Mycoscience (2008) 49:66–74
DOI 10.1007/s10267-007-0390-4 © The Mycological Society of Japan and Springer 2008

FULL PAPER

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Phylogeny and taxonomy of the Asian grapevine leaf rust fungus, *Phakopsora euvitis*, and its allies (Uredinales)

Received: August 17, 2007 / Accepted: September 26, 2007

Abstract Heteroecious *Phakopsora euvitis*, *P. vitis*, and *P. ampelopsidis*, autoecious *P. meliosmae*, and an unconnected *Aecidium* on *Meliosma* are closely allied. A total of 45 collections representing the five rust fungi from Japan, Australia, and East Timor were subjected to molecular phylogenetic analyses: the D1/D2 region of nuclear large subunit rDNA and nuclear small subunit internal transcribed spacer 2 (ITS2) region including 5.8S rDNA were analyzed. Tree topologies generated from parsimony and distance methods of the D1/D2 and ITS2 sequences were similar. The 45 collections (44 for ITS2 and 33 for D1/D2, with 32 common for both analyses) are grouped into seven clades: *P. ampelopsidis*, *P. vitis*, unconnected *Aecidium*, *P. euvitis* from Japan, *P. euvitis* from Australia and East Timor, *P. meliosmae* on *M. myriantha*, and *P. meliosmae* on *M. tenuis*. The results confirm the phylogenetic distinctness of *P. euvitis*, *P. ampelopsidis*, and *P. vitis* distributed in Japan. A grapevine leaf rust fungus in Australia and East Timor has genetically diverged from *P. euvitis* in Japan. The unconnected *Aecidium* is highly likely to be an aecial anamorph of a *Phakopsora* fungus. Autoecious *Phakopsora* fungi on *M. meliosmae* and *M. tenuis* need further host-specificity and morphological studies to confirm their taxonomic status.

Key words *Meliosma* · Molecular systematics · *Phakopsora* · Vitaceae · *Vitis*

Introduction

Rust is one of the serious fungal foliar diseases of grapevines in Asia. The Asian grapevine leaf rust is particularly severe in the late growing season just before harvest in temperate regions but is severe throughout the year in subtropical regions (Ono 2000). The causal fungus was determined as *Phakopsora euvitis* Y. Ono, a new species being separated from morphologically similar *P. ampelopsidis* Dietel & P. Sydow and *P. vitis* P. Sydow (Ono 2000).

Phakopsora euvitis produces its uredinial-telial stage on *Vitis* species and *P. vitis* on *Parthenocissus* species; and both fungi share *Meliosma myriantha* Sieb. & Zucc. as the spermogonial-aecial host. *Meliosma myriantha* harbors an additional *Phakopsora* species, *P. meliosmae* Kusano, with an autoecious macrocyclic life cycle. *Meliosma tenuis* Maxim., which occurs sympatrically with *M. myriantha*, was once reported to be the spermogonial-aecial host of the Asian grapevine leaf rust fungus; however, the report has not been confirmed (Ono 2000). *Meliosma tenuis* harbors *P. meliosmae* and an unconnected *Aecidium* fungus, and the latter fungus is assumed to be an aecial anamorph of a phakopsoroid species (Ono 2000). *Phakopsora ampelopsidis* produces its uredinial-telial stage on *Ampelopsis* (and possibly other vitaceous genera); however, its enigmatic life cycle has not been disclosed (Ono 2000).

The spermogonial/aecial and uredinial/telial host relationships together with morphological similarities in each of the comparable life cycle stages indicate close phylogenetic relationships among the three heteroecious *Phakopsora* species, autoecious *P. meliosmae*, and the *Aecidium* fungus on *M. tenuis* (Ono 2000). Thus, it becomes of great interest how the five fungi are related and how they have speciated with host and life cycle changes.

Grapevine leaf rust was recently detected in Darwin, Northern Territory, Australia in 2001 (Weinert et al. 2003) and in Brazil (Paraná in 2001, Mato Grosso do Sul and São Paulo in 2003: Tessmann et al. 2004; and Rio Grande do Sul: Bayer and Costa 2006). The causal fungus was identified as *P. euvitis*, and it was considered to be of recent introduction. Caution must be taken, however, when interpreting the recent detection of the Asian grapevine leaf rust in Australia and Brazil because the fungus might have invaded there long before its detection and survived on indigenous vitaceous plants with cryptic infection.

