FULL PAPER

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Phylogenetic relationships among *Puccinia hemerocallidis*, *P. funkiae*, and *P. patriniae* (Uredinales) inferred from ITS sequence data

Abstract *Puccinia hemerocallidis* and *P. funkiae* resemble each other morphologically; however, they are biologically and taxonomically distinct, with telia of the former being restricted to species of *Hemerocallis* and the latter to *Hosta* species. However, both fungi share a macrocyclic and heteroecious life cycle with *Patrinia villosa* as the spermogonial and aecial host. An additional microcyclic rust fungus, *P. patriniae*, is also known on *P. villosa*. This microcyclic fungus is similar to the two macrocyclic fungi in its telial structure and teliospore morphology. These similarities in morphology and host relationships suggest the three fungi may also share a close evolutionary relationship. To determine the phylogenetic relationships of the three species, a portion of the nuclear ribosomal DNA repeat encoding the ITS and 5.8S subunit regions was amplified by PCR, sequenced, and analyzed. The resulting phylogenetic trees showed that *P. hemerocallidis* and *P. funkiae* share a recent common ancestor and that *P. patriniae* is closely allied with *P. hemerocallidis*. The results suggest a possible evolutionary derivation of microcyclic *P. patriniae* from macrocyclic heteroecious *P. hemerocallidis*, which fits the evolutionary interpretation of correlated species known as Tranzschel's law.

Key words *Hemerocallis* · *Hosta* · *Patrinia* · Rust fungus · Tranzschel's law

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Introduction

Daylilies (*Hemerocallis* spp.: Liliaceae) and plantainlilies (*Hosta* spp.: Liliaceae) are native to eastern Asia but are globally distributed as wild and horticulturally modified plants in botanical, horticultural, and residential gardens. Leaf rust is one of the few fungal diseases of daylilies (Hiratsuka et al. 1992; Kishi 1998; The Phytopathological Society of Japan 2000), and can be serious under certain circumstances, as recently experienced in North America (Williams-Woodward et al. 2001; Hernández et al. 2002). *Puccinia hemerocallidis* Thümen is the causal fungus of the daylily leaf rust and was once believed to be the same species as the plantainlily leaf rust fungus because of similarities in their telial structure and teliospore morphology (Hiratsuka et al. 1992; Kishi 1998; The Phytopathological Society of Japan 2000). However, Ono (2003, 2005) proved that the daylily rust and the plantainlily rust were caused by two distinct species and determined the latter causal fungus as *Puccinia funkiae* Dietel.

The two fungi are separated mainly based on the host specificity of the uredinial and telial stages, *P. hemerocallidis* on *Hemerocallis* spp. and *P. funkiae* on *Hosta* spp. However, they share *Patrinia villosa* Juss. (Valerianaceae) as the spermogonial and aecial host. An additional microcyclic rust fungus, *P. patriniae* Hennings, is known on *P. villosa*. *Puccinia patriniae* resembles *P. hemerocallidis* and *P. funkiae* in its telial structure and teliospore morphology. From the structural and morphological similarities in the telial stage and the shared spermogonial/aecial host relationships, the question was raised as to the phylogenetic relationships among the three rust fungi, particularly in regard to the origin of *P. patriniae* (Ono 2005).

This article examines phylogenetic relationships among *P. hemerocallidis*, *P. funkiae*, and *P. patrinia* by molecular phylogenetic methods recently applied to rust systematics studies (Zambino and Szabo 1993; Roy et al. 1998; Vogler and Bruns 1998; Pfunder et al. 2001; Weber et al. 2003; Smith et al. 2004; Pei et al. 2005).

Table 1. The specimens used in phylogenetic analyses of internal transcribed spacer (ITS) regions including 5.8S rDNA

Species	Voucher specimens	Host plant	Locality	GenBank accession no.
Puccinia hemerocallidis Thümen	IBA-8745	*Hemerocallis fulva* L. var. *longituba* (Miq.) Maxim.	Gumma	AB232544
	IBA-8749	*H. fulva* var. *longituba*	Tochigi	AB232546
	IBA-8759	*H. fulva* var. *longituba*	Ibaraki	AB232547
	IBA-8878	*H. fulva* var. *longituba*	Yamanashi	AB232545
P. funkiae Dietel	IBA-8875	*Hosta montana* F. Maekawa	Yamanashi	AB232541
	IBA-8876	*H. montana*	Yamanashi	AB232540
	IBA-8877	*H. albo-marginata* (Hook.) Ohwi	Yamanashi	AB232539
P. patriniae Hennings	IBA-8346	*Patrinia villosa* (Thunb.) Juss.	Tochigi	AB232542
	IBA-8568	*P. villosa*	Gumma	AB232543

