner 1950, 1966, 1969, 1970; Petersen 1971b, 1981, 1988; Marr & Stuntz 1973; Schild 1998; Roberts 1999; Giachini 2004).

DNA extraction, amplification, and sequencing

DNA sequence data were obtained from three independent loci: LR0R–LR3 region for nuclear large subunit ribosomal DNA (nuc-LSU-rDNA), MS1–MS2 region for mitochondrial small subunit ribosomal DNA (mt-SSU-rDNA), and ATPase subunit 6 (*atp6*). The primers and PCR protocols have been described previously (summarized in Assembling the Fungal Tree of Life website; <http://aftol.org/primers.php>).

Phylogenetic analyses were conducted for the concatenated three-locus dataset under Bayesian and parsimony criteria. Maximum parsimony analyses were conducted by PAUP*4.0b10 (Swofford 2002), with nodal supports tested by bootstrap analysis. Analyses were conducted with 10 000 random additions of heuristic search with TBR and Multrees option on. All MPTs recovered were subsequently compared to each other under the maximum likelihood criterion (Kishino & Hasegawa 1989). Significant topological differences under the maximum parsimony criterion and the combinability of the data were estimated via the Shimodaira & Hasegawa (1999) likelihood test ($p \leq 0.05$). Bayesian analysis was conducted by use of MrBayes ver. 3.0b4 (Huelsenbeck and Ronquist 2001), with 3 000 000 generations of MCMCMC. Every 100th tree was sampled, to produce 30 000 trees. Four chains were applied (one cold and three heated; temperature set to the default value of 0.2). We applied independent models for each partition using general time reversible (GTR) and gamma (δ) distribution (burn-in period of 15 000 trees). Stationarity was determined when chains reached the arithmetic mean likelihood value of $-94\,331.77$.

Alternative phylogenetic hypotheses reflecting different classifications and species relationships were constructed in MacClade version 3.03 (Maddison & Maddison 1992) and PAUP* version 4.0b10 (Swofford 2002). These trees were used as constraint starting topologies in maximum parsimony analyses in the heuristic search option (100 random sequence additions, TBR, and MULPARS off). Most parsimonious trees recovered with and without constraints were compared by

the Kishino–Hasegawa (K–H) test implementing the likelihood model described above (Table 2). Character state reconstruction for substrate affinity, basidiomata morphology, and presence/absence of clamp connections was performed in Mr Bayes (MC³) version 3.0 (Huelsenbeck & Ronquist 2001); equal weights for all character state transformations were assumed.

Character mapping

For this study, the GTR (likelihood) model of character evolution fit our data best. Adopting GTR with Multistate ver. 0.8 (Pagel 2003), we calculated trait evolution for characters representing substrate affinity, presence/absence of clamp connections, and basidiomatal morphology for the node/clade denoting the order Gomphales.

Results

Sequence alignment and nucleotide sequence variation

Alignment over a broad taxonomic sampling (84 taxa) was not attainable for a few hypervariable, indel-rich regions: those were removed from the analyses. A few remaining single-gap regions occurring in only one or few sequences were removed due to the possibility that they represented sequencing errors. In contrast, several gap regions with short indels were recorded as phylogenetically informative. In all, 314 positions corresponding to regions with problematic alignments were removed, and 120 indel positions were recorded. After removal of the 5' and 3' positions (incomplete for several taxa), 2063 positions remained in the final analyses. Of these, 758 were constant, 328 variable characters were parsimony-uninformative, and 685 were parsimony-informative. The final alignment is available in the web as a NEXUS file (SN1858).

Phylogenetic trees

The analyses of the three combined loci yielded three MPTs of 4967 steps (Fig 1). For those trees, the CI was 0.287, the RI 0.566, and the RC 0.163. Fig 2 depicts the consensus tree obtained for the taxa studied. For this tree, Bayesian posterior probability values (MC³) are presented above branches and bootstrap values greater than 50 below branches (consensus and Bayesian produced identical trees). The results support the monophyletic status and the close evolutionary relationship of the orders Geastrales, Gomphales, Hysterangiales and Phallales, corroborating results previously obtained by Hibbett et al. (1997), Hosaka et al. (2006), Humpert et al. (2001), and Pine et al. (1999). These results agree with the findings of Giachini (2004) on the paraphyletic status of *Gomphus sensu lato* and confirm the monophyletic status of *Gloeocantharellus*, *Gomphus*, *Phaeoclavulina*, and *Turbinellus*. Furthermore, the phylogenetic analyses corroborate previous results on the paraphyletic status of *Clavariadelphus*, *Kavinia*, and *Ramaria*, and the monophyletic status of *Gautieria*, *Lentaria*, and *Ramaria* subgenus *Ramaria* (Humpert et al. 2001). In the Geastrales they support a monophyletic *Sphaerobolus* (Fig 1), a result corroborated by Hosaka et al. (2006).

Table 1 – Taxa included in the phylogenetic analyses.

