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Phylogeny of *Phellinus* and Related Genera Inferred from Combined Data of ITS and Mitochondrial SSU rDNA Sequences

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Abstract To elucidate phylogenetic relationships of *Phellinus* and its related genera, nuclear internal transcribed spacer and mitochondrial small subunit ribosomal DNA sequences from 65 strains were determined and compared. The combined dataset of two sequences increased informative characters and led to the production of trees with higher levels of resolution. Phylogenetic analysis of the combined dataset revealed thirteen evolutionary lineages and several unresolved species that were together subdivided into two large clusters consisting of oligonucleate species and binucleate species. These results coincided with previous cytological, morphological, and molecular studies. It is newly recognized that the *Phellinus linteus* complex forms a sister clade to *Inonotus*, and that *Fulvifomes* is somehow related to *Inocutis*. The *Phellinus linteus* complex of dimitic perennial taxa made an independent clade from *Inonotus* and suggested that hyphal miticity and fruitbody permanence had enough phylogenetic significance to keep the complex within the traditional genus *Phellinus*. Taxa lacking setae were clustered into *Fulvifomes*, *Phylloporia*, *Inocutis*, and *Fomitiporia*, and the first three were closely related sister groups, but *Fomitiporia* was a genus distantly related to them. Several taxa with branched setae were shown among distantly related genera. Molecular evidence indicated that the ancestral nuclear type could be a binucleate feature, and that there might be parallel gains of branched setae and parallel losses of setae in the Hymenochaetales.

Key words: Hymenochaetaceae, *Inonotus*, karyotypes, setae

Members in the family Hymenochaetaceae are primarily composed of lignicolous species and play a significant role in the ecological functioning of forests. Owing to great decaying abilities, certain *Phellinus* species have been reported to be more active in degrading lignin than *Phanerochaete chrysosporium* Burds, which has been extensively used in clearing pollutants [66], and to have the peculiar lignocellulose biotransformation activity during the solid-state fermentation process of oil-bearing crop wastes [10]. Some members play scavenger roles in forest ecosystems, but others vigorously kill dominant trees, altering the various structures and composition of the forest and causing great economic loss to wood industries [21].

Many species in *Phellinus* Quél. and related genera have been used as folk medicines because of their biochemical or pharmaceutical actions [29]. Twelve species of *Phellinus* have been used as Indian folk medicines [79], and *Inonotus obliquus* (Pers.) Pilát and *P. nigricans* (Fr.) P. Karst. have been used as folk medicines in West Siberia [73]. Chaga or birch fungus that is reputed in Russian folklore to have anticarcinogenic properties is *I. obliquus*, a common circumboreal fungus that causes decay in living birches and develops black clinker-like sterile conks with brown inner tissue [19]. *Phellinus linteus* (Berk. & M.A. Curt.) Teng, called sangwhang in Korea, is also known in Korea, China, and Japan for its anticarcinogenic properties [26, 41, 47, 74]. Aqueous extracts from *P. rhabarbarinus* (Berk.) G. Cunn exhibit strong anti-HIV-1 activity without toxicity on Molt-4 lymphocytic cells [84].

Phellinus, *Inonotus* P. Karst., *Hymenochaete* Lév., *Hydnochaete* Bres., and *Onnia* P. Karst., well-known white rotters, are included in the family Hymenochaetaceae, which was first recognized by Patouillard [64] as a natural group, and the family was then formally described by Donk [11]. These genera are characterized by brown tissues permanently darkening on KOH application (xanthochroic reaction), the absence of clamps, and the presence of a

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continuous parenthesome in the dolipore apparatus [5, 20, 38, 48, 49, 50, 62]. Nowadays, many mycologists accept this family as an order (Hymenochaetales) or as a natural taxonomic unit (Hymenochaetoid clade) [5, 9, 24, 59, 71, 80]. Phylogenetic analyses based on molecular data show that certain members of Corticiaceae (*Basidioradulum* Nobles, *Hyphodontia* J. Erikss.) and Polyporaceae [*Oxyporus* (Bourdot & Galzin) Donk, *Schizopora* Velen., *Trichaptum* Murrill] are also closely related to the Hymenochaetoid clade corresponding to the Hymenochaetales sensu Oberwinkler [23, 28, 33, 34, 37, 65]. Members of this clade appear to be phylogenetically united by the possession of an imperforate parenthesome [36, 48, 78].

However, many homoplasious characters could lead to artificial or incorrect taxonomic conclusions for this seemingly natural taxonomic unit. For this reason, there have been several attempts to split them into a number of smaller subgroups, based on both morphological and molecular characters. From the morphological point of view, the first attempt to split *Phellinus* was carried out by Murrill [51–54], and most of his subgroups have been reconfirmed by Fiasson and Niemelä [16], Ryvarden [71], and Dai [6]. Some subgroups of European *Phellinus* were erected as monophyletic taxa based on molecular approaches [18, 57, 81, 82]. Traditional European taxa of *Inonotus* s. lat. were subdivided into four sections by Donk [12, 13] and Wagner and Fischer [80]. *Hymenochaete* was split into three genera by Ryvarden [69, 70] and three or four sections by Escobar [14] and Léger [43]. However, phylogenetic analysis based on nuclear large subunit ribosomal DNA (nuc-lsu rDNA) showed that *Hymenochaete* was divided into two groups [81].

Onnia is characterized by stipitate to substipitate basidiocarps, duplex consistency of the context, and white pocket rot on conifers, which made it a separate genus. Ryvarden and Gilbertson [72] and Dai and Niemelä [7] included *Onnia* into *Inonotus* s. lat. but their suggestions were not supported by the molecular analysis of Wagner and Fischer [81]. Until recently, molecular sequence data for this natural group, Hymenochaetales or Hymenochaetoid clade, have been confined to several studies, mostly based on European taxa [2, 23, 33, 34, 57, 65, 80, 82, 83]. In this study, using the combined dataset of nuclear internal transcribed spacer (nuc ITS) and mitochondrial small subunit ribosomal DNA (mt-ssu rDNA) sequences, we expanded the taxon sampling and conducted new phylogenetic analyses for *Phellinus*, *Inonotus*, *Hymenochaete*, and *Onnia*.