Table 2. Details of GenBank sequences used in phylogenetic comparisons

Species/host (strain)	Source	GenBank accession no.
Puccinia hemerocallidis on *Hemerocallis* sp. (Japan)	Hernández JR, Palm ME, Castlebury LA (2002)	AF479743
P. hemerocallidis on *Hemerocallis* sp. (Russia)	Hernández JR, Palm ME, Castlebury LA (2002)	AF479744
P. hemerocallidis on *Hemerocallis* sp. (Costa Rica)	Hernández JR, Palm ME, Castlebury LA (2002)	AF479740
P. hemerocallidis on *Hemerocallis* sp. (Costa Rica)	Hernández JR, Palm ME, Castlebury LA (2002)	AF479741
P. hemerocallidis on *Hemerocallis* sp. (USA)	Hernández JR, Palm ME, Castlebury LA (2002)	AF479739
P. hemerocallidis on *Hemerocallis* sp. (USA)	Hernández JR, Palm ME, Castlebury LA (2002)	AF479742
P. sessilis strain PUR N4542	Szabo LJ (unpublished)	AY217134
P. sessilis strain PUR N4543	Szabo LJ (unpublished)	AY217135
P. allii strain isolate HSZO162/PUR N2537	Anikster Y, Szabo LJ, Eilam T, Manisterski J, Koike ST, Bushnell WR (2004)	AF511077
P. allii isolate HSZ0344	Anikster Y, Szabo LJ, Eilam T, Manisterski J, Koike ST, Bushnell WR (2004)	AF511078
P. hordei Otth isolate 22-81	Anikster Y, Szabo LJ, Eilam T, Manisterski J, Koike ST, Bushnell WR (2004)	AF511086
P. hordei strain CDL64-2B	Anikster Y, Szabo LJ, Eilam T, Manisterski J, Koike ST, Bushnell WR (2004)	AY187089
Uromyces scillarum isolate YA3464	Anikster Y, Szabo LJ, Eilam T, Manisterski J, Koike ST, Bushnell WR (2004)	AY302495
U. scillarum isolate 3465	Anikster Y, Szabo LJ, Eilam T, Manisterski J, Koike ST, Bushnell WR (2004)	AF511085
P. graminis on *Agropyron repens*	Weber RWS, Webster J, Engel G. (2003)	AF468044
P. graminis f. sp. *tritici* strain CDL 78-21-BB463	Szabo LJ (unpublished)	AY114289
P. striiformis f. sp. *tritici* strain PST-17	Szabo LJ (unpublished)	AY114292
P. coronata f. sp. *avenae* strain 93MN437	Szabo LJ (unpublished)	AY114290
P. consimilis on *Arabis holboellii* var. *pinetorum*	Roy BA (2001)	AF182992
P. monoica on *Arabis microphylla*	Roy BA (2001)	AF182996
P. sorghi strain HI1	Szabo LJ (unpublished)	AY114291
Melampsora epitea on *Salix arctica*	Smith JA, Blanchette RA, Newcombe G (2004)	AY471620

Materials and methods

Four *P. hemerocallidis*, three *P. funkiae*, and two *P. patriniae* samples were subjected to molecular phylogenetic analyses; DNA was extracted from dried herbarium specimens deposited in the Herbarium of Systematic Mycology, the Faculty of Education, Ibaraki University (IBA) (Table 1). The GenBank sequences of *P. allii* Rudolphi (AF511077 and AF511078), *P. consimilis* Ellis & Everhart (AF182992), *P. coronata* Corda (AY114290), *P. graminis* Persoon (AF468044 and AY114289), *P. hordei* Otth (AF511086 and AY187089), *P. monoica* Arthur (AF182996), *P. sessilis* Schneider (AY217134 and AY217135), *P. sorghi* Schweinitz (AY114291), *P. striiformis* Westendorp (AY114292), and *Uromyces scillarum* G. Winter (AY302495 and AF511085) were included as in-groups together with *P. hemerocallidis*

(AF479739, AF479740, AF479741, AF479742, AF479743, and AF479744) for the combined analyses; and *Melampsora epitea* Thüm. (AY471620) was included as an outgroup for the ITS alignment (Table 2).

