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Phylogenetic positions of rust fungi parasitic on ferns: Evidence from 18S rDNA sequence analysis

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Molecular phylogenetic analyses of fern rusts were carried out based on 18S rDNA sequences. We sequenced the 18S rDNAs of fern rusts (*Hyalopsora polypodii* and *Uredinopsis intermedia*) and non-fern rusts (*Aecidium epimedii, Coleosporium asterum, Ochropsora kraunhiae, Puccinia suzutake* and *Physopella ampelopsidis*) and analyzed their phylogenetic relationships with other members of the Urediniomycetes. Our bootstrapped neighbor-joining tree obtained from these analyses showed that rust fungi were apparently monophyletic at high confidence level (100% bootstrap confidence). In this molecular phylogenetic tree, the two fern rusts did not occupy the basal position within the rust fungal lineage and did not form a monophyletic lineage. Two species of the Cronartiaceae (*Peridermium harknessii, Cronartium ribicola*) and one species of the Coleosporiaceae (*Coleosporium asterum*) grouped with the fern rusts. Therefore, our results suggested that the two fern rusts were not primitive. On the other hand, *Mixia osmundae*, which is parasitic on the primitive fern *Osmunda*, was phylogenetically far from the fern rusts.

Key Words——18S rDNA; fern parasites; molecular phylogenetics; rust fungi; Urediniomycetes.

There are 5,000–6,000 species of rust fungi belonging to 12 families and 100–150 genera (Alexopoulos et al., 1996; Cummins and Hiratsuka, 1983). In the phylogeny of rust fungi, their coevolution with host plants has been suggested, and three genera parasitic on ferns (*Uredinopsis* Magnus, *Milesina* Magnus and *Hyalopsora* Magnus) have been considered as the most primitive rusts (Ando, 1984; Arthur, 1923; Leppik, 1953; Savile, 1955). Of these, *Uredinopsis* was considered to be the most primitive extant rust because it has the simplest teliospores and is the only genus that includes a species parasitic on *Osmunda*, a more primitive fern genus than the other genera of the Polypodiaceae (Arthur, 1923; Savile, 1955). Ando (1984), however, hypothesized that *Milesina* was the most primitive of these three genera, based mainly on spermogonial and telial morphology. Savile (1955, 1968) also speculated that the rusts were derived from *Taphrina*-like ancestors parasitizing ancient ferns or fern ancestors. Leppik (1961) and Donk (1972, 1973), on the other hand, speculated that the rusts evolved from parasitic Auriculariales. Several other hypotheses on the origin of rusts have also been proposed (Hiratsuka and Sato, 1982; Petersen, 1974).

Hart (1988) studied the coevolution of rust fungi and host plants using cladistic analyses of both hosts and parasites. He suggested that the fern rusts were not the basal group of rust phylogeny, that rusts and their hosts have not necessarily undergone a long period of coevolution, and that host transfer has probably occurred as frequently as coevolution.

Nevertheless, evolutionary relationships between fern rusts and *Mixia osmundae* (T. Nishida) Kramer parasitic on *Osmunda japonica* are suggested by the fact that *M. osmundae* was transferred from the Ascomycota to the Basidiomycota and accommodated within the simple septate basidiomycete lineage (Urediniomycetes, sensu Swann and Taylor, 1995a) based on evidence from the 18S rDNA phylogeny and morphology (Nishida et al., 1995). Nishida et al. (1995) also reported that the major phylogenetic lineage including *M. osmundae* contained the two species of rust fungi, *Cronartium ribicola* J. C. Fischer and *Peridermium harknessii* J. P. Moore.

The interrelationships of rust fungi and their phylogenetic relationships to other fungi based on molecular phylogenetic analyses are poorly understood because rust fungi are obligate biotrophs that are impossible or difficult to obtain or maintain in pure cultures. However, the polymerase chain reaction (PCR) technique has allowed analyses of sequences from unculturable organisms such as members of the Uredinales, Erysiphales, and Laboulbeniales. Such molecular data perhaps may provide a precise tool with which to estimate the "true" phylogeny of rust fungi.

Therefore, we analyzed the 18S rRNA gene sequences of seven species of rust fungi including two fern rust fungi, *Hyalopsora polypodii* (Dietel) Magnus and *Ure-*

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dinopsis intermedia Kamei, to clarify their phylogenetic positions in the Urediniomycetes.