MATERIALS AND METHODS

Cultures, DNA Extraction, Amplification, and Sequencing

The 63 strains used in this study are listed in Table 1. Cultures were grown on 1.5% malt extract agar (MEA) at 24°C for several days in a biological oxygen demand (BOD) incubator. Voucher specimens were deposited in Seoul National University Fungus Collection (SFC), Seoul, Korea. Total DNA was extracted from herbarium specimens and petri dish-grown mycelia [40] with some modifications [31, 32, 42, 60]. ITS regions including the 3' flanking region of nuclear small subunit ribosomal DNA (nuc-ssu rDNA) and the 5' flanking region of nuc-lsu rDNA were amplified using primers NS7 (5' GAGGCAATAACAGGTCTGTG-ATGC 3') [85] and LW2 (5' CATTCCCAAACAACTCG-ACTC 3') [28]. To amply the internal regions of the mt-ssu rDNA gene corresponding to U2 to U5, primers MSU215 (5' CAAGAATATTAGTCAATGCTC 3') and MSU573 (5' AACATGCTTCACTTCGTTTGCTC 3') were used [28]. Amplifications were carried out in 50 μl of reaction mixture after Lee *et al.* [42] using the PTC-100 Programmable Thermal Cycler (MJ Research, Inc.). Amplified products were purified using Wizard PCR Prep (Promega), and purified PCR products were then directly sequenced by the thermal cyclic termination method with ^{35}S-labeled ATP [25, 86]. The ITS region was sequenced using primers ITS4 and ITS5. The partial mt-ssu rDNA region was sequenced, using forward primers MSU215 and MSU435 and reverse primers MSU413 and MSU573 [28]. Both strands were sequenced with the Top™ DNA sequencing kit (Bioneer Corp.). The sequences obtained in this study were deposited in GenBank (Table 1).

Phylogenetic Analyses

ITS and partial mt-ssu rDNA sequences were aligned using the CLUSTALX program [77] and then finally optimized using the PHYDIT program version 3.1 (http://plaza.snu.ac.kr/~jchun/phydit/)[4]. Parsimony analyses of two sequences were conducted for separate data and combined datasets using PAUP*4.0b4a [76]. The heuristic search options were applied with simple addition sequence, with MULPARS on, and with tree bisection-reconnection (TBR) branch swapping [76]. Combinability of the ITS and mt-ssu rDNA sequence data for a total of 65 taxa was assessed using the partition homogeneity test (PHT) (simple addition sequence, TBR, 1,000 random repartitions, MAXTREES set to 5,000) implemented within PAUP*4.0b4a [76]. Mt-ssu and ITS sequences of three strains, *Ganoderma lucidum* (Curtis) P. Karst. (mt-ssu U27040, ITS AF079584), *Schizopora paradoxa* (Schrad.) Donk (mt-ssu AF026654, ITS AF145571), and *S. radula* (Pers.) Hallenb. (mt-ssu AF069636, ITS AF145565), were retrieved from GenBank as outgroups to root the main tree. For detailed analyses of major groups, only the combined dataset was used because of the small numbers of parsimony-informative characters, and all tree data were deposited at TreeBASE. Ambiguous regions of the alignment were excluded from analyses. Clade stability was assessed by 1,000 bootstrap replications. For the purpose of discussions, we named clades

Table 1. List of species, sources, localities, and GenBank accession numbers of taxa used in this study.