Whole-cell DNA was extracted from about 150–200 teliospores obtained from a single telium. Spores were crushed between two sterile glass slides and suspended in 20 μl extraction buffer [10 mM Tris-HCl pH 8.3, 1.5 mM MgCl$_2$, 50 mM KCl, 0.001% sodium dodecyl sulfate (SDS), 0.01% Proteinase K], and incubated at 37°C for 60 min and then at 95°C for 10 min (Suyama et al. 1996; Virtudazo et al. 2001). This crude extract (3 μl) was amplified directly by polymerase chain reaction (PCR). Internal transcriber spacer (ITS)1 and ITS2 regions including the 5.8S subunit of the nuclear rDNA repeat were separately amplified by PCR. The primers were ITS5 (5′-GGAAGTAAAAG TCGTAACAAGG-3′; White et al. 1990) and ITS2bl (5′-

Table 3. Sequence differences in ITS regions among *Puccinia hemerocallidis* specimens

GenBank accession no.	Base position												
	11	19	57	97	123	127	158	163	164	208	209	486	538
AB232544 (IBA-8745)	T	A	T	G	C	–	A	T	–	–	–	–	–
AB232545 (IBA-8878)	T	A	C	G	C	–	A	T	–	T	A	–	–
AB232546 (IBA-8749)	T	A	C	G	C	–	A	T	–	T	A	–	–
AB232547 (IBA-8759)	C	A	C	G	C	–	A	T	–	T	A	–	–
AF479743 (Japan)	T	A	Y[a]	G	C	–	A	T	–	–	–	–	C
AF479744 (Russia)	T	A	T	G	T	T	A	T	–	–	–	–	–
AF479742 (U S A)	T	T	T	A	T	T	T	T	T	–	–	T	–
AF479739 (U S A)	T	T	T	A	T	T	T	T	T	–	–	T	–
AF479741 (Costa Rica)	T	W[a]	T	A	T	T	T	T	T	–	–	T	–
AF479740 (Costa Rica)	T	W[a]	T	A	T	T	T	–	–	–	–	T	–

Only positions where differences occurred are shown. All other positions are identical. Dashes represent alignment gaps

[a] As shown in Hernández et al. (2002; Fig. 3)

CTGTGTTCTTCATCGATGTGA-3′; Vogler and Bruns 1998) for the ITS1 region and primers ITS3r (5′-ATCGATGAAGAACACAG-3′; Vogler and Bruns 1998) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White et al. 1990) for the ITS2 region. Reaction mixtures (40 µl) included 4 µl 10× PCR buffer, 1 unit Taq DNA polymerase (Takara, Tokyo, Japan), 0.2 mM of each dNTP, and 2 µM of each primer in pairs. The PCR was performed under the conditions employed by Virtudazo et al. (2001): 95°C for 3 min, then 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, and a final extension step of 72°C for 10 min. The PCR products were electrophoresed in 1.0% agarose gels in Tris-acetate-EDTA (TAE) buffer. The DNA bands of expected size were excised from the ethidium bromide-stained gel and purified using Geneclean III Kit (Qbiogene, Irvine, CA, USA) following the manufacturer's instructions. The purified DNA was subsequently prepared for sequencing with a Big Dye Terminator ver. 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) with the same primers used for PCR amplification under the following conditions: 96°C for 1 min, then 25 cycles of 96°C for 30 s, and 50°C for 1 min, 60°C for 3 min. Cycle sequencing reaction products (10 µl) were purified by ethanol/ethylenediaminetetraacetic acid (EDTA) precipitation and then sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