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To elucidate possible relationships among the four *Phakopsora* and one *Aecidium* fungi of assumed phylogenetic closeness and possible relationships of the newly detected grapevine leaf rust fungus populations in Australia to *P. euvitis* in temperate Asian regions, molecular phylogenetic techniques could serve as a powerful tool, having been successfully applied in various rust taxa (e.g., Roy et al. 1998, for the *Puccinia (Puc.) monoica* complex; Pfunder et al. 2001, for the *Uromyces pisi* complex; Smith et al. 2004, for *Melampsora epitea* Thümen; Chatasiri et al. 2006, for the *Puc. hemerocallidis* complex; and Szabo 2006, for the *Puc. andropogonis* complex and the *Puc. coronata* complex).

Beside the Asian grapevine leaf rust fungus, two American grapevine leaf rust fungi, *P. uva* Buriticá & Hennen (Buriticá 1994) and *P. muscadiniae* Buriticá (Buriticá 1999), have been known in southern North America and South America. The life cycle is not known for either species. Their pathogenicity to cultivated grapevines is not as severe as that of the Asian grapevine leaf rust fungus in Australasia. Thus, it is of horticultural importance how the Asian and American grapevine leaf rust fungi are related and what would be the fate of the former fungus if it were introduced in the Americas.

This study reports the phylogenetic distinctness of *P. ampelopsidis*, *P. vitis* species, and the *Aecidium* fungus on *M. tenuis*, the possible taxonomic division of autoecious *P. meliosmae* by host specificity, and the possible genetic differentiation of the grapevine leaf rust fungus in Australia and East Timor from *P. euvitis* in eastern Asia. The relationships between the Asian and the American grapevine leaf rust fungi are also discussed.

Materials and methods

Rust fungus collections

Forty-five collections representing *P. euvitis*, *P. vitis*, *P. ampelopsidis*, *P. meliosmae*, and an unconnected *Aecidium* were used in this study; their details are listed in Table 1. The collections of *P. euvitis*, *P. vitis*, and *P. meliosmae* included those obtained by artificial inoculation to confirm the host specificity and life cycle. All voucher specimens have been deposited in the Herbarium of Systematic Mycology, the Faculty of Education, Ibaraki University (IBA). For D1/D2 analyses, the GenBank sequences of *P. pachyrhizi* Sydow & P. Sydow (DQ354537) and *P. tecta* H.S. Jasks. & Holw. (DQ354535) were used as the outgroup; for internal transcribed spacer (ITS) analyses, the GenBank sequences of *P. pachyrhizi* (AF333488 and AF333491) and *P. meibomiae* (Arthur) Arthur (AF333501 and AF333502) were used as the outgroup (Table 2).

Polymerase chain reaction and DNA sequencing

Whole-cell DNA was extracted either from approximately 200 spores obtained either from a single aecium or uredinium, depending on which spore stage was formed on the specimens. The 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, 0.01% 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). The D1/D2 region of the large subunit (LSU) rDNA was amplified using the primer set NL1 (5′-GCATATCAATAAGCGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O'Donnell 1993). The complete ITS regions including 5.8S rDNA were amplified with the primer sets ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al. 1990)/ ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990) or ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns 1993)/ LR15 (5′-TAAATTACAACTCGGAC-3′). Reaction mixtures (40 μl) included 4 μl 10× polymerase chain reaction (PCR) buffer, 1 unit Taq DNA polymerase (Takara, Tokyo, Japan), 0.2 mM of each dNTP, 2 μM each primer in a pair, and 10 μl crude extract. A negative control lacking DNA template was included for each set of reactions. The PCR was performed with an iCycler DNA thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 94°C for 10 min, then 40 cycles of 94°C for 1 min, 50°C for 90 s, and 72°C for 2 min, and a final step of 72°C for 10 min. The PCR products were electrophoresed in 1.0% agarose gels in 1× TAE buffer. The DNA band was excised from the ethidium bromide-stained gel and purified using Geneclean III Kit (Q-Biogene), following the manufacturer's instructions.