Species	Source	Locality	Taxonomy[a]	GenBank	
				ITS	mt-ssu
Hydnochaete japonica Lloyd	CBS 499.76	Japan	*Hymenochaete* (4)	AY558596	AY558658
Hymenochaete adusta (Lév.) Pat.	CBS 759.91		*Hymenochaete* (4)	AY558594	AY558656
H. denticulata J.C. Léger & Lanq.	CBS 789.91		*Hymenochaete* (4)	AY558595	AY558657
H. spreta Peck	CBS 213.91	Canada	–	AY558597	AY558659
H. tabacina (Sowerby) Lév.	IFO 4969		*Pseudochaete* (4)	AY558598	AY558660
Inonotus andersonii (Ellis & Everh.) Černý	SFCC 50025	Korea	*Inonotus* (3)	AY558599	AY558661
I. cuticularis (Bull.) P. Karst.	IFO 9788		*Inonotus* (3)	AY558600	AY558662
I. dryadeus (Pers.) Murrill	IFO 9352		*Pseudoinonotus* (3)	AY558601	AY558663
I. hispidus (Bull.) P. Karst.	CBS 386.61	UK	*Inonotus* (3)	AY558602	AY558664
I. obliquus (Pers.) Pilát	IFO 8681		*Inonotus* (3)	AY558593	AY558655
I. porrectus Murrill	CBS 296.56	USA	–	AY558603	AY558665
I. tamaricis (Pat.) Maire	CBS 384.72	Turkmenia	*Inocutis* (1, 3, 4)	AY558604	AY558666
Onnia orientalis (Lloyd) Imazeki	IFO 30386		–	AY558606	AY558668
O. tomentosa (Fr.) P. Karst.	CBS 278.55	Germany	*Onnia* (3)	AY558607	AY558669
Phellinus badius (Berk.) G. Cunn.	CBS 449.76	India	*Fulvifomes* (2*)	AY558609	AY558671
P. baumii Pilát	SFCC 50029	China	*Fulvifomes* (2*), *Inonotus* (5)	AY558608	AY558670
P. bicuspidatus Lombard & M. J. Larsen	KCTC 6651	USA	–	AY558610	AY558672
P. caryophylli (Racib.) G. Cunn.	CBS 448.76	India	*Fulvifomes* (2*)	AY558611	AY558673
P. chrysoloma (Fr.) Donk	CFMR 5406	USA	*Porodaedalea* (1, 3)	AY558612	AY558674
P. cinchonensis (Murrill) Ryvarden	CBS 447.76	India	*Fuscoporia* (5)	AY558613	AY558675
P. conchatus (Pers.) Quél.	CBS 167.29	Canada	*Porodaedalea* (1), *Phellinus* (2*, 3)	AY558614	AY558676
P. fastuosus (Lév.) S. Ahmad	CBS 213.36	Philippines	*Fulvifomes* (2*)	AY558615	AY558677
P. ferreus (Pers.) Bourdot & Galzin	CBS 444.48	Canada	*Fuscoporia* (1, 2*, 3)	AY558617	AY558679
P. ferrugineo-velutinus (Henn.) Ryvarden	CBS 218.48		–	AY558618	AY558680
P. ferruginosus (Schrad.) Pat.	KCTC 6652	India	*Fuscoporia* (1, 2*, 3)	AY558616	AY558678
P. fragrans M. J. Larsen & Lombard	CBS 202.90	USA	–	AY558619	AY558681
P. gilvus (Schwein.) Pat.	KCTC 6653		*Fuscoporia* (2*, 5)	AY558620	AY558682
P. hartigii (Allesch. & Schnabl) Pat.	CBS 162.30	Russia	*Fomitiporia* (1, 2, 3)	AY558621	AY558683
P. hippophaeicola H. Jahn	CBS 252.50	Finland	*Fomitiporia* (1, 2, 3)	AY558622	AY558684
P. igniarius (L.) Quél.	CFMR 5698	USA	*Ochroporus* (1), *Phellinus* (2*, 3)	AY558623	AY558685
P. igniarius var. *trivialis* (Bres.: Killerm.) Niemelä	CBS 512.63	Sweden	*Ochroporus* (1), *Phellinus* (2*, 3)	AY558624	AY558686
P. johnsonianus (Murrill) Ryvarden	ATCC 60051	USA	–	AY558625	AY558687
P. laevigatus (Fr.) Bourdot & Galzin	CFMR 5640	USA	*Ochroporus* (1), *Phellinus* (2*, 3)	AY558626	AY558688
P. linteus (Berk. & M.A. Curt.) Teng	SFCC 10208	Korea	*Inonotus* (5)	AY558627	AY558689
	SFCC 10209	Korea	*Inonotus* (5)	AY558628	AY558690
	SFC 990520-2	Costa Rica	*Inonotus* (5)	AY558629	AY558691
P. lundellii Niemelä	CBS 540.72	Finland	*Ochroporus* (1), *Phellinus* (2*, 3)	AY558630	AY558692
P. nigricans (Fr.) P. Karst.	CBS 213.48	Canada	*Ochroporus* (1)	AY558631	AY558693
P. nigrolimitatus (Romell) Bourdot & Galzin	CBS 214.48	Canada	*Ochroporus* (1), *Fuscoporia* (2*), *Phellopilus* (6)	AY558632	AY558694
P. nilgheriensis (Mont.) G. Cunn.	CBS 209.36	USA	–	AY558633	AY558695
P. occidentalis (Overh.: Lombard, R.W. Davidson & Gilb.) Gilb.	CBS 196.55	USA	–	AY558634	AY558696
P. pachyphloeus (Pat.) Pat.	CBS 193.37		*Phellinidiopsis* (2*)	AY558635	AY558697
P. pini (Brot.) A. Ames	KCTC 6655	Canada	*Porodaedalea* (1, 2*, 3)	AY558636	AY558698
	SFCC 50030	China	*Porodaedalea* (1, 2*, 3)	AY558637	AY558699
P. populicola Niemelä	CBS 638.75	Finland	*Ochroporus* (1), *Phellinus* (3)	AY558638	AY558700
P. punctatus (Fr.) Pilát	CBS 386.66	Germany	*Fomitiporia* (1, 2, 3)	AY558640	AY558702
P. repandus (Overh.) Gilb.	CBS 616.89	USA	–	AF534076	AY558703
P. rhabarbarinus (Berk.) G. Cunn.	CBS 282.77		*Fuscoporia* (2*)	AY558642	AY558704
P. ribis f. *ulicis* (Bourdot & Galzin) Pilát	CBS 579.50		*Phylloporia* (2, 3, 7)	AY558644	AY558706
P. robiniae (Murrill) A. Ames	CBS 211.36	USA	*Fulviformes* (1)	AY558646	AY558708

Table 1. Continued.

Species	Source	Locality	Taxonomy[a]	GenBank	
				ITS	mt-ssu
P. robustus (P. Karst.) Bourdot & Galzin	KCTC 6657		*Fomitiporia* (1, 2, 3)	AY558645	AY558707
P. senex (Nees & Mont.) Imazeki	CBS 442.76	India	*Fuscoporia* (2*)	AY558647	AY558709
P. spiculosus (W.A. Campb. & R.W. Davidson) Niemelä	KCTC 6658	USA	-	AY558648	AY558710
P. torulosus (Pers.) Bourdot & Galzin	CBS 182.34	USA	*Fuscoporia* (2*, 3)	AY558649	AY558711
P. tremulae (Bondartsev) Bondartsev & Borissov	CBS 123.40	USA	*Ochroporus* (1), *Phellinus* (2*, 3)	AY558650	AY558712
P. tropicalis M. J. Larsen & Lombard	CBS 617.89	Costa Rica	*Inonotus* (5)	AF534077	AY558713
P. tuberculosus (Baumg.) Niemelä	CBS 171.32	UK	*Ochroporus* (1), *Phellinus* (2*, 3)	AY558652	AY558714
P. viticola (Schwein.) Donk	CBS 381.82	Germany	*Fuscoporia* (1, 2*, 3)	AY558653	AY558715
P. weirianus (Bres.) Gilb.	CBS 618.89	USA	*Inonotus* (5)	AY558654	AY558716
P. weirii (Murrill) Gilb.	KCTC 6646	USA	-	AY558605	AY558667

[a]Current generic positions and parenthesized citations: 1. Fiasson and Niemelä [16]; 2. Dai [6]; 3. Wagner and Fischer [80]; 4. Wagner and Fischer [81]; 5. Wagner and Fischer [82]; 6. Niemelä *et al.* [57]; 7. Wagner and Ryvarden [83]. Asterisked numbers indicate the references of subgeneric use for the currently acknowledged genus.

corresponding to the genus nomenclature that is currently acknowledged [80].

