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FULL PAPER

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Phylogenetic relationships on 14 morphologically similar species of *Pucciniastrum* in Japan based on rDNA sequence data

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Abstract We analyzed the phylogenetic relationships of 49 specimens comprising 14 morphologically similar species of *Pucciniastrum* distributed in Japan based on the sequence data of the large subunit rDNA (D1/D2), 5.8S rDNA, and internal transcribed spacer (ITS) regions. Neighbor-joining and parsimony analyses generated six major groups for both the D1/D2 and ITS regions. *Pucciniastrum circaeae* and *P. epilobii* formed a single group. *P. hydrangeae-petiolaris*, *P. coryli*, *P. fagi*, *P. hikosanense*, *P. tiliae,* and *P. boehmeriae* were each a distinct clade, and *P. fagi* formed a close relationship with *P. hikosanense.* However, these analyses did not support the monophyly of the following species: *P. kusanoi*, *P. actinidiae*, *P. corni*, *P. styracinum*, *P. yoshinagai*, and *P. miyabeanum.*

Key words ITS1–5.8S–ITS2 · LSU rDNA · Molecular phylogeny · *Pucciniastrum* · Rust fungus

Introduction

The genus *Pucciniastrum* was established by Otth in 1861 based upon its type species, *Pucciniastrum epilobii* Otth on *Epilobium angustifolium* L. Many additional species were thereafter described (Hiratsuka 1936). Hiratsuka (1958) revised the taxonomy of the Pucciniastreae, emphasizing the position of the telia in the plant tissue as an important taxonomic characteristics, separated *Thekopsora* Magnus and *Calyptospora* J.G. Kühn from the genus *Pucciniastrum*. Recently, Cummins and Hiratsuka (2003) also recognized *Pucciniastrum* as a distinct genus based on the following characteristics: ostiolar peridial cells in uredinia, subepidermal telia, and 2–4-celled teliospores divided by vertical or oblique septa without pedicels (Hiratsuka 1958; Sato et al. 1993).

Hiratsuka (1958) accepted 23 species in *Pucciniastrum*. In following years, Laundon (1963) described 1 species, *P. magnisporum* Laundon on *Acer davidi* Franch. and *A. rubescens* Hayata. Liang et al. (2005) described a new species, *Pucciniastrum hakkodaense* Y.M. Liang & Kakishima on *Enkianthi campanulati* (Miq.) G. Nicholson. To date, of 25 species listed under *Pucciniastrum,* most species are heteroecious and have macrocyclic life cycles (Cummins and Hiratsuka 2003).

Of the 25 species of *Pucciniastrum*, 22 species have been reported in Japan (Hiratsuka et al. 1992; Liang et al. 2005). These species can be divided into four morphological groups based on the characteristics of ostiolar peridial cells in the uredinia. One of these groups has smooth ostiolar cells and contains 16 species (referred to as group III in Hiratsuka et al. 1992), which are separated into different species mainly based on the shape and size of urediniospores. However, there are no clear morphological distinctions among these species. Therefore, they were identified based on uredinial and telial host ranges (Hiratsuka 1958; Hiratsuka et al. 1992).

In recent years, there have been many molecular phylogenetic studies of rust fungi that were shown to be very useful in establishing phylogenetic relationships in fungi. Sjamsuridzal et al. (1999) utilized molecular methods to determine relationships among the rusts that infect ferns. Maier et al. (2003) analyzed sequences of large subunit (LSU) rDNA to discuss suprageneric relationships of the rust fungi. Wingfield et al. (2004) employed the sequence data from the small subunit (SSU) rRNA to infer phylogenetic relationships in the Uredinales. Moreover, molecular phylogenetic studies on the genus have also been successfully applied to solve the phylogenetic relationships among rust fungi that were morphologically similar, for example, in

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the genera *Puccinia* (Zambino and Szabo 1993; Weber et al. 2003; Chung et al. 2004), *Melampsora* (Smith et al. 2004; Tian et al. 2004; Pei et al. 2005), *Melampsoridium* (Kurkela et al. 1999), and *Cronartium* (Vogler and Bruns 1998).