Materials and Methods

Fungal materials Fresh materials of the rust fungi were collected in the field and identified based on their morphological characteristics (Hiratsuka et al., 1992). Their states, hosts, locality and date of sampling are listed in Table 1.

Polymerase Chain Reaction (PCR) amplification of the 18S rRNA gene The spots of rust pustules on host leaves were removed with sterile toothpicks and suspended in sterile distilled water. This spore suspension was used directly for the amplification of 18S rRNA coding region sequence by PCR. The following primers were used for PCR: NS1, NS2, NS3, NS4, NS5, NS6, NS7 (White et al., 1990), M13 Forward (-21) Primer 5′-TTCAAAGTAAAAGTCCTGGTT-3′ and M13 Reverse Primer 5′-GATCCTTCTGCAGGTTCACC-3′. The PCR reaction was carried out using PCR Beads (Pharmacia) under the following conditions: 30 cycles of 94°C for 1 min (first cycle, 4 min), 53°C for 1 min, and 72°C for 2 min (last cycle, 8 min).

DNA sequencing The fresh PCR products were cloned using TA Cloning Kit (Invitrogen) (Andres et al., 1993). Plasmids were isolated and purified using a QIAprep Spin Miniprep Kit (QIAGEN), and sequenced using a Dye Primer Cycle Sequencing Ready Reaction -21 M13 (Perkin-Elmer Applied Biosystems). Two PCR products were directly sequenced without TA cloning using a Dye Terminator Cycle Sequencing Ready Reaction. Data collection was performed on an Applied Biosystems 373A automated DNA sequencer.

Phylogenetic analysis Multiple alignment was performed using CLUSTAL W ver. 1.7 (Thompson et al., 1994). We then omitted the positions with gaps and manually adjusted the sequences for phylogenetic analysis. We also included other published 18S rDNA sequence data in our analysis (Table 2). The aligned sequence data file is obtainable from the corresponding author. The distance matrix for the aligned sequences was calculated by using the two-parameter method (Kimura, 1980). The neighbor-joining (NJ) method (Saitou and Nei, 1987) was used in order to construct all phylogenetic trees. Bootstrap resamplings (Felsenstein, 1985) were used to estimate the reliability of the inferred tree.

Results and Discussion

As shown in Table 1, we determined nearly full sequences of the 18S rRNA gene of fern rusts (three sequences of *H. polypodii* on two different host plants, *Leptogramma mollisima* and *Athyrium yokoscense*, and one sequence of *U. intermedia* on *Athyrium* sp.), and partial sequences of the 18S rRNA gene of non-fern rusts (*Aecidium epimedii* Hennings & Shirai, *Coleosporium asterum* (Dietel) H. & P. Sydow, *Ochropsora kraunhiae* (Dietel) Dietel, *Puccinia suzutake* Kakishima & S. Sato, and *Physopella ampelopsidis* (Dietel & P. Sydow) Cummins & Ramachar). Table 2 shows the sequence lengths. In addition we retrieved another four sequences of the 18S rDNA of non-fern rusts from a DNA database and used them in the phylogenetic analysis. They were *C. ribicola*, *Gymnoconia nitens* (Schweinitz) F. Kern & H. W. Thurston, *Nyssopsora echinata* (Leveille) Arthur and *P. harknessii* (Table 2).

The 18S rDNA of *H. polypodii* on *A. yokoscense* revealed two different sequences, *Hp*1 and *Hp*2, which differed in 9 of the 1737 nucleotides compared. Both were isolated from the same individual host frond. Hiratsuka et al. (1992) reported morphological variations of *H. polypodii* in the size and wall thickness of urediniospores, and in the presence of paraphyses. However, we cannot determine whether this was caused by species diversity (or species convergency) or ribosomal DNA polymorphism.

The phylogenetic tree (Fig. 1) for selected taxa of the Urediniomycetes (sensu Swann and Taylor, 1995a) was constructed using the NJ method. The Urediniomycetes are divided into four major clusters (with $>95\%$ boot-

Table 1. The rust fungi used in this study and their hosts.