RESULTS

DNA Amplification and Sequence Analysis

Sequences of the ITS region ranged from 560 to 890 bps. Such difference resulted from the size variation of the ITS1 region. Two kinds of fragments were observed in the size of partial mt-ssu rDNAs produced by primers MSU215 and MSU573. Short fragments of 600–700 bps were amplified in most species, whereas long fragments of 2.2 kb were produced in *P. baumii* Pilát, *P. badius* (Berk.) G. Cunn., *P. johnsonianus* (Murrill) Ryvarden, *P. linteus*, *P. repandus* (Overh.) Gilb., *P. weirianus* (Bres.) Gilb., and *I. porrectus* Murrill, indicating a possible presence of an intron. Heuristic searches produced 481 equally parsimonious trees of 1,572 steps for ITS sequence data and 384 equally parsimonious trees of 887 steps for partial mt-ssu rDNA sequences (both ITS and mt-ssu rDNA trees not shown). Low resolution was exhibited for *I. dryadeus* (Pers.) Murrill, *I. hispidus* (Bull.) P. Karst., *I. obliquus*, *I. porrectus*, *Onnia tomentosa* (Fr.) P. Karst., *P. bicuspidatus* Lombard & M. J. Larsen, *P. cinchonensis* (Murrill) Ryvarden, *P. ferreus* (Pers.) Bourdot & Galzin, *P. ferruginosus* (Schrad.) Pat., *P. punctatus* (Fr.) Pilát, and *P. spiculosus* (W.A. Campb. & R.W. Davidson) Niemelä in the ITS tree; and for *O. orientalis* (Lloyd) Imazeki, *P. bicuspidatus*, and *P. spiculosus* in the mt-ssu rDNA tree.

The PHT result for incongruence between two kinds of sequence data was not so significant (P=0.33>0.05), and we concluded that ITS and partial mt-ssu rDNA data could be combined together. The combined dataset of 63 strains comprised 919 characters, out of which 417 were constant and 353 were parsimony-informative. The combined dataset generated 216 most parsimonious trees (MPTs) (TL=2,184 steps, CI=0.365, RI=0.583) (Fig. 1). Analysis of the two individual sequence data produced trees with different

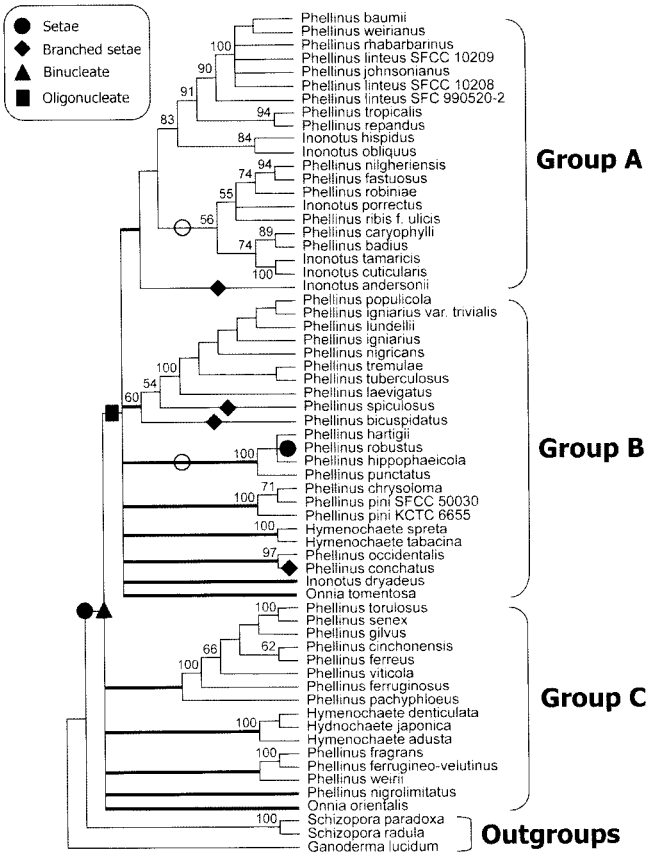

Fig. 1. Strict consensus tree of *Phellinus* and related genera.
This is one of 216 most parsimonious trees inferred from 63 combined data of ITS and mt-ssu rDNA sequences. *Ganoderma lucidum* and two *Schizopora* species were used as outgroups to root the tree. Thirteen evolutionary lineages are shown as thick lines. Bootstrap percentage (1,000 replicates) is indicated for branches supported by more than 50%. Filled symbols indicate gain of setal characters and empty symbols loss of those characters.

levels of resolution, but their topologies were similar in general. Although the ITS region appeared to have more noise than the mt-ssu rDNA, separate sequence data had less informative sites (238 for the ITS region and 158 for the partial mt-ssu rDNA) to infer proper phylogenetic relationships. However, the combined dataset provided enough informative sites (353 for combined ITS and partial mt-ssu rDNAs) and proved to be more confident in phylogenetic inferences. Even though basal relationships were not resolved enough, the strict consensus tree constructed from all taxa revealed thirteen evolutionary lineages that were grouped into two large main clades (Fig. 1). One clade (Groups A and B) included species with an oligonucleate karyotype and the other clade (Group C) included those with a binucleate karyotype in vegetative mycelia [80].

Reanalysis of Main Clades

To increase the resolution of relationships between clades, these three clades were re-analyzed. The re-analyzed Group A of 23 combined sequences yielded 27 most parsimonious trees (TL=482, CI=0.606, RI=0.602). Figure 2 shows the resulting tree, and phylogenetic analysis demonstrated that Group A formed five monophyletic subclades. The grouping branch of these subclades was supported by an 88% bootstrap value. *Phellinus linteus* complex (91% bootstrap value) was characterized by the fact that basidiospores are hyaline or slightly pigmented and negative to Melzer's reagent. *Inonotus* subclade (99% bootstrap value) formed a sister clade to the *P. linteus* complex. However, based on the results of nuc-lsu rDNA sequence data, Wagner and Fischer [82] included dimitic perennial taxa of *Phellinus* [*P. baumii*, *P. linteus*, *P. pachyphloeus* (Pat.) Pat., *P. tropicalis* M. J. Larsen & Lombard, *P. vaninii* Ljub., *P. weirianus*] in the monomitic annual genus, *Inonotus* s. str. (sensu Wagner and Fischer) [80], and combined those six *Phellinus* species into *Inonotus* s. str. Nevertheless, according to our present study, which was based on the ITS and mt-ssu rDNA combined dataset, the *P. linteus* complex containing *P. baumii*, *P. linteus*, *P. tropicalis*, and *P. weirianus* along with *P. johnsonianus*, *P. rhabarbarinus*, and *P. repandus* made a well-supported clade independent from *Inonotus* and other *Phellinus* members, suggesting that hyphal miticity and fruitbody durability have phylogenetic significance and are good enough to keep dimitic perennial taxa of *Phellinus* within the traditional genus *Phellinus*. The *Fulvifomes* [55]