Maier et al. (2003) reported a phylogenetic analysis of genera in the Uredinales, which included two species of *Pucciniastrum* group III. These two species are phylogenetically very close to each other although their host plants are different. Therefore, phylogenetic relationships among morphologically similar species of *Pucciniastrum* are important to evaluate the systematics of these species. The sequence data of the LSU rDNA (D1/D2) region of the 28S rDNA and the internal transcribed spacer (ITS) region including the 5.8S rDNA were used to analyze the phylogenetic relationships among the morphologically similar species of *Pucciniastrum* group III in Japan.

Materials and methods

Specimens examined

Forty-nine specimens, constituting 14 species of group III were used for the phylogenetic analysis. Two species were not included because specimens were not available. Some specimens were freshly collected from different districts in Japan by the authors, although most specimens were loaned from the following herbaria: the Hiratsuka Herbarium (HH) in Tokyo; the Herbarium of Systematic Mycology, the College of Education, Ibaraki University (IBA), Mito; and the Mycological Herbarium of the Graduate School of Life and Environmental Sciences, University of Tsukuba (TSH), Tsukuba. All specimens' voucher number and accession numbers of the DNA sequences (DDBJ, EMBL, and GenBank) are listed in Table 1.

DNA extraction

DNA was extracted from about 150–200 urediniospores obtained from a single uredinium. Spores were crushed between two sterile glass slides and suspended in 20 µl extraction buffer [10 mM Tris-HCI pH 8.3, 1.5 mM $MgCI_2$, 50 mM KCI, 0.01% sodium dodecyl sulfate (SDS), 0.01% Proteinase K], and incubated at 37°C for 60 min and then in 95°C for 10 min (Suyama et al. 1996; Virtudazo et al. 2001), followed by a 4°C soak. From this crude extract, 3 µl was used directly for each polymerase chain reaction (PCR) amplification.

PCR amplification and sequencing

Double-stranded DNA spanning the D1/D2 region of the LSU rDNA and the entire ITS1–5.8S–ITS2 (ITS) region of the rDNA was amplified by PCR, using the primer pairs NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O'Donnell 1993), and ITS1F (5′- CTTGGTCATTTAG AGGAAGTAA-3′) (Gardes and Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990), respectively. DNA was amplified using a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA); reaction mixtures of 40-µl volumes were composed of 1 unit of Taq DNA polymerase (Takara, Tokyo, Japan), a commercial deoxynucleoside triphosphate (dNTP) mixture (containing 2.5 mM of each dNTP), Taq reaction buffer (containing 2 mM Mg^{2+}), and 0.2 µM of each primer. PCR was run under the following conditions: 3 min initial denaturation at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 55°C, 1 min at 72°C, and the reaction was terminated after a final extension at 72°C for 10 min.

PCR products were first purified with MicroSpin S-400 HR columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and prepared for sequencing using a Big Dye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) with the same primers used for PCR amplification under the following conditions: 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Cycle sequencing reaction products were finally purified using Centri-Sep spin columns CS-901 (Princeton Separations, Adelphia, NJ, USA), and then sequenced using an ABI Prism 310 Automated DNA Sequencer (Applied Biosystems).

Sequence alignments and analyses

DNA sequence alignments were generated with Clustal X multiple program version 1.8 (Thompson et al. 1997). Then manual alignment was done in Se-Al version 2.07a (Rambaut 2001). *Pucciniastrum pyrolae* Dietel ex Arthur (AF426233) and *P. goeppertianum* (Kühn) Klebahn (L76508, L76509) were included as outgroup in D1/D2 and ITS analyses, respectively (see Table 1). In addition, for comparison, two sequence data of *P. circaeae* (Winter) Spegazzini ex de Toni (AF426227) and *P. epilobii* (Otth) (AF522179), from GenBank, were also included in the analyses (see Table 1). The program PAUP version 4.0b10 (Swofford 2002) was used to construct a neighbor-joining (NJ) topology under the Kimura two-parameter model, with transition/transversion rate 2.0 (Kimura 1980), excluding positions with gaps and correcting for multiple substitutions. Maximum-parsimony analysis was also performed using the heuristic search option with 100 random stepwise addition sequences to search for the most parsimonious tree. Bootstrap (Felsenstein 1985) values were determined using 1000 replicates to estimate support for clade stability of the consensus tree using the same program.