Taxa ^a	Collection ^b	Herbarium ^c	GenBank accession numbers		
			nuc-25S-rDNA	mit-18S-rDNA	mit-atp6-rDNA
<i>Beenakia fricta</i> Maas Geest.	2083	K	AY574693	AY574766	AY574833
<i>Clathrus cibarius</i> (Tul.) E. Fisch.	107 652	OSC	AY574641	AY574715	AY574783
<i>Clavariadelphus ligula</i> (Schaeff.) Donk	67 068	OSC	AY574650	AY574723	AY574793
<i>Clavariadelphus occidentalis</i> Methven	37 018	OSC	AY574648	AY574721	AY574791
<i>Clavariadelphus truncatus</i> Donk	67 280	OSC	AY574649	AY574722	AY574792
<i>Gallacea scleroderma</i> (Cooke) Lloyd	59 621	OSC	AY574645	AY574719	AY574787
<i>Gautieria monticola</i> Harkn.	65 121	OSC	AY574651	AY574724	AY574794
<i>Gautieria parksiana</i> Zeller & C.W. Dodge	58 907	OSC	AY574652	AY574725	AY574795
<i>Gastrum saccatum</i> Fr.	23 765	Trappe	AY574646	AY574720	AY574788
<i>Gloeocantharellus dingleyae</i> (Segedin) Giachini	30 179*	PDD	AY574668	AY574741	–
<i>Gloeocantharellus novae-zelandiae</i> (Segedin) Giachini	44 960*	PDD	AY574666	AY574739	AY574809
<i>Gloeocantharellus pallidus</i> (Yasuda) Giachini	54 917*	BPI	AY574673	AY574746	AY574815
<i>Gloeocantharellus papuanus</i> Giachini, Bougher, Castellano & Trappe	06 707 114*	PERTH	AY574667	AY574740	AY574810
<i>Gloeocantharellus purpurascens</i> (Hesler) Singer	12 793	TENN	AY574683	AY574756	AY574823
<i>G. purpurascens</i> (Hesler) Singer	14 265*	TENN	AY574684	AY574757	AY574824
<i>Gomphus brunneus</i> (Heinem.) Corner	034 190-46	BR	AY574680	AY574753	AY574821
<i>Gomphus clavatus</i> (Pers.) Gray	97 616	OSC	AY574664	AY574737	AY574807
<i>G. clavatus</i> (Pers.) Gray	s.n.	UPS	AY574665	AY574738	AY574808
<i>Hysterangium coriaceum</i> R. Hesse	64 939	OSC	AY574686	AY574759	AY574826
<i>Hysterangium crassum</i> (Tul. & C. Tul.) E. Fisch.	110 447	OSC	AY574687	AY574760	AY574827
<i>Hysterangium occidentale</i> Harkn.	47 048	OSC	AY574685	AY574758	AY574825
<i>Kavinia albovidis</i> (Morgan) Gilb. & Budington	102 140	O	AY574692	AY574765	AY574832
<i>Kavinia himantia</i> (Schwein.) J. Erikss.	102 156	O	AY574691	AY574764	AY574831
<i>Lentaria pinicola</i> (Burt) R.H. Petersen	M89**	SUC	AY574688	AY574761	AY574828
<i>L. pinicola</i> (Burt) R.H. Petersen	M46	SUC	AY574689	AY574762	AY574829
<i>L. pinicola</i> (Burt) R.H. Petersen	M560	SUC	AY574690	AY574763	AY574830
<i>Mutinus elegans</i> (Mont.) E. Fisch.	107 657	OSC	AY574643	AY574717	AY574785
<i>Phaeoclavulina africana</i> (R.H. Petersen) Giachini	39 621*	TENN	AY574653	AY574726	AY574796
<i>Phaeoclavulina cokeri</i> (R.H. Petersen) Giachini	36 030*	TENN	AY574701	AY574774	AY574843
<i>Phaeoclavulina curta</i> (Fr.) Giachini	8711	OSC	AY574713	–	AY574858
<i>Phaeoclavulina cyanocephala</i> (Lév.) Giachini	37 827	TENN	AY574710	AY574779	AY574854
<i>Phaeoclavulina eumorpha</i> (P. Karst.) Giachini	36 218	TENN	AY574712	AY574781	AY574856
<i>P. eumorpha</i> (P. Karst.) Giachini	37 842	TENN	–	AY574782	AY574857
<i>Phaeoclavulina gigantea</i> (Pat.) Giachini	109*	FH	AY574703	AY574776	AY574845
<i>Phaeoclavulina grandis</i> (Corner) Giachini	073 158-06*	BR	AY574678	AY574751	AY574820
<i>Phaeoclavulina guadelupensis</i> (Pat.) Giachini	120*	FH	AY574682	AY574755	–
<i>Phaeoclavulina guyanensis</i> (Pat.) Giachini	84*	FH	AY574706	–	AY574848
<i>Phaeoclavulina insignis</i> (Pat.) Giachini	104*	FH	AY574704	–	AY574846
<i>Phaeoclavulina longicaulis</i> (Pat.) Giachini	33 826	TENN	AY574700	AY574773	AY574842
<i>Phaeoclavulina ochraceo-virens</i> (Jungh.) Giachini	23 475	OSC	AY574714	–	AY574859
<i>Phaeoclavulina panaribbea</i> (R.H. Petersen) Giachini	31 836*	TENN	AY574707	–	AY574849
<i>Phaeoclavulina subclaviformis</i> (Berk.) Giachini	073 159-07*	BR	AY574679	AY574752	–
<i>Phaeoclavulina viridis</i> (Pat.) Giachini	97 708	OSC	AY574675	AY574748	AY574817
<i>P. viridis</i> (Pat.) Giachini	1853	FH	AY574676	AY574749	AY574818
<i>P. viridis</i> (Pat.) Giachini	4302	PERTH	AY574677	AY574750	AY574819
<i>Phallus impudicus</i> L.	107 655	OSC	AY574642	AY574716	AY574784
<i>Protuberia nothofagi</i> Castellano & Beever	59 699	OSC	AY574644	AY574718	AY574786
<i>Pseudocolus fusiformis</i> (E. Fisch.) Lloyd	96-033	DSH	AF518641	AF026666	–
<i>Ramaria apiculata</i> (Fr.) Donk	23 549	OSC	AY574695	AY574768	AY574836
<i>R. apiculata</i> var. <i>brunnea</i> R.H. Petersen	53 935	TENN	AY574696	AY574769	AY574837
<i>Ramaria araiospora</i> var. <i>araiospora</i> Marr & D.E. Stuntz	M739*	SUC	AF213068	AF213141	AY574838
<i>R. araiospora</i> var. <i>araiospora</i> Marr & D.E. Stuntz	M556	SUC	AY574697	AY574770	AY574839
<i>Ramaria botrytis</i> var. <i>botrytis</i> (Pers.) Ricken	M457	SUC	AY574698	AY574771	AY574840
<i>R. botrytis</i> var. <i>botrytis</i> (Pers.) Ricken	M740	SUC	AY574699	AY574772	AY574841
<i>Ramaria circinans</i> (Peck) Marr & D.E. Stuntz	s.n.	NYS	AY574702	AY574775	AY574844
<i>R. circinans</i> var. <i>anceps</i> Marr & D.E. Stuntz	M615*	SUC	AY574711	AY574780	AY574855
<i>Ramaria gelatiniaurantia</i> var. <i>violetingens</i> Marr & D.E. Stuntz	M830	SUC	AY574708	AY574777	AY574851
<i>Ramaria rainieriensis</i> Marr & D.E. Stuntz	M231	SUC	AF213115	AF213135	AY574834
<i>R. rainieriensis</i> Marr & D.E. Stuntz	M431	SUC	AY574694	AY574767	AY574835
<i>Ramaria rubribrunnescens</i> Marr & D.E. Stuntz	M844*	SUC	AF213098	AF213142	AY574852
<i>Ramaria stuntzii</i> Marr	M214	SUC	AF213102	AF213134	AY574850