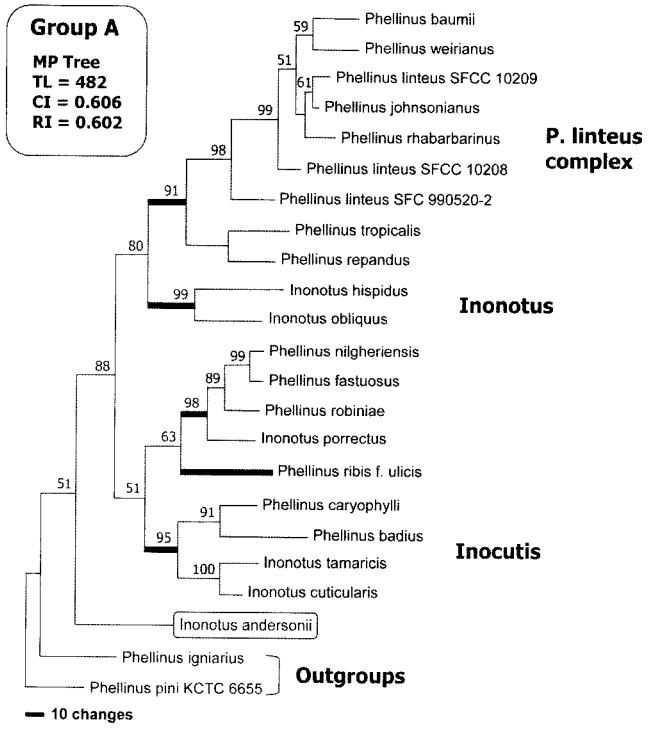

Fig. 2. Most parsimony tree of re-analyzed Group A.
This is one of 27 most parsimonious trees inferred from 23 combined data of ITS and mt-ssu rDNA sequences. *Phellinus igniarius* and *P. pini* were selected as outgroups from Group B. Branches corresponding to generic levels are shown as thick lines and their subclades as shaded areas. Bootstrap percentage (1,000 replicates) is indicated for branches supported by more than 50%.

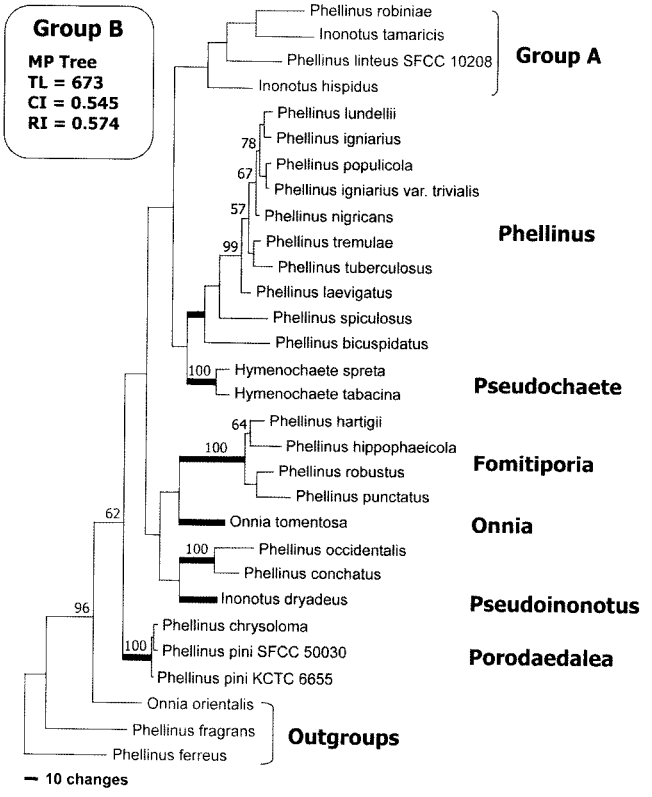

Fig. 3. Most parsimony tree of re-analyzed Group B.
This is one of 31 most parsimonious trees inferred from 30 combined data of ITS and mt-ssu rDNA sequences. *Onnia orientalis*, *P. fragrans*, and *P. ferreus* were selected as outgroups from Group C. Branches corresponding to generic levels are shown as thick lines and their subclades as shaded areas. Bootstrap percentage (1,000 replicates) is indicated for branches supported by more than 50%.

subclade included *P. fastuosus* (Lév.) S. Ahmad, *P. nilgheriensis* (Mont.) G. Cunn., *P. robiniae* (Murrill) A. Ames, and *I. porrectus*. This group has been recognized as a unique one, because all members have similar rusty brown spores, no setae, and ungulate basidiocarps that rapidly become black and rimose. *Phellinus ribis* f. *ulicis* (Bourdot & Galzin) Pilát (*Phylloporia* subclade) [51] was basal to the *Fulvifomes* subclade, and both again formed a sister clade to the *Inocutis* [16] subclade (Fig. 2). *Inonotus andersonii* (Ellis & Everh.) Černý formed a unique branch in the trees (Figs. 1 and 2) and was a basal taxon to Group A containing the *P. linteus* complex, *Inonotus*, *Fulvifomes*, *Phylloporia*, and *Inocutis*.