Results

Analysis of D1/D2 region

The data for the D1/D2 region analysis comprised 47 samples, which included GenBank accessions AF522179

Table 1. Specimens of some *Pucciniastrum* rusts and their DNA database accession no. used for phylogenetic analysis

Rust species	Host plant	Voucher specimens[a]	Locality in Japan	Collection	Database accession no.[b]	
					D1/D2	ITS
Pucciniastrum fagi Yamada						
	Fagus crenata	TSH-R10724	Tochigi	2001, W.H. Chung & Y. Okuchi	AB221378	AB221425
	F. crenata	TSH-R21254	Akita	2003, Y.M. Liang & C.M. Tian	AB221375	AB221424
	F. crenata	TSH-R21242	Kitaibaraki	2003, Y.M. Liang & C.M. Tian	AB221374	AB221420
	F. japonica	TSH-R4238 (IBA6307)	Niigata	1992, Y. Ono	AB221376	AB221421
	F. crenata	TSH-R4245 (IBA8447)	Shizuoka	1999, Y. Ono & K. Ishimiya	AB221377	AB221423
	F. crenata	TSH-R4243 (IBA8372)	Gunma	1999, Y. Ono & K. Ishimiya	–	AB221422
P. kusanoi Dietel						
	Clethra barbinervis	TSH-R21252	Kitaibaraki	2003, Y.M. Liang & C.M. Tian	AB221401	AB221430
	C. barbinervis	TSH-R3847	Shizuoka	1998, W. Asano et al.	AB221398	AB221428
	C. barbinervis	TSH-R21299	Nagano	2003, C.M. Tian & M. Imazu	AB221399	AB221427
	C. barbinervis	21509 (HU)	Miyazaki	1992, Y. Harada	AB221402	AB221426
	C. barbinervis	HH98635	Hyogo	1975, N. Hiratsuka & S. Kaneko	AB221400	AB221429
P. actinidiae Hiratsuka, f.						
	Actinidia rufa	TSH-R4267 (IBA7716)	Okinawa	1995, Y. Ono	AB221404	AB221447
	A. arguta	TSH-R23801	Okinawa	2003, Y. Ono	AB221403	AB221446
	A. rufa	TSH-R4266 (IBA7700)	Okinawa	1995, Y. Ono	AB221405	AB221448
	A. rufa	TSH-R4268 (IBA8002)	Okinawa	1997, Y. Ono	AB221407	AB221445
	A. rufa	HH102310	Okinawa	1955, S. Sato & N. Hiratsuka	AB221406	–
P. coryli Komarov						
	Corylus sieboldiana	TSH-R4236 (IBA7603)	Tochigi	1995, Y. Ono	AB221380	–
	C. heterophylla	TSH-R4233 (IBA2582)	Yamanashi	1982, Y. Ono	AB221379	–
	C. sieboldiana	TSH-R4237 (IBA8641)	Fukushima	2000, Y. Ono & H. Mori	AB221381	AB221419
P. corni Dietel						
	Cornus kuosa	TSH-R13510	Tottori	1971, S. Kaneko & I. Ohira	AB221409	AB221437
	C. kuosa	TSH-R4273 (IBA7671)	Miyazaki	1995, Y. Ono	AB221408	AB221436
P. hikosanense Hiratsuka, f.						
	Acer rufinerva	TSH-R4287 (IBA2565)	Yamanashi	1982, Y. Ono	AB221388	AB221441
	A. rufinerva	TSH-R4289 (IBA8441)	Shizuoka	1999, Y. Ono & K. Ishimiya	AB221389	AB221440
	A. rufinerva	TSH-R4288 (IBA2569)	Yamanashi	1982, Y. Ono	AB221390	–
P. styracinum Hiratsuka						
	Styrax japonica	TSH-R t015	Tsukuba	2002, C.M. Tian & M. Kakishima	AB221416	AB221431
	S. japonica	TSH-R1527	Toyama	1995, Y. Sato	AB221417	AB221433
	S. japonica	TSH-R1583	Toyama	1996, T. Kobayashi et al.	AB221418	AB221432
P. yoshinagai Hiratsuka, f.						
	Stewartia monadelpha	TSH-R4272 (IBA8430)	Nara	1999, Y. Ono & K. Ishimiya	AB221411	AB221434
	S. monadelpha	TSH-R4270 (IBA8404)	Nara	1999, Y. Ono & K. Ishimiya	AB221410	AB221435
P. miyabeanum Hiratsuka						
	Viburnum furcatum	TSH-R4281 (IBA8721)	Yamagata	2001, Y. Ono	AB221394	AB221442
	V. furcatum	TSH-R4279 (IBA7888)	Aomori	1997, Y. Ono	AB221395	–
	V. furcatum	TSH-R4278 (IBA7659)	Miyazaki	1995, Y. Ono	AB221396	–
	V. furcatum	TSH-R3849	Shizuoka	1998, W. Asano et al.	–	AB221444
	V. furcatum	TSH-R10202	Akita	1997, Y. Yamaoka et al.	AB221397	AB221443