DNA sequence alignments were generated with Clustal X multiple program version 1.8 (Thompson et al. 1997). The alignment was manually refined in Sequence Alignment (Se-Al) Editor v 2.0 (Rambaut 2000). For the parsimony analysis, we used the maximum-parsimony method with heuristic searches using PAUP 4.0b10 (Swofford 2002). This search was repeated 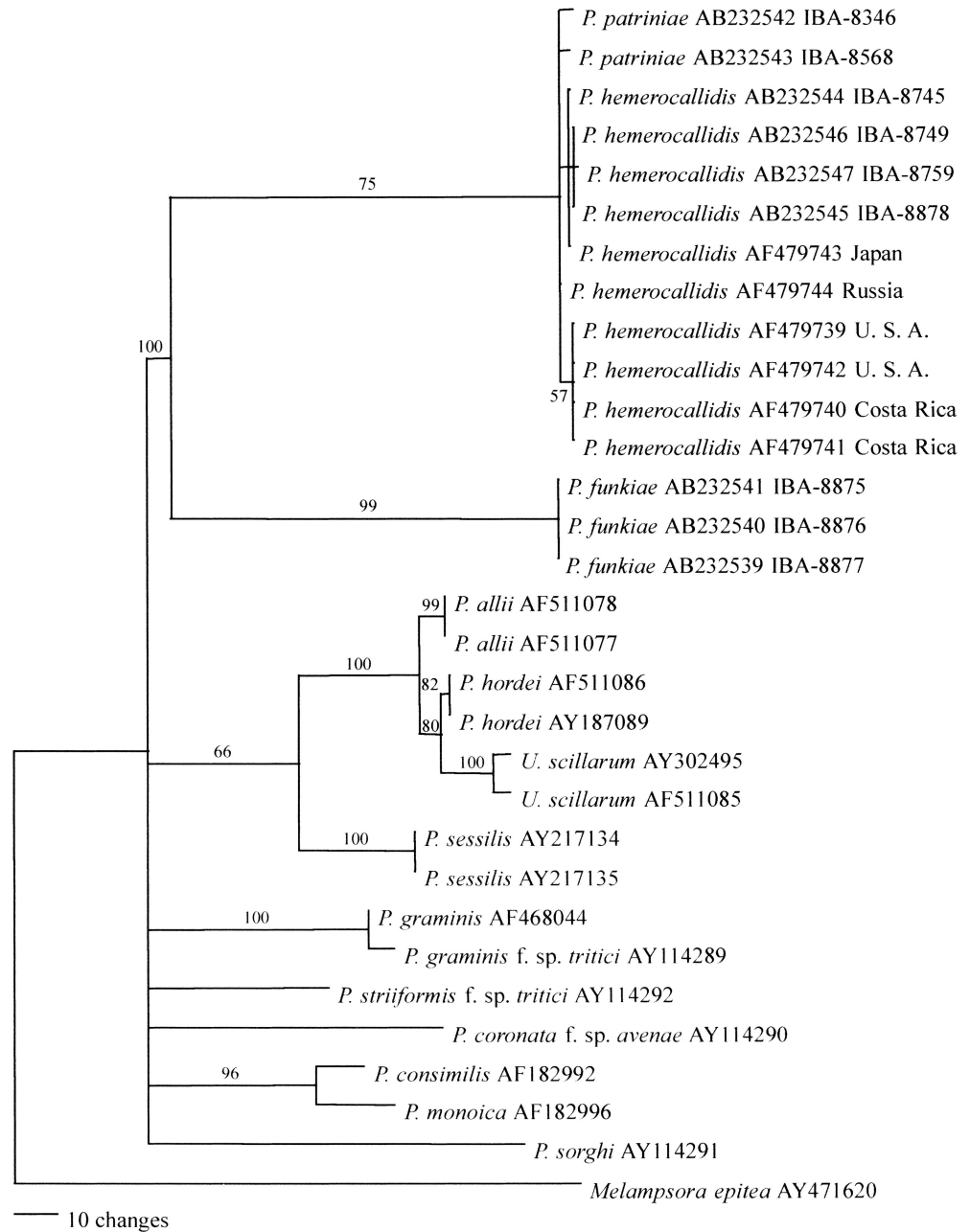100 times with random stepwise addition of operational taxonomic units (OTUs) and branch-swapping by tree-bisection-reconnection. All characters were treated as unordered and weighted equally. Gaps were treated as missing data. For the NJ analysis (Saitou and Nei 1987), we calculated the evolutionary distance matrix by Kimura's two-parameter method (Kimura 1980) and inferred the phylogeny using PAUP. The relative support for each node was estimated by bootstrap analysis (Felsenstein 1985) of 1000 replicates both in MP and NJ analyses.

Results

The DNA sequences of the entire ITS region, including the 5.8S subunit, of *P. hemerocallidis*, *P. funkiae*, and *P. patriniae* ranged from 542 to 545 base pairs (bp): 197–199 bp in the ITS1, 158–159 bp in the 5.8S rDNA, and 186–189 bp in the ITS2 region. The 5.8S subunit sequences were highly conserved among all 31 specimens whereas the sequences of the ITS1 and ITS2 regions were variable. Sequences were the same among *P. funkiae* and *P. patriniae* specimens, respectively. However, base differences were detected at 13 positions between Japanese and American specimens of *P. hemerocallidis* (Table 3).