Parsimony analysis from 30 combined sequences of Group B yielded 31 MPTs (TL=673, CI=0.545, RI=0.574). One of the resulting trees (Fig. 3) for Group B demonstrated six subclades and two species. In Group B of Fig. 1, *P. spiculosus* and *P. bicuspidatus* were basal to the fully supported subclade (100% bootstrap value) of *Phellinus* s. str. (sensu Wagner and Fischer) [80] one by one, but were supported by low bootstrap values (54% and 60%, respectively). *Inonotus dryadeus* and *O. tomentosa* fell within Group B (Fig. 3), where *O. tomentosa* was the sister group of the *Fomitiporia* [54] subclade and *I. dryadeus* (*Pseudoinonotus* subclade) [80] the sister group of *P. occidentalis* (Overh.: Lombard, R.W. Davidson & Gilb.) Gilb. and *P. conchatus* (Pers.) Quél. (Fig. 3), but the bootstrap support of these groups was less than 50%. Without proper bootstrap support, the *Pseudochaete* [81] subclade and the *Phellinus* subclade formed sister groups that again had a sister relationship to Group A. The *Porodaedalea* [27] subclade was the basal lineage to Group B with 62% bootstrap support and eventually to Group A (Fig. 3).

Group C containing binucleate species in the vegetative mycelium was a paraphyletic group. Parsimony analysis of 28 combined sequences of Group C yielded five MPTs (TL=991, CI=0.465, RI=0.448). Figure 4 shows one of five resulting trees. Group C included three monophyletic subclades that were completely supported and four unresolved additional species. The *Hymenochaete* subclade was a well-supported monophyletic group (100%) and showed an adjacent relationship with Groups A and B. The *Fuscoporia* [54] subclade was a sister to the *Phellinidium* [16] subclade, but the bootstrap support for their node was negligible.

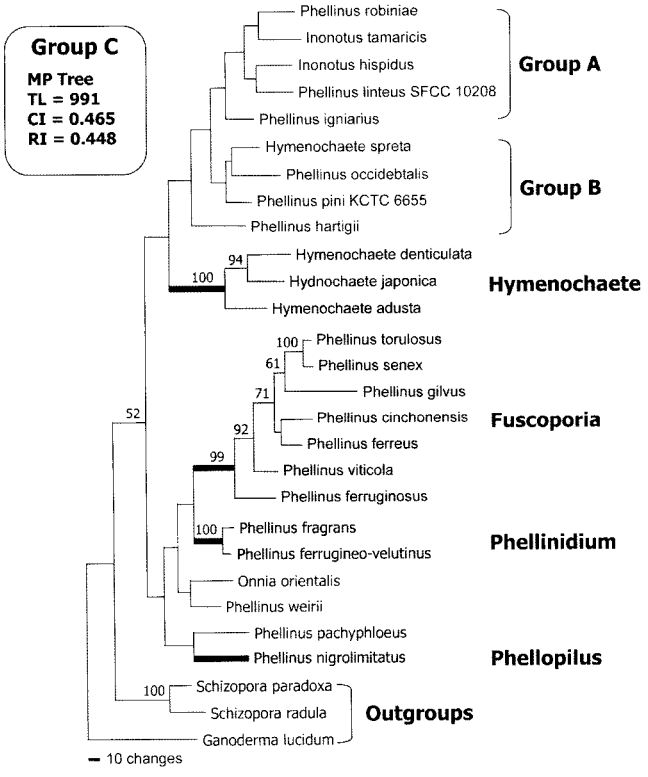

Fig. 4. Most parsimony tree of re-analyzed Group C.
This is one of five most parsimonious trees inferred from 28 combined data of ITS and mt-ssu rDNA sequences. *Ganoderma lucidum* and two *Schizopora* species were used as outgroups. Branches corresponding to generic levels are shown as thick lines and their subclades as shaded areas. Bootstrap percentage (1,000 replicates) is indicated for branches supported by more than 50%.

DISCUSSION

Although some inner branches were not resolved enough, the combined dataset of ITS and mt-ssu rDNA sequences showed that independent lineages were grouped into two large clusters, which correlated with nuclear types. This result coincided with a previous phylogenetic analysis by Wagner and Fischer [80], which was restricted to the European taxa of *Phellinus* and *Inonotus*. Oligonucleate species formed a monophyletic group (Groups A and B, cluster A of Wagner and Fischer [80]) and binucleate species a paraphyletic group (Group C, cluster B of Wagner and Fischer [80]). On trees that we constructed, the dataset analyses indicated that the ancestral karyotype might have been binucleate. This result supports Boidin's hypothesis that fungal evolution progressed from binucleate to multinucleate types [1].

Wagner and Fischer [82] recently established a definition on *Inonotus* s. str. that comprised taxa of annual or perennial basidiocarps and monomitic or dimitic hyphal structures by including some species like *P. baumii*, *P. linteus*, *P. tropicalis*, and *P. weirianus* of *Phellinus* s. lato. However, our results were incongruent with those of Wagner and Fischer [82] and, as shown in Fig. 2, their *Inonotus* s. str. was subdivided into two groups (*Phellinus linteus* complex and *Inonotus* subclade) by high bootstrap values. Some morphological characters also supported these findings. The *Phellinus linteus* complex is well defined by

hyaline to pigmented basidiospores with negative reaction to Melzer's reagent. Host specificity and the basidiocarp habit also could be used as demarcating characters for this complex. *Phellinus linteus* was originally described from Nicaragua, and its local habitats have been reported to range widely from tropical to subtropical regions. Owing to their morphological similarity and ambiguous species concept, *P. linteus* has been continuously confused with *P. baumii*.

Recently, the former fungus has medicinally been highlighted, since its anticarcinogenic effect on some cancer cell lines has been shown in several drug tests [26, 41, 47, 74]. However, Dai and Xu [8] revealed that the Asian fungus was in fact *P. baumii*, and this opinion was supported by the RFLP analysis of Lim *et al.* [44]. Molecular data of Jung *et al.* [30] and Park *et al.* [61] also showed that *P. linteus* from temperate regions was phylogenetically intermingled with *P. baumii*, but Costa Rican *P. linteus* (SFC 990520-2) of a tropical region was positioned outside those temperate taxa (Figs. 1 and 2). Dai [6] treated *P. baumii* within *Phellinus* subgenus *Fulvifomes* and *P. rhabarbarinus* within *Phellinus* subgenus *Fuscoporia*, and Murrill [54] treated *P. johnsonianus* as *Fomitiporella johnsoniana* Murrill. However, based on morphological and molecular analyses, several studies showed that *P. baumii* is closely related to *P. johnsonianus*, *P. linteus*, *P. lonicericola*, *P. lonicerinus*, *P. rhabarbarinus*, and *P. weirianus* [28, 30, 63]. *Phellinus repandus* and *P. tropicalis* have fairly different habitats and are found at different localities. Nevertheless, they formed a clade as a base of the *P. linteus* complex. Because of their microscopic features and molecular evidences, these two taxa need to be included within the *P. linteus* complex.