Species	State[a]	Host plant	Locality	Date of collection	Voucher specimen no.[b]
Aecidium epimedii	I	*Epimedium grandiflorum*	Nobeyama, Minamimakimura, Nagano Pref., Japan	26. vii. 1997	TSH-R1643
Coleosporium asterum	II	*Aster ageratoides*	Mt. Tsukuba, Ibaraki Pref., Japan	13. ix. 1997	TSH-R1644
Hyalopsora polypodii (*Hp*1, 2)	II	*Athyrium yokoscense*	Ikawa, Shizuoka-shi, Shizuoka Pref., Japan	5. vii. 1997	TSH-R1620
H. polypodii (*Hp*)	II	*Leptogramma mollisima*	Mt. Tsukuba, Ibaraki Pref., Japan	13. ix. 1997	TSH-R1625
Ochropsora kraunhiae	II	*Wisteria brachybotrys*	Mt. Tsukuba, Ibaraki Pref., Japan	13. ix. 1997	TSH-R1645
Physopella ampelopsidis	II	*Partenocissus tricuspidata*	Mt. Tsukuba, Ibaraki Pref., Japan	13. ix. 1997	TSH-R1647
Puccinia suzutake	I	*Hydrangea hirta*	Mt. Tsukuba, Ibaraki Pref., Japan	13. ix. 1997	TSH-R1646
Uredinopsis intermedia	II	*Athyrium* sp.	Hanazono Valley, Ibaraki Pref., Japan	27. viii. 1997	TSH-R1624

a) I, aecial state; II, uredinial state (Cummins and Hiratsuka, 1983).
b) TSH: Mycological Herbarium, Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba.

Table 2. Strains or specimens compared in this study and their 18S rRNA gene sequence accession numbers.

Taxon	Strain or specimen no.[a]	DNA database accession no.
Ingroup		
1. Urediniomycetes		
Aecidium epimedii	TSH-R1643	AB011050 (1,649 bp)[b]
Agaricostilbum hyphaenes	RJB 75-6661-A9	U40809
Bensingtonia yuccicola	CBS 7331	U40810
Coleosporium asterum	TSH-R1644	AB011052 (1,271 bp)[b]
Cronartium ribicola	Unknown	M94338
Erythrobasidium hasegawianum	IAM 12911	D12803
Gymnoconia nitens	Unknown	U41565
Helicobasidium corticioides	CBS 304.65	U75303
H. mompa	CBS 278.51	U77064
Hyalopsora polypodii	TSH-R1620 (Hp1)	AB011016 (1,737 bp)[b]
H. polypodii	TSH-R1620 (Hp2)	AB011017 (1,737 bp)[b]
H. polypodii	TSH-R1625 (Hp)	AB011015 (1,747 bp)[b]
Mixia osmundae	IFO 32408	D14163
Nyssopsora echinata	ECS 244	U77061
Ochropsora kraunhiae	TSH-R1645	AB011051 (1,423 bp)[b]
Peridermium harknessii	RUR-152	M94339
Physopella ampelopsidis	TSH-R1647	AB011054 (1,404 bp)[b]
Puccinia suzutake	TSH-R1646	AB011053 (1,249 bp)[b]
Rhodosporidium toruloides	IAM 13469	D12806
Rhodotorula graminis	NCYC502	X83827
R. lactosa	CBS 5826	D45366
R. minuta	CBS 319	D45367
R. mucilaginosa	NCYC 63	X84326
Sakaguchia dacryoidea (as '*dacryoides*')[c]	IAM 13522	D13459
Sporidiobolus johnsonii	41.1 Wells	L22261
Sporobolomyces roseus	MUCL 30251	X60181
Uredinopsis intermedia	TSH-R1624	AB011018 (1740 bp)[b]
Outgroups		
2. Hymenomycetes		
Coprinus cinereus	Unknown	M92991
Filobasidium floriforme	CBS 6241	D13460
3. Ustilaginomycetes		
Ustilago hordei	Unknown	U00973

a) CBS=Centraalbureau voor Schimmelcultures, Delft, The Netherlands; IFO=Institute for Fermentation, Osaka, Japan; IMI=CAB International Mycological Institute, Bakeham Lane, Egham, UK; MUCL=Mycotheque de l'Universite Catholique, Louvain-la-Neuve, Belgium; TSH=The Mycological Herbarium, Institute of Agriculture and Forestry, University of Tsukuba, Japan.

b) New sequences determined in this study.

c) See Index of Fungi **9**: 507. 1995.

strap support): the *Erythrobasidium/Sakaguchia* lineage (I), the *Agaricostilbum/Bensingtonia* lineage (II), the *Rhodosporidium/Sporidiobolus* lineage (III), and the rust fungi/*Helicobasidium* lineage (IV). This result agrees with the phylogenetic analyses by Swann and Taylor (1995a, b). However, the order of divergence of the major lineages was different. The *Erythrobasidium/Sakaguchia* lineage diverged earlier than other Urediniomycetes. In addition, the placement of *M. osmundae* by Nishida et al. (1995) is still uncertain within the Urediniomycetes.