The final data matrix consisted of 31 taxa and 714 characters, including gaps. Of these, 381 (53.36%) were constant, 121 (16.95%) were variable but uninformative, and 212 (29.69%) were parsimony informative. Parsimony analysis generated 100 equally parsimonious trees of 654 steps with a consistency index (CI) of 0.717, a retention index (RI) of 0.871, and a rescaled consistency index (RC)

Fig. 1. A phylogenetic tree with the highest log-likelihood among 100 equally parsimonious trees [consistency index (CI) = 0.717, retention index (RI) = 0.871, rescaled consistency index (RC) = 0.625] constructed from sequences of the internal transcribed spacer (ITS) regions including 5.8S rDNA. The bootstrap values of 1000 replications are given *above branches*

P. patriniae AB232542 IBA-8346
P. patriniae AB232543 IBA-8568
P. hemerocallidis AB232544 IBA-8745
P. hemerocallidis AB232546 IBA-8749
P. hemerocallidis AB232547 IBA-8759
P. hemerocallidis AB232545 IBA-8878
P. hemerocallidis AF479743 Japan
P. hemerocallidis AF479744 Russia
P. hemerocallidis AF479739 U. S. A.
P. hemerocallidis AF479742 U. S. A.
P. hemerocallidis AF479740 Costa Rica
P. hemerocallidis AF479741 Costa Rica
P. funkiae AB232541 IBA-8875
P. funkiae AB232540 IBA-8876
P. funkiae AB232539 IBA-8877
P. allii AF511078
P. allii AF511077
P. hordei AF511086
P. hordei AY187089
U. scillarum AY302495
U. scillarum AF511085
P. sessilis AY217134
P. sessilis AY217135
P. graminis AF468044
P. graminis f. sp. tritici AY114289
P. striiformis f. sp. tritici AY114292
P. coronata f. sp. avenae AY114290
P. consimilis AF182992
P. monoica AF182996
P. sorghi AY114291
Melampsora epitea AY471620

— 10 changes

of 0.625. The log likelihood of the most parsimonious trees was determined under the likelihood-based topology test using PAUP. The Kishino–Hasegawa test (Kishino and Hasegawa 1989) with normal test distribution was used, and two-tailed *P* values were selected. The number of substitution types was 2 (HKY85 variant), the transition/transversion ratio was 2 (kappa = 4.5671961) and nucleotide frequencies were unequal (A = 0.32247, C = 0.16778, G = 0.15315, and T = 0.35660). The tree with the highest log-likelihood was deemed as the "best tree" (Saenz and Taylor 1999). The phylogenetic tree with the highest log likelihood is shown in Fig. 1. The illustrated tree had the same topology as the strict consensus tree. *Puccinia funkiae* and *P. hemerocallidis* each formed a distinct lineage with a shared recent ancestor, with the microcyclic *P. patriniae* grouped

within the macrocyclic *P. hemerocallidis* clade (Fig. 1). However, these data could not resolve relationships between *P. patriniae* and *P. hemerocallidis* (Fig. 1). This analysis also showed that *P. hemerocallidis* isolates originating in the United States and Costa Rica are genetically distinct from those collected in Japan (also see Table 3). A tree topology generated by neighbor-joining analysis was essentially the same as that of parsimony analysis (Fig. 2).

Discussion

Before the work of Ono (2003, 2005), daylily rust fungus and plantainlily rust fungus were believed to be the same

Fig. 2. A neighbor-joining tree constructed from sequences of the ITS regions including 5.8S rDNA. Bootstrap values of 1000 replications are given *above branches*. Length of branches is proportional to number of base changes as indicated by *bar at bottom*