(continued on next page)

Table 1 (continued)

Taxa ^a	Collection ^b	Herbarium ^c	GenBank accession numbers		
			nuc-25S-rDNA	mit-18S-rDNA	mit- <i>atp6</i> -rDNA
<i>Ramaria suecica</i> (Fr.) Donk	s.n.	BPI	AY574705	–	AY574847
<i>Ramaria vinosimaculans</i> Marr & D.E. Stuntz	23 287	OSC	AY574709	AY574778	AY574853
<i>Sphaerobolus stellatus</i> Tode	96-015	DSH	AF393077	AF026662	AY574789
<i>S. stellatus</i> Tode	SS28	–	AY574647	AY488024	AY574790
<i>Turbinellus flabellatus</i> (Berk.) Giachini	191 [†]	FH	AY574674	AY574747	AY574816
<i>T. flabellatus</i> (Berk.) Giachini	1770*	K	AY574681	AY574754	AY574822
<i>Turbinellus floccosus</i> (Schwein.) Earle	MY-1839	OSA	AY574654	AY574727	AY574797
<i>T. floccosus</i> (Schwein.) Earle	MY-1840	OSA	AY574655	AY574728	AY574798
<i>T. floccosus</i> (Schwein.) Earle	69 167	OSC	AY574656	AY574729	AY574799
<i>T. floccosus</i> (Schwein.) Earle	33 233	TENN	AY574657	AY574730	AY574800
<i>T. floccosus</i> (Schwein.) Earle	21 238 [†]	SFSU	AY574658	AY574731	AY574801
<i>T. floccosus</i> (Schwein.) Earle	33 295 [†]	TENN	AY574659	AY574732	AY574802
<i>T. floccosus</i> (Schwein.) Earle	5588 [†]	MICH	AY574660	AY574733	AY574803
<i>T. floccosus</i> (Schwein.) Earle	10 721 [†]	MICH	AY574661	AY574734	AY574804
<i>T. floccosus</i> (Schwein.) Earle	759 902 [†]	UC	AY574662	AY574735	AY574805
<i>T. floccosus</i> (Schwein.) Earle	924 302 [†]	UC	AY574663	AY574736	AY574806
<i>Turbinellus fujisanensis</i> (S. Imai) Giachini	MY-1841	OSA	AY574670	AY574743	AY574812
<i>T. fujisanensis</i> (S. Imai) Giachini	MY-1842*	OSA	AY574669	AY574742	AY574811
<i>Turbinellus kauffmanii</i> (A.H. Sm.) Giachini	10 069*	MICH	AY574671	AY574744	AY574813
<i>T. kauffmanii</i> (A.H. Sm.) Giachini	97 590	OSC	AY574672	AY574745	AY574814
Outgroup					
<i>Bondarzewia berkeleyi</i> (Fr.) Bondartsev & Singer	93–190	DSH	SARn	U27026	–
<i>Cortinarius iodes</i> Berk. & M.A. Curtis	JM96/23	–	AF042613	AF026675	AF388826
<i>Russula</i> sp.	s.n.	–	U11926	U27074	AF002148

a Epithets according to Giachini (2004), Humpert et al. (2001), Marr & Stuntz (1973), and Petersen (1981, 1988).