Our molecular phylogeny (Fig. 1) clearly indicates the monophyly of members of the rust fungi, which is statistically supported (100% bootstrap confidence), and of this group with saprobic yeasts in the Urediniomycetes. The rusts and two species of *Helicobasidium* (*H. mompa* Tanaka and *H. corticioides* Bandoni) clustered together in a monophyletic group which reflects the plant parasitic fungal lineage. These *Helicobasidium* species are cause of violet root rot in a number of plants and are

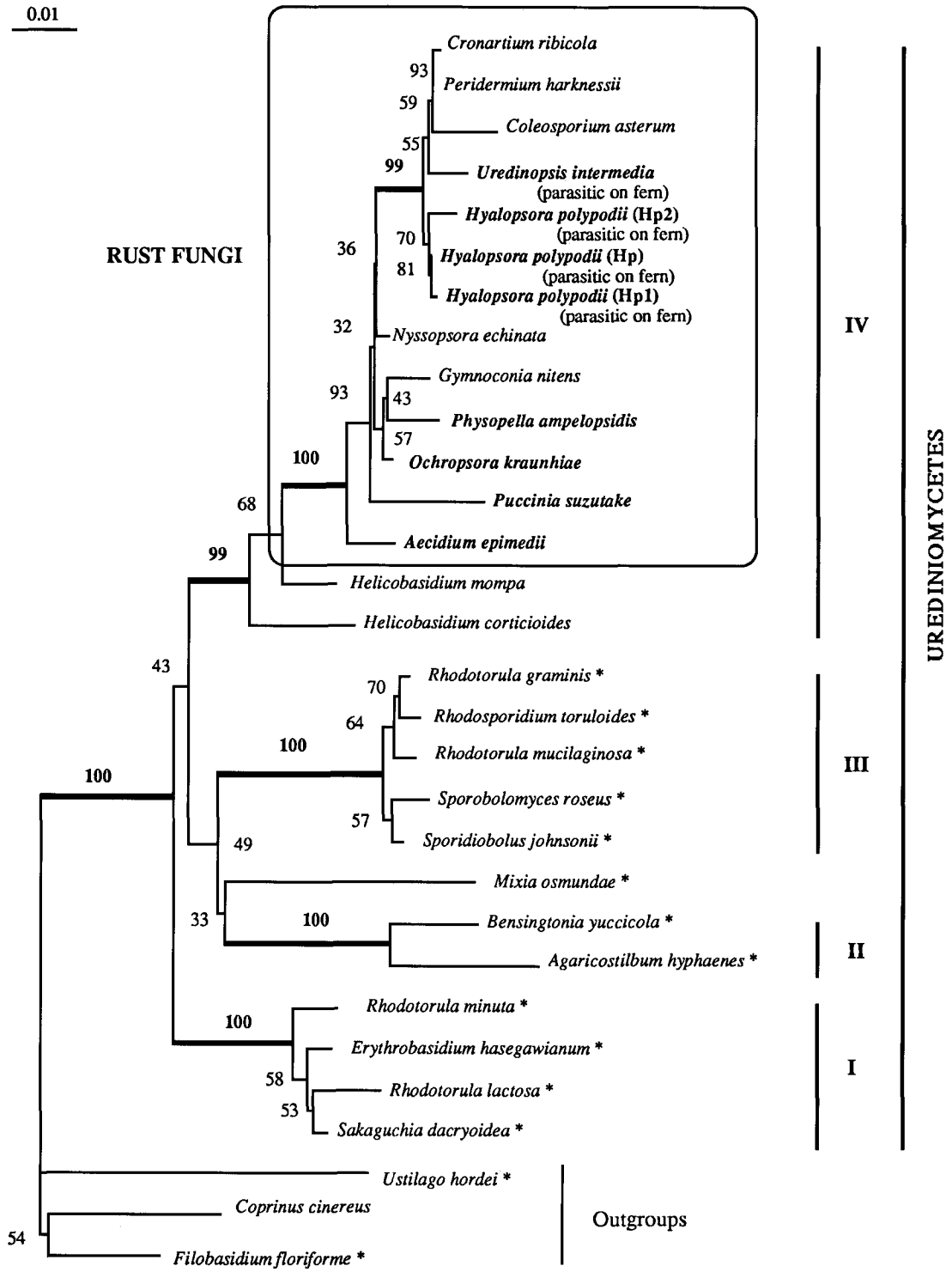

Fig. 1. Neighbor-joining tree, inferred from 1,052 sites of the 18S rDNA sequences, shows the relationships between rust fungi and other related basidiomycetes.
The species, newly sequenced in this study, are indicated in boldface. The scale bar indicates one base change per 100 nucleotide positions. *Coprinus cinereus*, *Filobasidium floriforme* and *Ustilago hordei* were used as outgroups. Thick internodes indicate ≧99% bootstrap support of 1,000 replicates. I=*Erythrobasidium/Sakaguchia* lineage; II=*Agaricostilbum/Bensingtonia* lineage; III=*Rhodosporidium/Sporidiobolus* lineage; IV=rust fungi/*Helicobasidium* lineage. Asterisk (*) indicates species with a yeast state.