— 0.01 substitutions/site
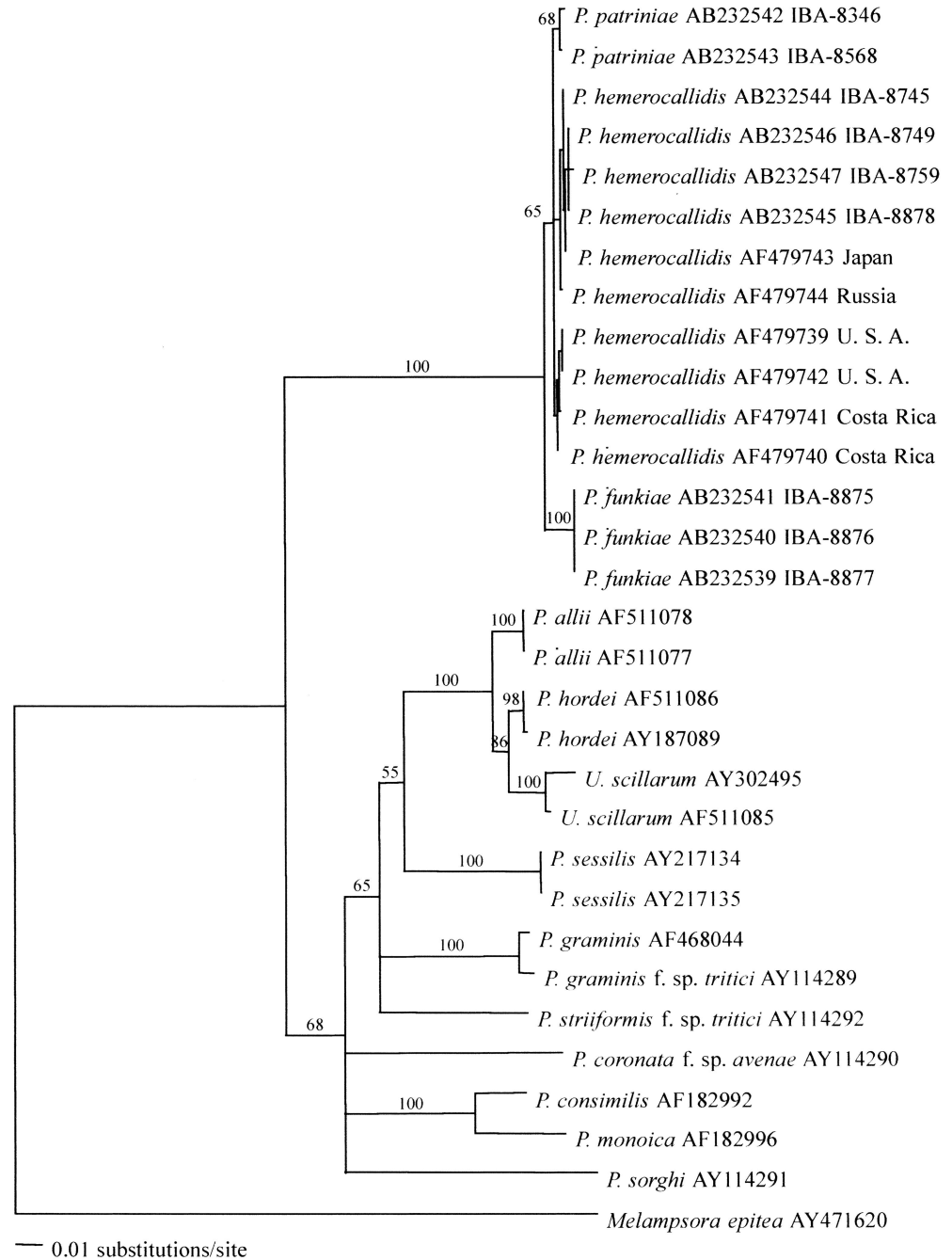
species (Farr et al. 1989; Hiratsuka et al. 1992; Kishi 1998; The Phytopathological Society of Japan 2000). Although both have a heteroecious life cycle, sharing the same spermogonial-aecial host (*Patrinia villosa*), consistent differences in the uredinial-telial host specificity and uredinial morphology suggested their reproductive isolation. Consequently, Ono (2003, 2005) showed conclusively that the causal agents of daylily rust and plantainlily rust are the separate and distinct taxa, *P. hemerocallidis* (von Thümen, 1880) and *P. funkiae* (Dietel, 1898), respectively.