b *Holotype specimens; **Paratype specimens; s.n. = no number; [†]type for older name (see Giachini 2004 for details).

c Herbarium for source of collections: BPI = U.S. National Fungal Collections – Beltsville; BR = Herbarium of the National Botanical Garden of Belgium – Meise; DSH = Personal collection of Dr David S. Hibbett, Biology Department, Clark University – Worcester; FH = Farlow Herbarium of Cryptogamic Botany – Cambridge; K = Royal Botanic Gardens Herbarium – Kew; MICH = University of Michigan Fungus Collection – Ann Arbor; NYS = Herbarium of the New York State Museum – Albany; O = Herbarium of the Botanical Museum of Oslo – Oslo; OSA = Osaka Museum of Natural History – Osaka; OSC = Oregon State University Herbarium – Corvallis; PDD = New Zealand Plant Diseases Division Herbarium – Auckland; PERTH = CSIRO Forestry and Forest Products Herbarium – Perth; SFSU = Harry D. Thiers Herbarium at San Francisco State University – San Francisco; SUC = State University of New York Herbarium – Oneonta; TENN = University of Tennessee Herbarium – Knoxville; Trappe = Personal collection of Dr James M. Trappe, Department of Forest Science, Oregon State University – Corvallis; UC = University of California Herbarium – Berkeley; UPS = Herbarium of the Uppsala Botanical Museum of Uppsala University – Uppsala.

Table 2 – Kishino–Hasegawa likelihood test results.

Topology ^a	Trees ^b	–ln likelihood	p ^c
Unconstrained	3	20 283.09185	Better
Monophyletic <i>Gomphus sensu lato</i>	2	205 92.30503–20 597.07762	<0.0001*
Monophyletic <i>Phaeo</i> + <i>Gloeo</i> + <i>Gom</i>	1	20 630.80136	<0.0001*
Monophyletic <i>Phaeo</i> + <i>Gloeo</i> + <i>Turb</i>	8	20 608.04892–20 623.80385	<0.0001*
Monophyletic <i>Phaeo</i> + <i>Gom</i> + <i>Turb</i>	10	20 595.90882–20 614.11428	<0.0001*
Monophyletic <i>Phaeo</i> + <i>Gloeo</i>	1	20 550.19484	<0.0001*
Monophyletic <i>Phaeo</i> + <i>Gom</i>	1	20 612.56469	<0.0001*
Monophyletic <i>Phaeo</i> + <i>Turb</i>	3	20 648.44839–20 657.53649	<0.0001*
Monophyletic <i>Gloeo</i> + <i>Gom</i> + <i>Turb</i>	2	20 549.91723–20 554.45501	<0.0001*
Monophyletic <i>Gloeo</i> + <i>Gom</i>	1	20 571.14433	<0.0001*
Monophyletic <i>Gloeo</i> + <i>Turb</i>	7	20 534.76597–20 553.41940	<0.0001*
Monophyletic <i>Gom</i> + <i>Turb</i>	1	20 578.88126	<0.0001*

a *Gloeo* = *Gloeocantharellus*; *Gom* = *Gomphus*; *Phaeo* = *Phaeoclavulina*; *Turb* = *Turbinellus*.

b The best –ln likelihood tree from the maximum parsimony analyses.

c Probability of getting a more extreme t-value under the null hypothesis of no difference between the two trees (two-tailed test); * statistically significant at $p < 0.05$.