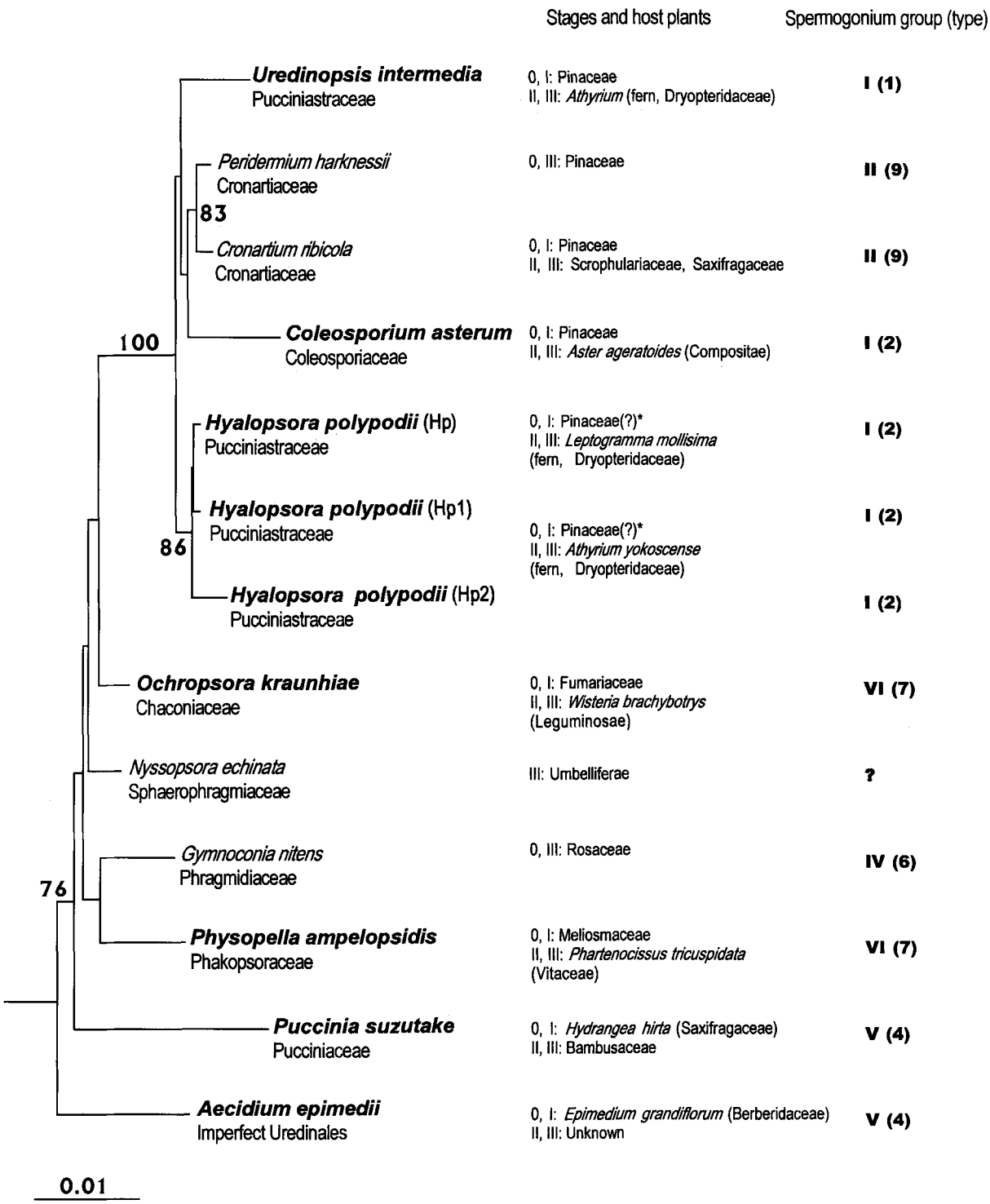

Fig. 2. Neighbor-joining tree, inferred from 1,166 sites of 18S rDNA sequences, shows the relationships among rust fungi. The species newly sequenced in this study are indicated in boldface. The scale bar indicates one base change per 100 nucleotides positions. *Helicobasidium corticioides* and *H. mompa* were used as outgroups. Family names, terminology of spore states and spermogonium group (type) are based on Cummins and Hiratsuka (1983): O=spermogonia; I=aecia; II=uredinia; III=telia; IV=basidia. *=Spermogonial and aecial states are unknown but it is suggested to produce these states on species of *Abies* (Pinaceae).

basal to the rusts. The genus *Helicobasidium* has been accommodated within the family Platygloeaceae in the order Platygloeales within the Ustomycetes (Hawksworth et al., 1995). This genus was previously placed in the family Auriculariaceae in the class Hymenomycetes (McNabb, 1973). This suggests the need for further reconsideration of the phylogenetic hypotheses concerning the origin of rust fungi, as proposed by many authors (Donk, 1972, 1973; Hennen and Buritica, 1980; Hiratsuka and Sato, 1982; Leppik, 1961; Petersen, 1974; Savile, 1955, 1968).

Our tree (Fig. 1) supports the previously published molecular-based phylogenies showing that members of the Uredinales are more advanced taxa arising from the simple-septate Auriculariales s. lat. on mosses, ferns, and some angiosperms, such as *Jola* Moller, *Eocronartium* Atk., *Helicobasidium* Pat., *Helicogloea* Pat., *Herpobasidium* Lind, *Platycarpa* Couch and *Septobasidium* Pat. (McLaughlin et al., 1995; Swann and Taylor, 1995a, b). To date, however, Swann and Taylor's 18S rDNA sequence data (except for *Helicobasidium* spp.) are not available in the DNA database.