Table 1. *Continued*

Rust species	Host plant	Voucher specimens[a]	Locality in Japan	Collection	Database accession no.[b]	
					D1/D2	ITS
P. boehmeriae P. et H. Sydow						
	Boehmeria platanifolia	TSH-R4253 (IBA8481)	Tokyo	1999, Y. Ono	AB221391	AB221451
	B. spicata	TSH-R4254 (IBA8571)	Tochigi	2000, Y. Ono	AB331392	–
	B. tricuspis	TSH-R21289	Aomori	2003, Y.M. Liang & C.M. Tian	AB221393	AB221450
	B. tricuspis	TSH-R21290	Aomori	2003, Y.M. Liang & C.M. Tian	–	AB221449
	B. tricuspis	TSH-R21307	Nikko	2003, Y.M. Liang & C.M. Tian	–	AB221452
P. tiliae Miyabe						
	Tilia japonica	TSH-R12717	Tochigi	1963, K. Sugimoto	AB221413	–
	T. mandshurica	TSH-R19878	Niigata	2003, C.M. Tian & M. Imasu	AB221412	AB221455
	T. japonica	TSH-R4295 (IBA7878)	Aomori	1997, Y. Ono	AB221415	AB221454
	T. japonica	TSH-R4294 (IBA7670)	Miyazaki	1995, Y. Ono	AB221414	AB221453
P. hydrangeae-petiolaris Hiratsuka, f.						
	Hydrangea petiolaris	TSH-R4263 (IBA6660)	Kitaibaraki	1992, K. Higuchi et al.	AB221382	–
	H. petiolaris	TSH-R4264 (IBA7881)	Aomori	1997, Y. Ono	AB221385	AB221439
	H. petiolaris	TSH-R4265 (IBA8377)	Gunma	1999, Y. Ono & K. Ishimiya	AB221384	AB221438
	H. petiolaris	TSH-R4261 (IBA2367)	Yamanashi	1981, Y. Ono	AB221383	–
P. circaeae (Winter) Spegazzini ex de Toni						
	Circaea erubescens	TSH-R10187	Aomori	1997, Y. Yamaoka et al.	AB221387	AB221456
	C. lutetiana	RB 2098		R. Bauer	AF 426227[d]	–
P. epilobii Otth						
	Epilobium cephalostigma	TSH-R4285 (IBA2253)	Nagano	1981, Y. Ono	AB221386	–
	E. angustifolium				AF522179[d]	–
P. pyrolae Dietel ex Arthur (outgroup)						
	Pyrolae minor L.	HeRB 4570		R. Berndt	AF 426233[d]	–
P. goeppertianum (Kühn) Klebahn[c] (outgroup)						
	Abies grandis	PgW-1			–	L 76508[d]
	Abies grandis	PgW-2			–	L 76509[d]

[a] TSH, Mycological Herbarium, University of Tsukuba, Japan; IBA, Herbarium of Systematic Mycology, Ibaraki University, Japan; HH, Hiratsuka Herbarium, Tokyo, Japan; HU, Hirosaki University, Japan

[b] DDBJ, EMBL, and GenBank database accession number of the nucleotide sequence data

[c] According to Hiratsuka (1958) and Cummis and Hiratsuka (2003), it is described as *Calyptospora goeppertiana*

[d] From GenBank sequence

(*P. epilobii*) and AF426227 (*P. circaeae*) for comparison to original data, plus *P. pyrolae* (AF426233) as the outgroup.