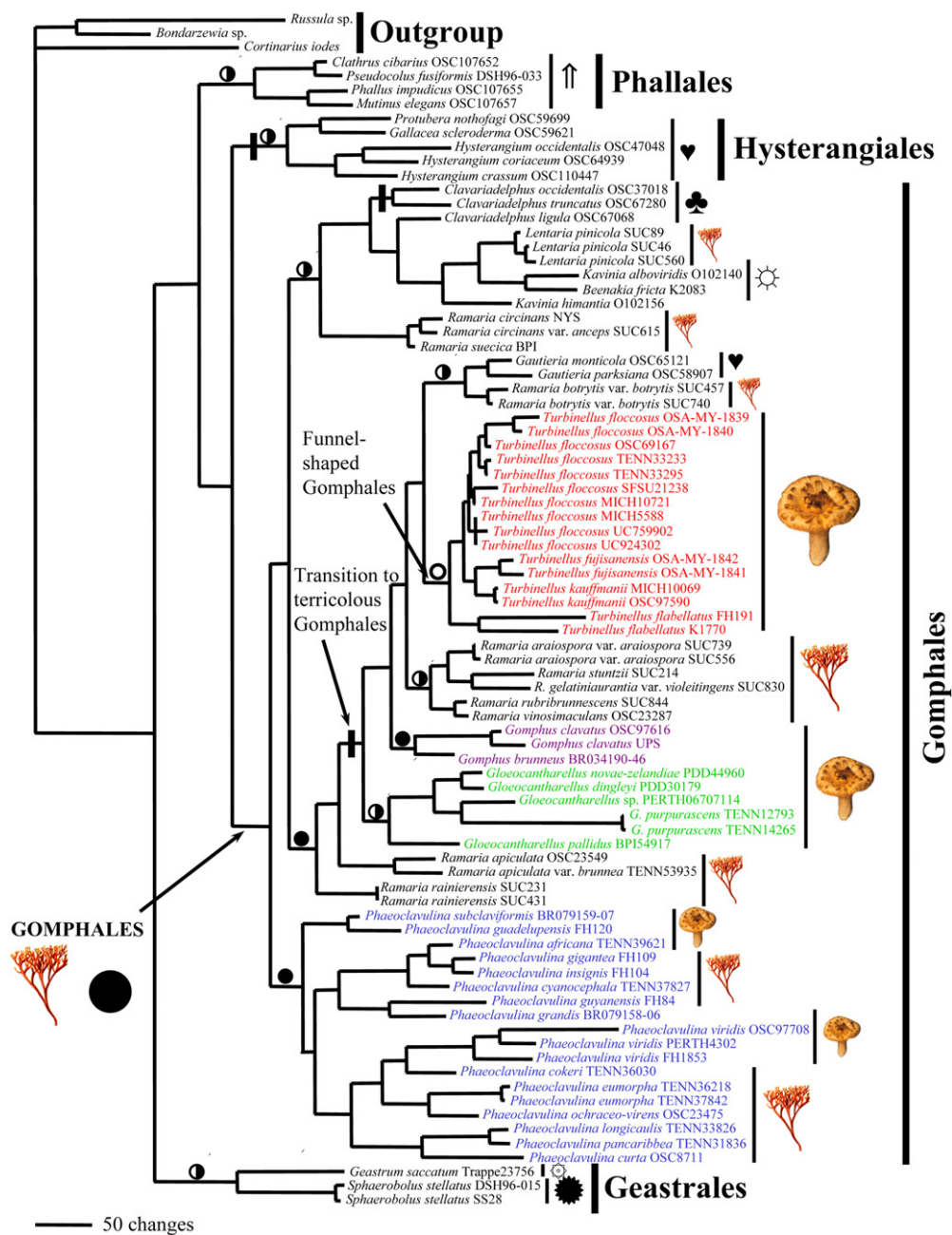


Fig 1 – Cladogram of 1 of 3 most parsimonious (MP) trees recovered from phylogenetic analyses of the nuc-25S-rDNA, mit-12S-rDNA, and mit-atp6-DNA combined sequences. Tree length = 4967; CI = 0.287, RI = 0.566, RC = 0.163. Basidiomata morphology: ☞ = ramarioid; ♣ = club; ☞ = cantharelloid-gomphoid; ↑ = stink horn; ♥ = sequestrate; ⊙ = earth-star; ⊙ = cannon ball; ☼ = odontoid/resupinate. Transition to a terricolous substrate affinity is indicated by the symbol ¶. Clamp connections are indicated with the following symbols: ● = clamp connection present; ○ = clamp connections absent; ◐ = clamp connections present in some species while absent in others.

Geastrales, Gomphales, Hysterangiales, and Phallales

The results of the three combined loci support the hypothesis of a Geastrales–Hysterangiales–Phallales relationship to the Gomphales (Colgan et al. 1997; Hibbett et al. 1997; Humpert et al. 2001; Giachini 2004; Hosaka et al. 2006). Both Bayesian MC³ and bootstrap values indicate a consistent and confident resolution for the evolutionary placement of Geastrales, Hysterangiales, and Phallales in relation to the Gomphales. The

placement of the three genera sampled for the Hysterangiales (Gallacea, Hysterangium, and Protuber) indicates a close relationship of the Hysterangiales to the Gomphales (Fig 1). Hysterangium, represented by North American species, was recovered as monophyletic, disagreeing with the work of Hosaka et al. (2006). Our study, however, sampled a much smaller portion of the order, as well as only three loci compared to five of Hosaka et al. (2006); accordingly we accept their conclusion that Hysterangium is paraphyletic. We sampled four genera of