The purified DNA was subsequently prepared for sequencing using a Big Dye Terminator v. 3.1 Cycle Sequencing 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, 50°C for 1 min, 60°C for 3 min. Cycles sequencing reaction products (10 μl) were purified by ethanol/EDTA precipitation and then sequenced using an ABI Prism 3100 genetic analyzer (Applied Biosystems).

Alignment and phylogenetic analyses

DNA sequences were initially aligned using the CLUSTAL X multiple program, version 1.8 (Thompson et al. 1997) and then manually refined using Sequence Alignment (Se-Al) Editor version 2.0 (Rambaut 2000). Sequence data have been deposited in GenBank and alignment data in TreeBASE. For parsimony analyses, we used the maximum-parsimony method with the heuristic search using PAUP 4.0b10 (Swofford 1999). This search was repeated 100 times with different random starting points, using the stepwise addition option to increase the likelihood of finding the most parsimonious tree. All characters were treated as unordered and weighted equally. Gaps were treated as missing data. Branch swapping by tree-bisection-reconnection was performed with branches collapsing if the maximum branch length was zero. The log-likelihood of the most parsimonious was determined under the likelihood-based topology test using PAUP. The Kishino–Hasegawa test

Table 1. Rust fungus collections examined in this study

Rust/host	Specimen data	Herbarium accession no.	GenBank accession no.	
			D1/D2	5.8S + ITS2
Phakopsora ampelopsidis Diet. & P. Syd.				
Ampelopsis brevipedunculata (Maxim.) Trautv.	JAPAN: Kagoshima, 29 Oct 2000, Y. Ono	IBA-8597	AB354738	AB354771
	JAPAN: Okinawa, 10 Nov 1997, Y. Ono	IBA-7988	AB354739	AB354772
	JAPAN: Kagoshima, 30 Oct 2000, Y. Ono	IBA-8613	AB354740	AB354773
	JAPAN: Kagoshima, 30 Oct 2000, Y. Ono	IBA-8618	AB354741	AB354774
	JAPAN: Kagoshima, 31 Oct 2000, Y. Ono	IBA-8633	AB354742	AB354775
	JAPAN: Okinawa, 11 Nov 1997, Y. Ono	IBA-7999	–	AB354776
	JAPAN: Nara, 1 Oct 1999, Y. Ono and K. Ishimiya	IBA-8419	AB354743	AB354777
Phakopsora euvitis Y. Ono				
Meliosma myriantha Sieb. & Zucc.	JAPAN: Ibaraki, 28 Jun 1992, K. Higuchi et al.	IBA-6612	–	AB354778
	JAPAN: Ibaraki, 7 Jun 1998, Y. Ono and K. Ishimiya	IBA-8084	AB354744	AB354779
	JAPAN: Ibaraki, 4 Jul 2001, Y. Ono and H. Mori	IBA-8695	AB354745	AB354780
	JAPAN: Ibaraki, 8 Jun 2006, Y. Ono and S. Kodato	IBA-9679[a]	AB354746	AB354781
	JAPAN: Ibaraki, 8 Jun 2006, Y. Ono and S. Kodato	IBA-9680[a]	AB354747	AB354782
Vitis amurensis Rupr.	JAPAN: Ibaraki, 26 Sep 1994, Y. Ono	IBA-7289[a]	–	AB354783
V. coignetiae Pulliat.	JAPAN: Niigata, 5 Oct 1992, Y. Ono	IBA-6282	–	AB354784
	JAPAN: Tochigi, 28 Sep 2000, Y. Ono	IBA-8584	AB354748	AB354785
	JAPAN: Ibaraki, 11 Oct 2005, Y. Ono and S. Kodato	IBA-9605[a]	AB354749	AB354786
	JAPAN: Ibaraki, 4 May 2006, Y. Ono and S. Kodato	IBA-9668[a]	AB354750	AB354787
Vitis cv. Kyoho	JAPAN: Ibaraki, 10 Jun 1998, Y. Ono	IBA-8106[a]	AB354751	AB354788
Unidentified *Vitis* cultivar	JAPAN: Okinawa, 8 Dec 1995, Y. Ono	IBA-7720	AB354752	AB354789