All specimens produced a single fragment 545–566 bases long from the nuclear LSU rDNA region. The final LSU rDNA (D1/D2) sequence alignment included a total of 576 characters, of which 495 sites were constant, 20 sites were variable and parsimony uninformative, and 61 sites were parsimony informative. A parsimony analysis using PAUP obtained one most parsimonious tree with 104 steps [consistency index (CI) = 0.846, retention index (RI) = 0.940, and rescaled consistency index (RC) = 0.795; tree not shown]. The NJ tree (Fig. 1) was also obtained through the distance phylogenetic analysis using the NJ method, which was nearly same as the parsimony tree.

All the specimens of *Pucciniastrum* except for the outgroup taxon *P. pyrolae* separated into six groups (see Fig. 1). Four specimens of *P. circaeae* and *P. epilobii* on Oenotheraceae formed a distinct group (group A), which was strongly supported by the 100% bootstrap. Group B included 4 specimens of *P. hydrangeae-petiolaris* Hiratsuka, f. on genus *Hydrangea* of Hydrangeaceae, which formed a well-supported group (100% in both the NJ tree and the MP tree). Likewise, group C was also a distinct group (98% in the NJ tree, 99% in the MP), including 3 specimens of *P. coryli* Komarov on genus *Corylus* of Betulaceae. Group D, supported by 62% of the bootstrap replicates, consisted of two species, *P. fagi* Yamada on genus *Fugus* of Fagaceae and *P. hikosanense* Hiratsuka, f. on genus *Acer* of Aceraceae. Within group D, the species *P. hikosanense* and *P. fagi* further formed two subgroups (subgroups Da and Db). Group E only included specimens of *P. tiliae* Miyabe on genus *Tilia* of Tiliaceae, without bootstrap values. Group F consisted of 24 specimens from seven species, i.e., *P. corni* Dietel, *P. kusanoi* Dietel, *P. styracinum* Hiratsuka, *P. actinidiae* Hiratsuka, f., *P. boehmeriae* P. et H. Sydow, *P. miyabeanum* Hiratsuka, and *P. yoshinagai* Hiratsuka, f., which supported by the bootstrap values (66% in the NJ tree and 62% in the MP tree).

Analysis of ITS1–5.8S–ITS2 region

All samples produced a single fragment 724–729 bases long. The alignment data matrix consists of 40 samples, of which two sequences of *P. goeppertianum* (GenBank accession nos. L76508 and L76509) from GenBank were used as the outgroup. The final ITS region sequence alignment included a total of 752 characters, of which 575 sites were constant, 56 variable characters were parsimony uninformative, and 121 sites were parsimony informative. The parsimony analysis of the sequence data resulted in a single most parsimonious tree with 237 steps (CI = 0.873, RI = 0.907, RC = 0.792; tree not shown). The neighbor-joining consensus tree (Fig. 2) by distance phylogenetic analysis was identical to the maximum-parsimony tree.

The phylogenetic trees constructed from the ITS and 5.8S rDNA regions also separated the specimens into six groups with high bootstrap support (Fig. 2); these groups are similar to the groups of D1/D2 region analysis. Group A (*P. circaeae*), group B (*P. hydrangeae-petiolaris,* 100% in both NJ and MP), group C (*P. coryli*), and group E (*P. tiliae,* 85% in NJ and 73% in MP) were distinct groups, respectively, each composed of only one species. Group D was more clearly divided into two subgroups by 72% bootstrap support; subgroup Da included two specimens of *P. hikosanense* (100% in NJ and 98% in MP), whereas sister position subgroup Db consisted of six specimens of *P. fagi* (84% in NJ and 74% in MP). In contrast to the D1/D2 region analysis, group F was also clearly divided into two subgroups. Subgroup Fa included four specimens of *P. boehmeriae* (86% in NJ, 83% in MP), and subgroup Fb (64% in NJ, 96% in MP) consisted of six species: *P. kusanoi*, *P. styracinum*, *P. corni*, *P. actinidiae*, *P. miyabeanum*, and *P. yoshinagai*.