The plant genera *Hosta* Tratt. and *Hemerocallis* L. are classified in the Hostaceae and Hemerocallidaceae (both in order Agavales, class Liliopsida), respectively, or alterna-

tively in the Liliaceae (Watson and Dallwitz 1991; Judd et al. 2002), reflecting their close phylogenetic relationship. Assumed close relatedness of the uredinial-telial hosts and the shared spermogonial-aecial host (*Patrinia villosa*) between *P. hemerocallidis* and *P. funkiae* suggest their phylogenetic close relationship. This view is reinforced by the fact that the two species produce morphologically similar loculate telia, which are surrounded by laterally adherent paraphyses beneath host epidermis, in which two-celled, short pedicellate teliospores are produced (Ono 2003, 2005).

In addition to the two macrocyclic, heteroecious species, *Patrinia villosa* harbors another microcyclic species, *P. patriniae*, which produces only a telial stage in its life cycle

(Hiratsuka et al. 1992). Telial structure and teliospore morphology of *P. patriniae* are similar to those of *P. hemerocallidis* and *P. funkiae*, although paraphyses in the loculate telia are less pronounced in *P. patriniae*. Consequently, it is assumed that the two macrocyclic and one microcyclic species might have originated from a recent common ancestor.

Both maximum-parsimony and neighbor-joining trees support the distinctness of *P. funkiae* and *P. hemerocallidis* (see Figs. 1,2). Although both parsimony and neighbor-joining trees showed the common ancestry of *P. hemerocallidis* and *P. patriniae*, those did not explicitly show the evolutionary derivation of *P. patriniae* from *P. hemerocallidis* as initially expected. Nevertheless, *P. funkiae*, as well as the outgroup *M. epitea*, are macrocyclic heteroecious species; thus, the macrocyclic heteroecious life cycle could be considered as a basal state and the microcyclic life cycle, therefore, as derived. Consequently, one hypothesis for the origins of *P. patriniae* could be that it has derived from a (unidentified) macrocyclic heteroecious species that is a shared common ancestor with *P. hemerocallidis*.

Arthur (1934) used the term "correlated species" to describe the hypothetical relationship between macrocyclic and microcyclic species that share host species and produce morphologically similar teliospores. Thus, *Puccinia hemerocallidis*, *P. funkiae*, and *P. patriniae* represent a group of correlated species. The pattern of correlated species has been explained by Tranzschel's law: a microcyclic species is derived from macrocyclic, heteroecious species by the elimination of aecial and uredinial stages in the course of evolution with the telia of the derived microcyclic species produced on the aecial host (or closely related plants) of the parental species (Wilson and Henderson 1966; Shattock and Preece 2000; Cummins and Hiratsuka 2003).

The *Puccinia hemerocallidis* species complex is one of only a few cases that support Tranzschel's law by phylogenetic analysis. Another example is the *Uromyces pisi* species complex in which microcyclic *U. scutellatus* (Persoon) Léveillé and *U. striolatus* Tranzschel are suggested to be derived from macrocyclic *U. pisi* (DC.) G.H. Otth and *U. striatus* Schröter, although there is some ambiguity at the base of the phylogenetic tree in that study (Pfunder et al. 2001).

In contrast, Roy et al. (1998) and Roy (2001), working with various geographic populations of three apparently closely related "life-cycle species," *P. monoica* Arthur (macrocyclic), *P. consimilis* Ellis & Everhert (macrocyclic), and *P. thlaspeos* C. Schubert (microcyclic) on crucifers, showed a complex phylogenetic pattern among populations and species with different life cycles. The *P. monoica* species complex is the one case that is discordant with Tranzschel's law, and it is of great interest to see to what extent life-cycle plasticity is prevailing among apparently correlated species.

In the present study, four sequences of *P. hemerocallidis* from IBA specimens were aligned together with GenBank sequences of *P. hemerocallidis* from Japan, Russia, Costa Rica, and America. The results showed that *P.*

hemerocallidis specimens from Costa Rica and the United States were different from those from Japan (see Table 3, Figs. 1,2). *Puccinia hemerocallidis* was first detected in Georgia (USA) in 2000 and in Costa Rica in 2001 (Williams-Woodward et al. 2001; Hernández et al. 2002), and the fungus has since become widespread in North America. Costa Rica is one of the Central American countries from which daylily nursery stocks are shipped. It is certain that the fungus is of recent introduction from Asia, and it is unlikely that the fungus has genetically diverged very quickly for a short period of time after the introduction to the Americas. Currently, no pertinent interpretation is possible for the genetic difference observed between Asian and American populations. Extensive surveys for genetic variations of *P. hemerocallidis* in Asia are needed to identify the possible source of the current American populations of the fungus.

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