Our 18S rDNA sequence-based NJ tree (Fig.1) shows that *M. osmundae*, which is parasitic on the primitive fern *Osmunda*, is phylogenetically distant from the fern rusts. Our molecular phylogeny suggests that the fern rusts are not primitive phylogenetically (Fig. 2). None of the fern rusts connected directly to the origin of rusts. Therefore, we suggest reconsideration of the phylogenetic hypothesis, based mainly on host plants, morphology and life cycle (Ando, 1984; Arthur, 1923; Leppik, 1953; Savile, 1955), that fern rusts are most primitive among rust fungi. Hart (1988) carried out cladistic analyses based on morphological characters and suggested that tropical short-cycle rusts on angiosperms form the cladistically basal group of rusts, while the rusts on conifers and ferns form a nested terminal clade. This suggestion is very useful for our molecular analyses.

The fern rusts grouped with two species of the Cronartiaceae (*C. ribicola*, *P. harknessii*) and one species of the Coleosporiaceae (*C. asterum*). It is interesting that these rust fungi parasitic on Pinaceae were grouped together, suggesting that the rusts and their hosts plants are in someway related to one another. *Cronartium ribicola* and *Coleosporium asterum* produce spermogonia and aecia on species of *Pinus*, and *P. harknessii* produces peridermioid telia on species of *Pinus*. *Uredinopsis intermedia* produces spermogonia and aecia on species of *Abies*. The life cycle of *H. polypodii* is unknown, but it has been suggested that this species has spermogonia and aecia on species of *Abies* (Cummins and Hiratsuka, 1983). The close relationship between fern rusts and coniferous ones may be reflected in the host transfer via the spermogonial and aecial states suggested by Leppik (1953).

Hiratsuka and Cummins (1963) emphasized the significance of spermogonial morphology in rust taxonomy. The morphological types of spermogonia have been considered to be reliable characters for use in suprageneric taxonomy. Twelve types and six groups have been recognized by Hiratsuka and Cummins (1963) and Cummins and Hiratsuka (1983). They considered types 1, 2, 3, and 8 to be relatively basic types of spermogonia and proposed possible lines of evolution based on these spermogonial types. However, our phylogenetic tree (Fig. 2) did not support this hypothesis.

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