Discussion

In the present study, two species of *P. circaeae* and *P. epilobii* formed a highly coherent cluster in both NJ and MP and were well separated from other species of *Pucciniastrum*. Moreover, scanning electron microscopy (SEM) observation based on a large number of specimens on family Oenotheraceae from Japan, showed that *P. circaeae* on *Circaea* and *P. epilobii* on *Epilobium* did not have the ostiolar cells (these taxonomic characters will be discussed in another paper). The combined results of our study suggest that ostiolar cell characteristics may be important taxonomic characteristics. Furthermore, our specimens of *P. circaeae* and *P. epilobii* were clustered with the sequence of *P. circaeae* (AF426227) and *P. epilobii* (AF522179) from America and placed in the basal position in the D1/D2 tree (bootstrap 100%). Similarly, *P. circaeae* was separated from the other species by a long genetic distance based on ITS, although the sequence data of species *P. epilobii* was not obtained in the ITS region. In addition, Maier et al. (2003) examined the phylogenetic relationships of four species of *Pucciniastrum* based on 28S and reported that *P. circaeae* and *P. epilobii* form a highly supported cluster (100%) separated from other two species of *Pucciniastrum*. Therefore, it is suggested that these two species have a common ancestral lineage. On the other hand, *P. circaeae* and *P. epilobii* have closely related hosts and have morphologically similar urediniospores and teliospores (Hiratsuka et al. 1992), and possess very similar gymnopedunculate haustoria (Berndt and Oberwinkler 1995). These considerations suggest that *P. circaeae* and *P. epilobii* may be the same taxon at the species level.

Molecular phylogenetic trees showed that *P. hydrangeae-petiolaris*, *P. coryli*, and other species are distant from each other and separated from other species. Therefore, we considered these two species might be distinct taxa.

Comparing the divergence variation between the D1/D2 region and the ITS regions, the result of the D1/D2 region showed that groups D, E, and F each received low bootstrap