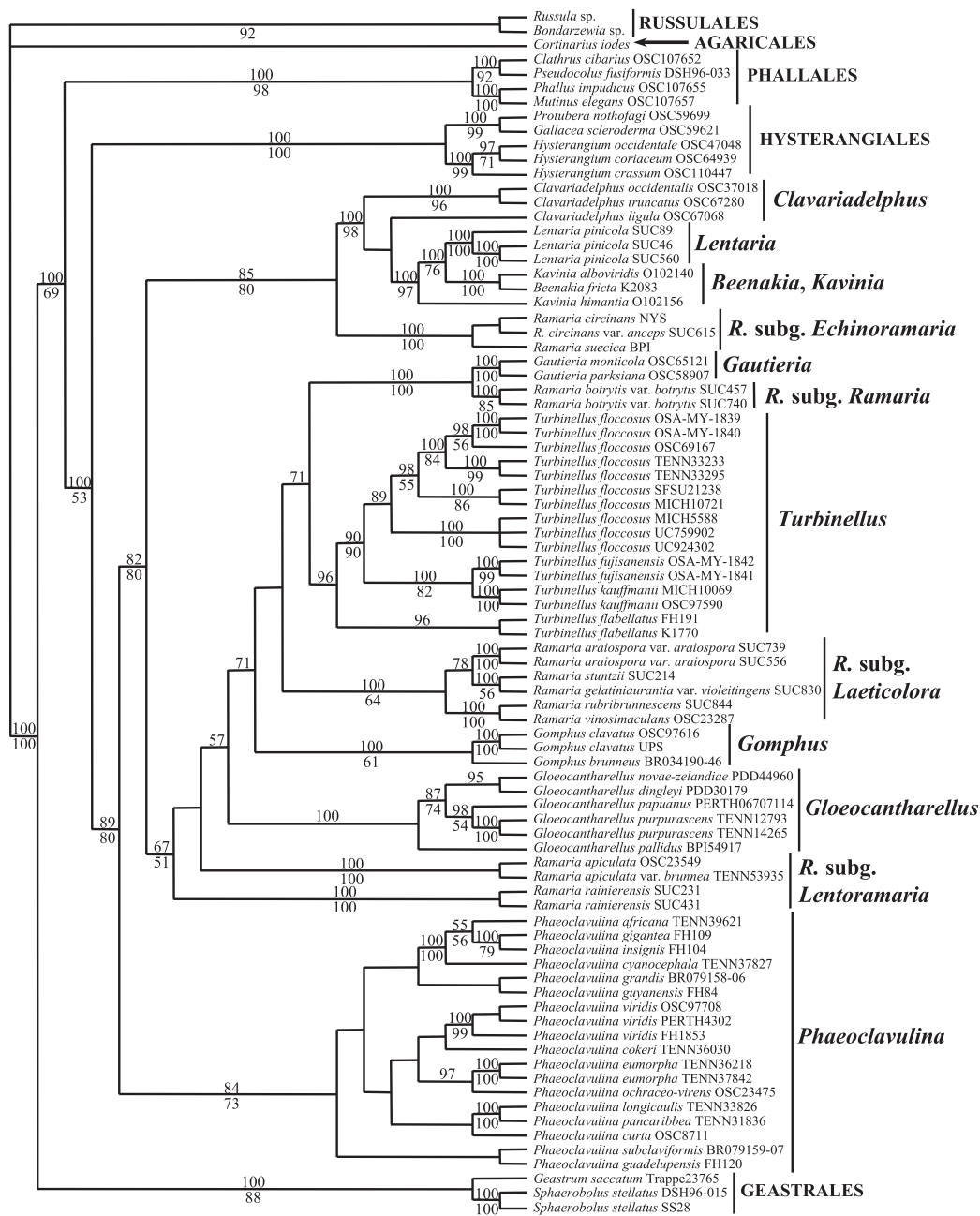


Fig 2 – Strict consensus cladogram of three equally parsimonious (MP) trees of 4967 steps based on nuc-25S-rDNA, mit-12S-rDNA, and mit-atp6-DNA combined sequences. MC³ and bootstrap values above 50 % are indicated above and below respective internode, respectively. CI = 0.287, RI = 0.566, RC = 0.163.

the Phallales: *Clathrus*, *Mutinus*, *Phallus*, and *Pseudocolus*. This order is shown as a sister group to the Hysterangiales (Fig 1). The Geastrales, represented in this study by *Geastrum* (earth-star fungus) and *Sphaerobolus* (cannon-ball fungus), was recovered as a basal, more primitive sister lineage (Fig 1). The placement of earth-star and cannon-ball fungi as an ancestral lineage for the Gomphales–Hysterangiales–Phallales has been shown by Hosaka et al. (2006), but beyond the basic hymenomycete features shared by these taxa, no other morphological characters have been identified to support this evolutionary line. Further sampling of the Geastrales might aid understanding of this evolutionary placement.

Character mapping

Characters for substrate affinity, presence/absence of clamp connections, and basidioma morphology are shown in Fig 1. Evolution of substrate affinity indicates both lignicolous and terricolous substrate affinities as ancestral for the Gomphales with one derivation of the strictly terricolous condition, for *Clavariadelphus* and the clade containing *Gloeocantharellus*, *Gomphus*, *Ramaria* subgenera *Laeticolora* and *Ramaria*, *Turbinellus*, and the sequestrate genus *Gautieria* (Fig 1). Evolution of basidioma macromorphology suggests that the ramarioid morphology is ancestral for the Gomphales with multiple

derivations of diverse basidiomata morphologies, i.e., club, gomphoid, odontoid, resupinate, and sequestrate (Fig 1). The mapping of presence or absence of clamp connections shows multiple gains and losses of this feature throughout the evolutionary history of the Gomphales (Fig 1). The results suggest an ancestral condition with clamp connections and multiple losses of this feature occurring in certain species of *Beenakia*, *Gautieria*, *Gloeocantharellus*, *Kavinia*, *Ramaria* subgenera *Laeticolora* and *Ramaria*, and *Turbinellus*.

Kishino–Hasegawa and Shimodaira–Hasegawa tests

The results of the Kishino–Hasegawa and Shimodaira–Hasegawa tests are presented in Table 2 and Fig 3, respectively. Hypotheses for the Kishino–Hasegawa test were (i) *Gomphus sensu lato* constrained (forced) to monophyly; and (ii) monophyly of combination sets involving at least two of the genera (at the time) *Gloeocantharellus*, *Gomphus*, *Phaeoclavulina* and *Turbinellus* on all possible fashions.