	JAPAN: Tokyo, 26 Oct 1998, Y. Ono	IBA-8210	AB354753	–
	AUSTRALIA: Northern Territory, Darwin, Jan 2002, M. Weinert	IBA-8750	–	AB354790
	AUSTRALIA: Northern Territory, Darwin, 25 Jan 2002, M. Weinert and Y. Ono	IBA-8755	AB354754	AB354791
	AUSTRALIA: Northern Territory, Darwin, 25 Jan 2002, M. Weinert and Y. Ono	IBA-8756	–	AB354792
	AUSTRALIA: Northern Territory, Darwin, 25 Jan 2002, M. Weinert and Y. Ono	IBA-8758	AB354755	AB354793
	EAST TIMOR: Dili, 9 May 2002, M. Weinert	IBA-8808	AB354756	AB354794
	EAST TIMOR: Dili, 9 May 2002, M. Weinert	IBA-8809	–	AB354795
	EAST TIMOR: Dili, 9 May 2002, M. Weinert	IBA-8810	AB354757	AB354796
Phakopsora meliosmae Kusano				
Meliosma myriantha Sieb. & Zucc.	JAPAN: Ibaraki, 4 Jul 1992, K. Higuchi et al.	IBA-6629	–	AB354797
	JAPAN: Fukushima, 2 Jun 1995, Y. Ono	IBA-7582	AB354759	AB354798
	JAPAN: Tochigi, 22 Sep 1995, Y. Ono	IBA-7612	AB354758	AB354800
	JAPAN: Ibaraki, 7 Jun 1998, Y. Ono and K. Ishimiya	IBA-8085	AB354760	AB354799
M. tenuis Maxim.	JAPAN: Ibaraki, 10 Jun 1996, Y. Ono	IBA-7758[b]	–	AB354801
Phakopsora vitis P. Sydow				
Meliosma myriantha Sieb. & Zucc.	JAPAN: Ibaraki, 10 May 1994, Y. Ono	IBA-7143[a]	AB354761	AB354802
	JAPAN: Ibaraki, 20 May 1994, Y. Ono	IBA-7144[a]	–	AB354803
	JAPAN: Ibaraki, 9 May 1998, Y. Ono and K. Ishimiya	IBA-8047	AB354762	AB354804
	JAPAN: Ibaraki, 30 May 1998, Y. Ono	IBA-8105[a]	AB354763	AB354805
Parthenocissus tricuspidata Planch.	JAPAN: Ibaraki, 4 Nov 1995, Y. Ono	IBA-7682	AB354764	AB354806
	JAPAN: Ibaraki, 10 Jun 1998, Y. Ono	IBA-8107[a]	AB354765	AB354807
	JAPAN: Yamagata, 29 Sep 2001, Y. Ono	IBA-8732	AB354766	AB354808
Unconnected *Aecidium*				
Meliosma tenuis Maxim.	JAPAN: Tochigi, 14 Jun 1995, Y. Ono	IBA-7591	–	AB354809
	JAPAN: Niigata, 29 Jul 2000, Y. Ono	IBA-8556	AB354767	AB354810
	JAPAN: Tochigi, 18 Aug 2005, Y. Ono et al.	IBA-9545	AB354768	AB354811
	JAPAN: Tochigi, 18 Aug 2005, Y. Ono et al.	IBA-9547	AB354769	AB354812
	JAPAN: Tochigi, 21 Jul 2006, Y. Ono et al.	IBA-9682	AB354770	AB354813
	JAPAN: Tochigi, 21 Jul 2006, Y. Ono et al.	IBA-9687	–	AB354814

ITS, internal transcribed spacer
[a] Life cycle and host specificity determined by artificial inoculations
[b] Life cycle determined by artificial inoculations
–, not determined

Table 2. Details of GenBank sequences used in the phylogenetic comparisons

Rust/host	Source	GenBank accession no.
Phakopsora tecta H. S. Jasks, & Holw. on *Commelina diffusa* Burm. f.	Aime MC (2006)	DQ354535
P. pachyrhizi Sydow & P. Sydow on *Glycine max* (L.) Merr.	Aime MC (2006)	DQ354537
P. pachyrhizi isolated in Hawaii 98	Frederick RD, Snyder CL, Peterson GL, Bonde MR (2002)	AF333491
P. pachyrhizi isolated in Australia 72-1	Frederick RD, Snyder CL, Peterson GL, Bonde MR (2002)	AF333488
P. meibomiae (Arthur) Arthur isolated in Brazil 82-1	Frederick RD, Snyder CL, Peterson GL, Bonde MR (2002)	AF333501
P. meibomiae isolated in Puerto Rico	Frederick RD, Snyder CL, Peterson GL, Bonde MR (2002)	AF333502