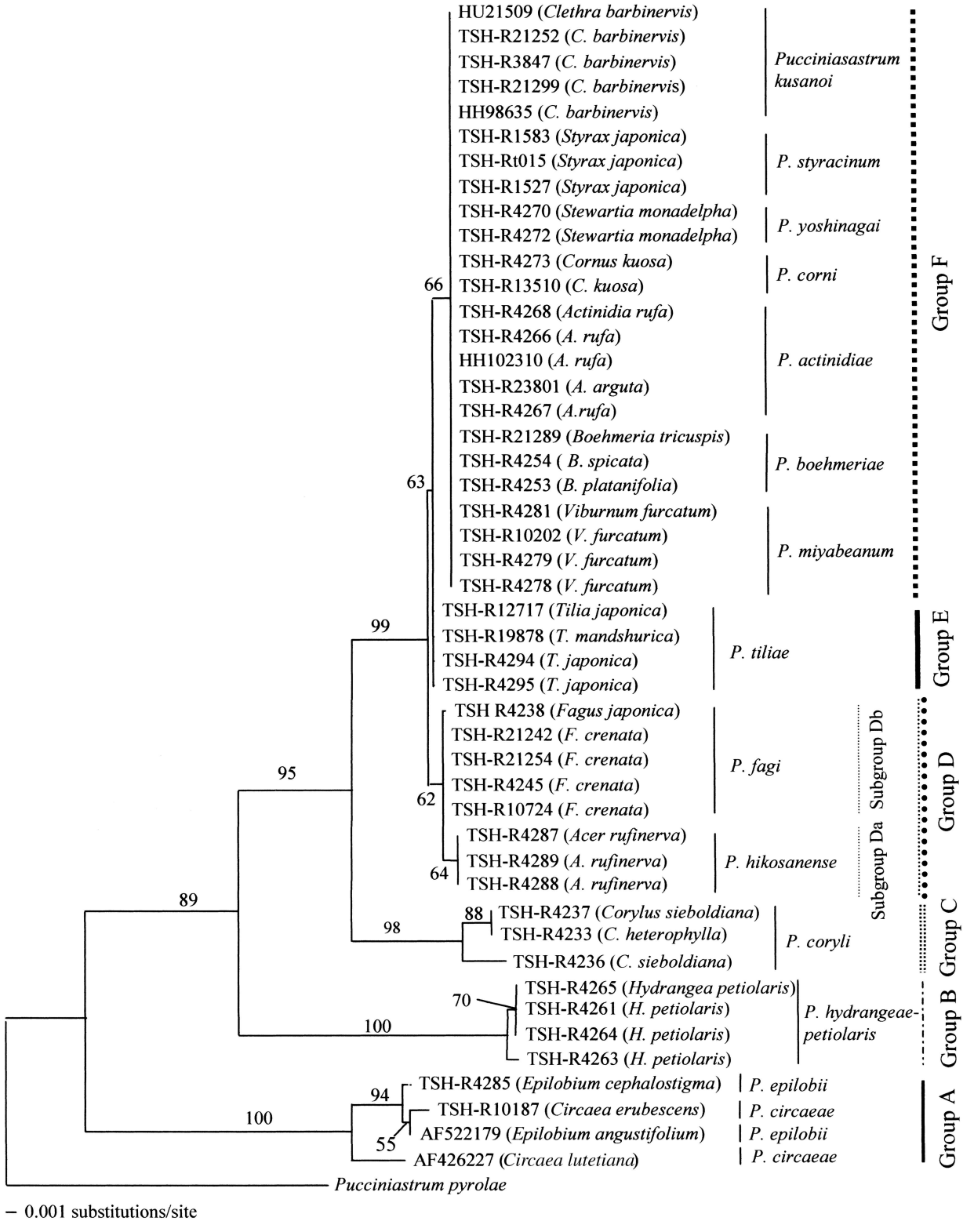

Fig. 1. A neighbor-joining tree inferred from sequences of D1/D2 region using PAUP. Bootstrap values above 50% from 1000 replicates are indicted for the corresponding branches. Length of branches is proportional to number of base changes, indicated by the scale bottom