The Shimodaira–Hasegawa test was employed to test the combinability of the three loci sampled. Although independent analyses of the three data sets produced somewhat different resolutions regarding a few weakly supported terminal nodes, the overall topological reconstructions obtained for each of the three independent loci were similar. The Shimodaira–Hasegawa test for data combinability showed that the nuc-25S-rDNA and mit-*atp6*-DNA are statistically combinable at $p = 0.111–0.574$ (Fig 3). Mit-12S-rDNA, on the other hand, seems to be the most divergent locus, not statistically combinable with either nuc-25S-rDNA ($p < 0.0001$) or mit-*atp6*-DNA ($p < 0.0001$). When forcing the data from mit-12S-rDNA into the topology of the best parsimonious tree obtained with the nuc-25S-rDNA data, the results indicate they are combinable ($p = 0.001–0.751$) (Fig 3). The divergence observed for the mit-12S-rDNA locus may be due to the faster or slower rate of evolution suspected for this genomic region when compared to the nuc-25S-rDNA and mit-*atp6*-DNA regions. The presence of large indels observed throughout the mit-12S-rDNA locus indicates that this genomic region

provides a different resolution than the nuc-25S-rDNA or mit-*atp6*-DNA. Since the results for the mit-12S-rDNA locus were marginal for some combination sets, we combined the three loci for further analyses.

Discussion

Support for a Geastrales–Gomphales–Hysterangiales–Phallales relationship

The three independent loci examined support a close phylogenetic relationship among the Geastrales, Gomphales, Hysterangiales, and the Phallales (Figs 1 and 2). Relationships among stinkhorns, earth-stars, the cannon-ball fungus, ramarioid-clavarioid, and cantharelloid-gomphoid fungi have only recently been proposed in the literature (Hibbett et al. 1997; Pine et al. 1999; Humpert et al. 2001; Hosaka et al. 2006). Evolutionary relationships for the fungi belonging to some of those groups have been proposed in the past, and the literature on some, i.e. the Gomphales, has been extensively reviewed, mostly in reference to morphological characters (Maire 1902, 1914; Donk 1964; Corner 1966, 1969; Petersen 1968, 1971a; Giachini et al. 2001). As for relationships among Geastrales, Gomphales, Hysterangiales, and Phallales, identified by Pine et al. (1999) and corroborated by Humpert et al. (2001) and Hosaka et al. (2006), no unifying morphological synapomorphies have been identified.

Rejection of a monophyletic *Gomphus sensu lato*

Phylogenetic analyses of the combined nuc-25S-rDNA, mit-12S-rDNA, and mit-*atp6*-DNA rejected the monophyletic condition for *Gomphus sensu lato* (Table 2). The results indicate *Gloeocantharellus*, *Gomphus*, *Phaeoclavulina*, and *Turbinellus* to be monophyletic genera (Table 2, Fig 1). *Gautieria* and *Ramaria* nested within *Gomphus sensu lato*, whereas *Beenakia*, *Clavariadelphus*, *Kavinia*, and *Lentaria* were recovered as a sister group to it. Clades represented by these genera received high likelihood (Bayesian posterior probability – MC³) and bootstrap support (Fig 2). The Kishino–Hasegawa test indicated significantly worse trees when *Gomphus sensu lato* or combinations of taxa within *Gomphus sensu lato* were constrained to the monophyletic condition (Table 2), indicating that a confident resolution of the phylogeny of the Gomphales was obtained after the combination of more than one locus. This suggests that a combination of fast evolving (mit-12S) and protein-coding (*atp6*) genes provided a consistent resolution for the placement and evolutionary history of this group.

Evolution of substrate affinity

The analyses indicated an ambiguous ancestral substrate affinity condition for the Gomphales (Fig 1). The most basal lineage of the Gomphales is composed of the lignicolous/terricolous genus *Phaeoclavulina* (Fig 1). Even though some taxa, including *Gautieria*, *Gomphus*, *Hysterangium*, *Ramaria*, and *Turbinellus*, are known mycorrhizal associates (Masui 1926, 1927; Castellano 1988; Miller & Miller 1988; Griffiths et al. 1991; Agerer et al. 1996a, b, c, d; Agerer et al. 1998), the mycorrhizal status of most lignicolous/terricolous species of the orders treated here is still unknown.

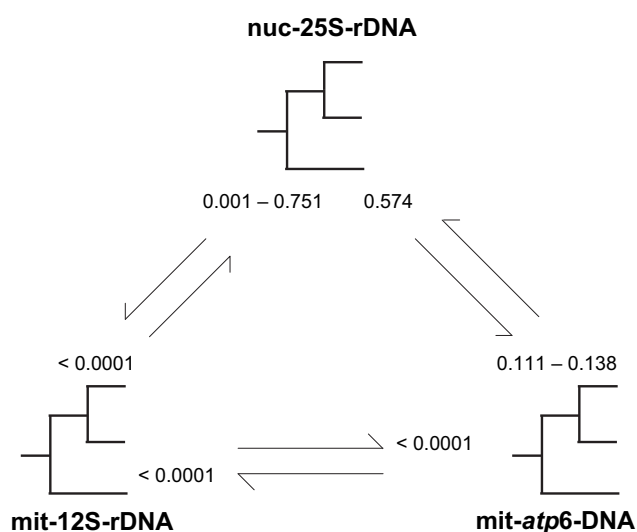


Fig 3 – Shimodaira–Hasegawa compatibility test.