Phylogenetic Analysis of Group A

In Group A, the *Inonotus* subclade was a sister group to the *P. linteus* complex by the bootstrap value of 80% (Fig. 2). Its generic status was reconfirmed by Fiasson and Niemelä [16] and Wagner and Fischer [80]. This genus is characterized by distinctly colored, non-cyanophilous and non-dextrinoid spores. *Fulvifomes* was treated by Dai as a subgeneric rank of *Phellinus* [6], who also included *P. baumii* and evaluated its status to be somewhat heterogeneous. The detailed description on *Fulvifomes* was originally made by Kotlaba and Pouzar [35], and characteristic features of *Fulvifomes* are large colored spores and complete absence of setae. *Inonotus porrectus* has a substipitate nature of basidiocarp, no setae, and contorted branching hyphae on the pileal surface, suggesting certain affinities with *Coltricia*; however, their relationship has not been supported by molecular data [16].

Phellinus ribis f. *ulicis* (*Phylloporia* lineage) formed a sister taxon to the *Fulvifomes* subclade by a 63% bootstrap value (Fig. 2). Although *Phylloporia* has many characters similar to *Fulvifomes*, it has been treated as a distinct genus [68], because of its rusty brown spores, absence of setae, monomitic hyphal system, and basidiocarps that are ungulate and rapidly become black and rimose. Based on morphological and anatomical characters as well as molecular data, Wagner and Ryvarden [83] suggested that *Phylloporia* had a monophyletic origin. In lineages of *Phellinus*, definitions between mitic systems are so vague that they often change from a monomitic system to a dimitic one or vice versa [80]. *Inocutis* was raised to a generic status from a section by Fiasson and Niemelä [16] and recently accepted by several authors [67, 80]. This genus is characterized by having no setae, a mycelial core, and brownish, non-dextrinoid, and weakly cyanophilous spores. According to the molecular phylogeny of nuc-lsu rDNA [80], three European species, *I. rheades* (Pers.) Bondartsev & Singer, *I. dryophilus* (Berk.) Murrill, and *I. tamaricis* (Pat.) Maire, were included in *Inocutis*. Ryvarden [68] grouped *P. caryophylli* (Racib.) G. Cunn. and *P. badius* into *Fulvifomes*; however, they have no setae, and their brownish spores and fibrous context of parallel arrangement suggest that these two species are closely related to the species of *Inocutis*. On the other hand, *I. cuticularis* (Bull.) P. Karst. has branched setal hyphae and hymenial setae and is distinctly different from other members of *Inocutis*, suggesting a possibility that the *I. cuticularis* IFO 9788 used in this study could be a misidentified species. *Fulvifomes*, *Phylloporia*, and *Inocutis* formed a monophyletic group sharing a common character of having no setae, but the bootstrap support of this group was low (56% in Fig. 1 and 51% in Fig. 2, respectively). *Inonotus andersonii* which develops under the outer layer of sapwood and ruptures the bark is similar in its growth habit and pathogenicity to *I. obliquus* [20]. It was phylogenetically basal to the taxa of other subclades, but its phylogenetic relationship was not yet clear in this study.

Phylogenetic Analysis of Group B

Phellinus s. str. is a well-defined genus by several morphological and molecular characters [15, 16, 80]. This genus is characterized by brown tissues darkening in KOH, formation of a crust on the pileal surface, distinct honeycomb structure in the hymenium, and non-dextrinoid basidiospores. Most species in this genus were phylogenetically confirmed by recent studies based on nuc-lsu rDNA data [80, 82]. In Group B, it was newly confirmed that *P. nigricans* also belongs to the *Phellinus* s. str. subclade (Fig. 3). This species almost always occurs on *Betula* [3] and has been quite similar to *P. lundellii* Niemelä and *P. igniarius* (L.) Quél. [56, 58]. *Phellinus spiculosus* and *P. bicuspidatus* appeared as basal taxa of this subclade, but their branches had low bootstrap values (54% and 60% in Fig. 1 but negligible in Fig. 3). Stalpers [75] showed that *P. spiculosus* had the same species code of Key G as those of *I. andersonii* and *I. nidus-pici* Pilát in cultural characters. However, Gilbertson and Ryvarden [20] reported that this

species is morphologically similar to *P. igniarius* and *P. laevigatus* (Fr.) Bourdot & Galzin. *Phellinus bicuspidatus* is closely related to *P. spiculosus* in pore sizes, hyphal dimensions, and apically branched setae. The occurrence of branched setae in the Hymenochaetales is not rare [45, 46]. Branched setae have been represented among several distantly related lineages of Groups A and B (Fig. 1). Our molecular evidence indicated that there had been parallel gains of branched setae in the Hymenochaetales. Although the phylogenetic positions of *P. spiculosus* and *P. bicuspidatus* were ambiguous because of their low bootstrap supports, these two species should be kept within the *Phellinus* s. str. for the time being.

Hymenochaete differs from *Phellinus* in that the hymenophore is smooth or slightly tuberculate and most of the species are annual. However, it formed polyphyletic groups that were intermingled with other subclades within the Hymenochaetales (Fig. 1). *Hymenochaete* members were subdivided into Groups B and C. *Hymenochaete spreta* Peck and *H. tabacina* (Sowerby) Lév. were included in Group B that has oligonucleate mycelial segments, and *H. adusta* (Lév.) Pat., *H. denticulate* J.C. Léger & Lanq., and *Hydnochaete japonica* Lloyd in Group C that has binucleate mycelial segments [1, 43]. Based on cytological and molecular data, Wagner and Fischer [81] suggested that *Hymenochaete*, *Hydnochaete*, *Stipitochaete* Ryvarden, and *Cyclomyces* Kunze should be placed together within the *Hymenochaete* group and then proposed a new genus *Pseudochaete* to accommodate holocoenocytic *H. tabacina*. *Pseudochaete* originally consisted of a single taxon separated from *Hymenochaete*; however, *H. spreta* was newly added into *Pseudochaete* in our study, and this clade was fully supported and phylogenetically a sister to *Phellinus* (Fig. 3).