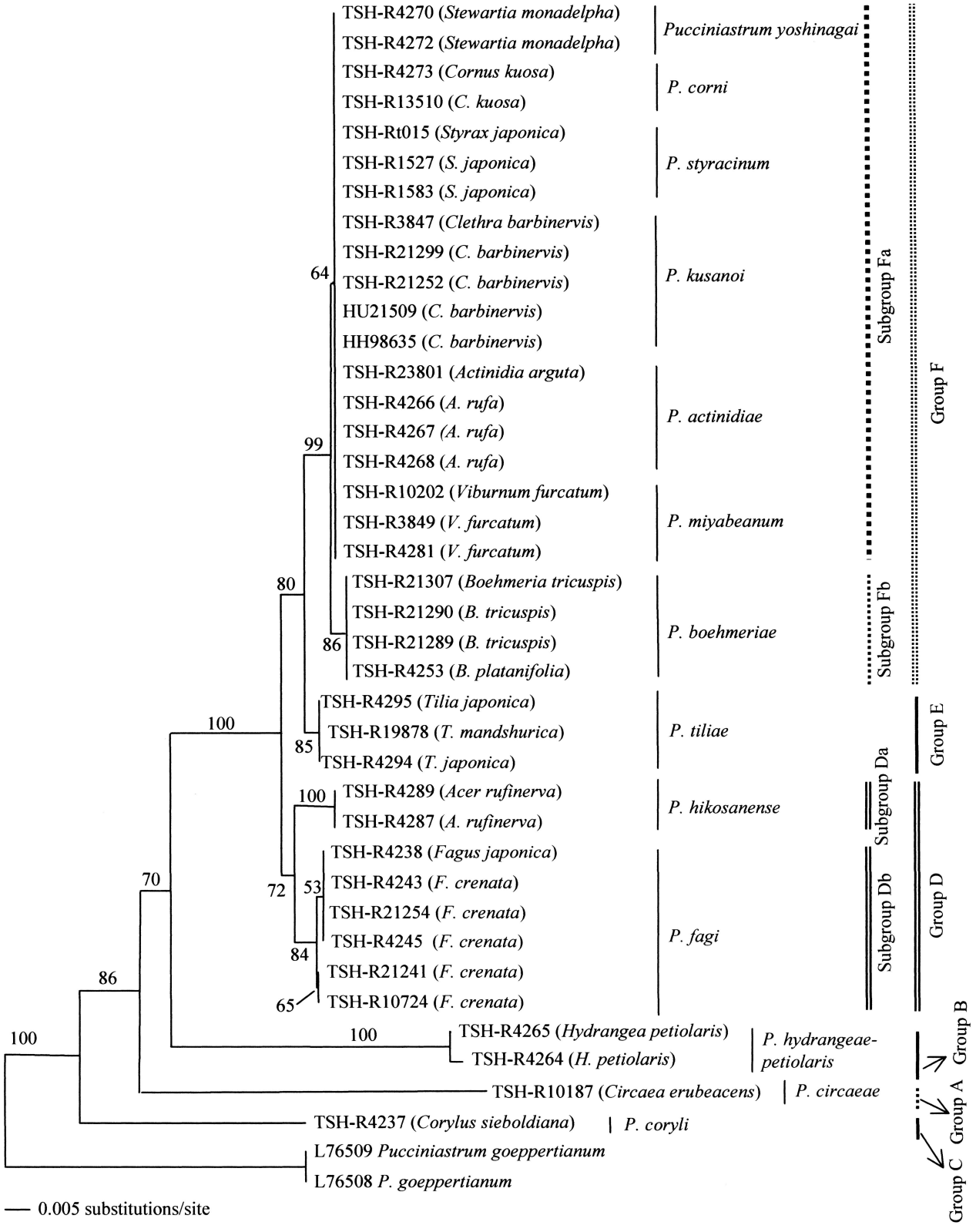

Fig. 2. A neighbor-joining tree inferred from sequences of ITS and 5.8S regions using PAUP. Bootstrap values above 50% from 1000 replicates are indicted for the corresponding branches. Length of branches is proportional to number of base changes, indicated by the scale bottom

values (see Fig. 1). However, it has been known that the mutation rate in D1/D2 regions of LSU rDNA is often slower than that in ITS regions. Therefore, the sequence variation in D1/D2 region is often insufficient to distinguish biological species (Maier et al. 2003), whereas sequence variation in the ITS region was commonly large enough to separate taxa at a species level (White et al. 1990). In this study, groups D, E, and F each received higher bootstrap support in the ITS region analysis (see Fig. 2) than D1/D2 region. Both the NJ and MP trees of ITS region clearly revealed that *P. fagi* (subgroup Db) and *P. hikosanense* (subgroup Da) are phylogenetically separated from each other, although they formed a sister-relationship. Similarly, *P. boehmeriae* (subclade Fb 86%) formed a sister-relationship to the six other species (subgroup Fa; Fig. 2). Consequently, these results suggest that *P. fagi*, *P. hikosanense*, *P. tiliae*, and *P. boehmeriae* might each be distinct taxa.

Twenty-four rust specimens from six species including *P. kusanoi* on *Clethra*, *P. styracinum* on *Styrax*, *P. corni* on *Cornus*, *P. actinidiae* on *Actinidia*, *P. miyabeanum* on *Viburnum*, and *P. yoshinagai* on *Stewartia* constitute a single cluster in both NJ and MP analyses based on both D1/D2 and ITS regions. In addition, they have identical nucleotide sequences in both the D1/D2 and the ITS regions. Although molecular data from wider regions of the rust genome and from a wider of species will provide a better understanding of evolutionary among different taxa of rust in Uredinales (Maier et al. 2003; Pei et al. 2005), the present results did not support that these six species are separate taxa in species level.

The present molecular phylogenetic analyses within genus *Pucciniastrum* clearly suggested that species systematics based on host plants does not support their phylogeny, and their revision is required.

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