Evolution of clamp connections

The presence of clamp connections is ancestral in the Gomphales with multiple losses of the clamped condition (Fig 1). This is consistent with Corner's (1966) evolution hypothesis for clamp connection in the Gomphales that suggests varying degrees of presence of clamp connections and assumes the "clampless" state as derived from the "clamped state." Humpert et al. (2001), however, showed species of *Ramaria* subgenus *Lentoramaria* without clamp connections to be evolutionarily more basal. Production of clamp connections varies among species of *Beenakia*, *Kavinia*, *Ramaria* subgenera *Laeticolora* and *Ramaria*, and within families of the Geastrales, Hysterangiales and Phallales. Invariable presence of clamp connections is only observed for species of *Clavariadelphus*, *Gomphus*, *Lentaria*, and *Phaeoclavulina*.

Polarity of basidioma morphology

Our data corroborate the hypothesis of Humpert et al. (2001) and Hosaka et al. (2006) of a ramarioid morphology as ancestral for the Gomphales, with multiple derivations of distinct basidiomatal morphologies, i.e., clavate, odontoid, gomphoid, resupinate, and sequestrate (Fig 1). Petersen (1971a) hypothesized the resupinate–odontoid genera *Kavinia* and *Ramaricum* to be derived from *Ramaria* subgenus *Lentoramaria*. Our results are consistent with the hypothesis that *Beenakia*, *Kavinia*, and *Lentaria* are derived from within the clavate, terricolous genus *Clavariadelphus*.

Evolution of sequestrate fungi from epigeous Gomphales

Gautieria is a sequestrate genus (Gautieriaceae) in the Gautieriales (Zeller & Dodge 1918). As shown by Humpert et al. (2001) and Hosaka et al. (2006), and corroborated here, *Gautieria* is closely related to *Ramaria*, more specifically a sister group to *Ramaria* subgenus *Ramaria*, and therefore a member of the Gomphales (Fig 1).

As discussed by Fischer (1933) and Cunningham (1942), *Gautieria* is regarded as coralloid/ramarioid with tramal plates growing outward from a central sterile base, resulting in the formation of pockets or locules. Alternatively, the development of *Gautieria* has also been described as forate by other authors, with formation of the branches occurring from inward growth of exterior tissue (Fitzpatrick 1913; Dring 1973; Miller & Miller 1988). The ridged spores of *Gautieria* were once thought to indicate a close evolutionary relationship with the Boletales (Smith 1973). Because *Gautieria* forms a true hymenium arising from the trama with its basidium tips exposed to an open chamber, it has also been suggested to have evolved from a hymenomycete (Dring 1973), more specifically from a ramarioid ancestor (Humpert et al. 2001). This finding supports the conclusion of Bruns et al. (1998), who showed that *Gautieria* was closely related to *Gomphus*, *Kavinia*, and *Ramaria*. It is also consistent with the findings of Agerer (1999), that the Gomphales, Geastrales, and Gautieriales all have a unique, amputate rhizomorphic morphology in common: they share the ramarioid, not the boletoid type. In addition, species of *Gautieria* and *Ramaria* subgenus *Ramaria* have similar ridged spores. However, the spores of *Gautieria* are statismosporic, whereas

those of *Ramaria* subgenus *Ramaria* are ballistosporic (Humpert et al. 2001). Other cases are known where epigeous and sequestrate taxa differ in the trait of spore symmetry but retain other characteristics such as size, shape and ornamentation (Thiers & Trappe 1969; Thiers 1984; Bruns & Szaro 1992; Mueller & Pine 1994; Hibbett et al. 1997; Lebel 1998).

Conclusion

Our data corroborate previous work rejecting the monophyly of *Gomphus sensu lato* (Giachini 2004; Hosaka et al. 2006). Furthermore, they support the monophyletic status of *Gloeocantharellus*, *Gomphus*, *Phaeoclavulina*, and *Turbinellus* (Fig 1). They also reinforce previous observations (Humpert et al. 2001; Hosaka et al. 2006) on the ramarioid ancestral condition for the Gomphales, and the independent derivations of clavate, gomphoid, odontoid, resupinate, and sequestrate morphologies. The coralloid–ramarioid morphology however, does not indicate a natural monophyletic group. Rather, this condition was gained and lost several times during the course of evolution. These data also suggest an ambiguous character state condition for substrate affinity in the Gomphales.

Fungi within the Geastrales, Gomphales, Hysterangiales and Phallales have been known for years to have important ecological roles (symbionts, decomposers, etc.). Although new information has been added, comparative studies on the anatomy and biochemistry of these taxa are still required to fully unveil the morphological features (synapomorphies) that unite these fungi.

Acknowledgments

This research was supported in part by the Forest Mycology Team, U.S. Forest Service Pacific Northwest Research Station (Forest Service and USDA, Corvallis). The senior author thanks the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of the Brazilian Ministry of Education for the doctoral fellowship. Special thanks to the following collections for loans: BPI, BR, CANB, CUP, DAOM, DAR, F, FH, GH, H, K, L, LPS, M, MICH, NCU, NY, NYS, OULU, OSA, OSC, PC, PDD, PERTH, PH, S, SFSU, TENN, TNS, UC, UPS, WTU, and ZT. Richard Halse of the Department of Botany, Oregon State University, unstintingly arranged loans from herbaria. Andy Taylor provided samples of *G. clavatus* from Sweden, and Leif Ryvarden for specimens of *Beenakia* and *Kavinia*. Thanks to Gi-Ho Sung and Sarah R. Dubrasich for editorial comments on this manuscript.

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