According to Wagner and Fischer [80], *Fomitiporia*, *Onnia*, *Pseudoinonotus*, and *Porodaedalea* were well-supported monophyletic groups, but their relationships were somewhat ambiguous. Although most members of *Fomitiporia* do not have setae, it seems not to be an important character for the genus. Absence of setae is found among clades that are distantly related, *Fulvifomes*, *Phylloporia*, *Inocutis*, and *Fomitiporia* (Figs. 2 and 3), indicating that there had been parallel losses of setae in the Hymenochaetales (empty symbols, Fig. 1). *Phellinus conchatus* was treated as a member of the subgenus *Phellinus* by Dai [6] and of the genus *Phellinus* by Wagner and Fischer [80]. However, Núñez and Ryvarden [58] separated it from other species of *Phellinus* s. str. for reasons of large and often deformed setae. *Phellinus conchatus* was also regarded as a species more related to *Porodaedalea* than to *Phellinus* [16, 17, 20, 22]. However, *P. conchatus* was fully related to *P. occidentalis* by 100% bootstrap value (Fig. 3). Grouping two species is morphologically supported by ovoid to subglobose, hyaline, smooth basidiospores, and large ventricose setae. Those species were included in the big clade containing *Fomitiporia*, *Onnia*, and *Pseudoinonotus*, but its position within the clade was still unresolved (Fig. 3).

Phylogenetic Analysis of Group C

In Group C, there were included three well-supported clades, *Fuscoporia*, *Phellinidium*, and *Hymenochaete*, and four unresolved species (Figs. 1 and 4). *Fuscoporia* is characterized by hyaline thin-walled spores and encrustations on generative hyphae. European taxa such as *P. torulosus* (Pers.) Bourdot & Galzin, *P. ferreus*, *P. viticola* (Schwein.) Donk, and *P. ferruginosus* were included in this group, based on morphological [16] and molecular [80] considerations. *Phellinus senex* (Nees & Mont.) Imazeki and *P. gilvus* (Schwein.) Pat. were treated by Dai [6] as members of this group. *Phellinus cinchonensis* is well characterized by hyaline, thin-walled, cylindrical to oblong-ellipsoid spores, soft, silky, reddish brown context with a thin superficial crust, and abundant, subulate hymenial setae. It was originally treated as a member of *Fomitiporia* [54], but its morphological features and molecular data supported *P. cinchonensis* to be grouped into the *Fuscoporia* group. *Phellinidium* comprised two taxa of *P. ferrugineo-velutinus* (Henn.) Ryvarden and *P. fragrans* M. J. Larsen & Lombard. Their most striking features are the macrosetae (also called setoid skeletal hyphae) that predominate in both trama and context and overshadow true hyphae. Their spores are ellipsoid to cylindrical, non-dextrinoid, and hyaline in common.

Characteristic features of *P. nigrolimitatus* (Romell) Bourdot & Galzin are the stratified structure of the context and the thin-walled spores of distinctive carrot shape. The curious habit to produce a soft-fibrous spongy context is also characteristic of this species. Niemelä *et al.* [57] confirmed that *P. nigrolimitatus* differs enough to be separated into a new genus, *Phellopilus* Niemelä, Wagner and Fischer. The phylogenetic position of *Phellopilus*, based on nuc-lsu rDNA analysis, was positioned at the basal region of *Fuscoporia* and *Phellinidium* [57], agreeing with one of our combined dataset analyses (Fig. 4). *Phellinus pachyphloeus* is taxonomically close to the genus *Phellinidium*, and both species and genus have a monomitic hyphal structure and hyphoid setae in both context and trama. However, colored spores and true hymenial setae differentiate *P. pachyphloeus* from *Phellinidium*; therefore, Dai [6] suggested a new *Phellinus* subgenus, *Phellinidiopsis* Dai, and placed *P. pachyphloeus* into this subgenus.

The light soft context and marginal tissue, hyaline ovoid spores, and conspicuous setal hyphae are important characters of *P. weirii* (Murrill) Gilb. It is similar to *P. ferrugineo-fuscus*, but the latter has narrow cylindrical spores and tramal setal hyphae that project into the hymenium with the tips curved and perpendicular to the axis of the tube [20]. *Phellinus weirii*, the causal agent of

laminated root rot in Douglas fir and other conifers, is considered to be the most economically damaging pathogen in the valuable Douglas fir forests west of the Cascade Mountains [21, 39]. No significant conclusion about phylogenetic relationships could be drawn from four taxa of Group C; *O. orientalis*, *P. weirii*, *P. pachyphloeus*, and *P. nigrolimitatus*. Morphological, cytological, and molecular features support their being put in Group C, but more strains of these species need to be analyzed in future studies.

When *Phellinus* and related genera are phylogenetically compared, there are thirteen evolutionary lineages and several unresolved species that are altogether subdivided into two large clusters according to karyotypes of the vegetative mycelium. Contrary to the recent conclusion based on the nuc-lsu rDNA sequence data [82], the *Phellinus linteus* complex of dimitic perennial taxa was newly recognized as an independent sister clade to *Inonotus* s. str., suggesting that hyphal miticity and fruitbody durability have phylogenetic significance, in addition to nuclear patterns, morphology, reactions and pigments of basidiospores, or setal features and characters, and also showing that it is recommendable to rely on multi-gene sequence data for inclusive phylogenetic conclusions. Setae have been suggested as one of the most important characters in *Phellinus* and related groups, and it is assumed that there might have been parallel gains of branched setae and parallel losses of ordinary setae during evolution. Traditional taxonomic systems or informations on phylogeny are often controversial, and taxonomic characters that are considered to be important for classification of these taxa often seem to be too homoplasious, thus leading to artificial or false systematic conclusions. For the present, additional taxa of systematic importance still need to be included and evaluated for comprehensive and reliable phylogenetic conclusions on the Hymenochaetales.

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