Table 3. Variation in sequence length (bp) among the rust collections examined in this study

Rust	Host	D1/D2	ITS1	5.8S	ITS2
Phakopsora ampelopsidis Diet. & P. Syd.	*Ampelopsis brevipedunculata* (Maxim.) Trautv.	559	236	154	239
P. euvitis Y. Ono	*Vitis* spp.	559	239–245	154	237–238
	Meliosma myriantha Sieb. & Zucc.	559	242	154	238
Phakopsora vitis P. Sydow	*Parthenocissus tricuspidata* Planch.	560	252	154	244
	M. myriantha	560	252	154	244
P. meliosmae Kusano	*M. myriantha*	561	224	154	263
	Meliosma tenuis Maxim.	562	235–236	154	249
Unconnected *Aecidium*	*M. tenuis*	559	243	154	238

(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), and the transition/transversion ratio was 2. The tree with the highest log-likelihood was considered as the best tree (Saenz and Taylor 1999).

For the distance analysis, the distance matrix for the aligned sequences was calculated using Kimura's two-parameter method (Kimura 1980) and was analyzed with the neighbor-joining method (Saitou and Nei 1987) using the program PAUP 4.0b10 (Swofford 1999). The relative nodal support was estimated by bootstrap analysis (Felsenstein 1985) of 1000 replicates in both the parsimony and distance analyses.

Results

D1/D2 sequences of the 33 fungal collections ranged from 559 to 562 bp (Table 3). Of the 586 aligned characters, 481 (82.08%) were constant, 26 (4.44%) were variable but uninformative, and 79 (13.48%) were parsimony informative. A parsimony analysis of D1/D2 sequence data generated only one parsimonious tree with a tree length of 123 steps, a consistency index (CI) of 0.902, a retention index (RI) of 0.955, and a rescaled consistency index (RC) of 0.862 (Fig. 1). The neighbor-joining tree was also obtained by the distance method, which topology was essentially identical with the parsimony tree (Fig. 1).

DNA sequences of the entire ITS region including the 5.8S rDNA of the 44 fungal collections ranged from 629 to 650 bp, with the ITS1 region ranging from 224 to 252 bp, ITS2 region from 237 to 263, and 5.8S rDNA being constant for all the collections (Table 3). Ambiguous sequences were found in the ITS1 region, which made definite alignment impossible. Thus, further analysis was not performed for the ITS1 sequences.

Of 441 aligned characters for the ITS2 including 5.8S rDNA, 263 (59.64%) were constant, 1 (0.23%) was variable but uninformative, and 177 (40.13%) were parsimony informative. A parsimony analysis of the ITS2 region including 5.8S rDNA sequence data generated two equally parsimonious trees with a tree length of 316 steps (CI = 0.854, RI = 0.964, and RC = 0.828). The parsimony tree with the highest log-likelihood value is shown in Fig. 2, together with the neighbor-joining tree generated from the distance method.

Both D1/D2 and ITS2 trees generated either from D1/D2 or ITS2 sequences were similar, and a total of 45 fungal collections were grouped into seven clades (Figs. 1, 2). Each of the seven clades corresponded to the fungal collections circumscribed by morphology, life cycle, host specificity (either proven or putative; see Table 1), and geographic distribution range, i.e., *P. ampelopsidis* (Ampelopsidis clade), unconnected *Aecidium* (Aecidium clade), *P. euvitis* distributed in Japan (Japanese Euvitis clade), *P. euvitis* distributed in Australia and East Timor (Australian-E. Timor Euvitis clade), *P. vitis* (Vitis clade), *P. meliosmae* on *M. myriantha* (Myriantha clade), and *P. meliosmae* on *M. tenuis* (Tenuis clade).

In both trees, an Ampelopsidis clade and a clade inclusive of Aecidium-Euvitis (both Japanese and Australian/East Timor collections) were in sister relationship, and this inclu-

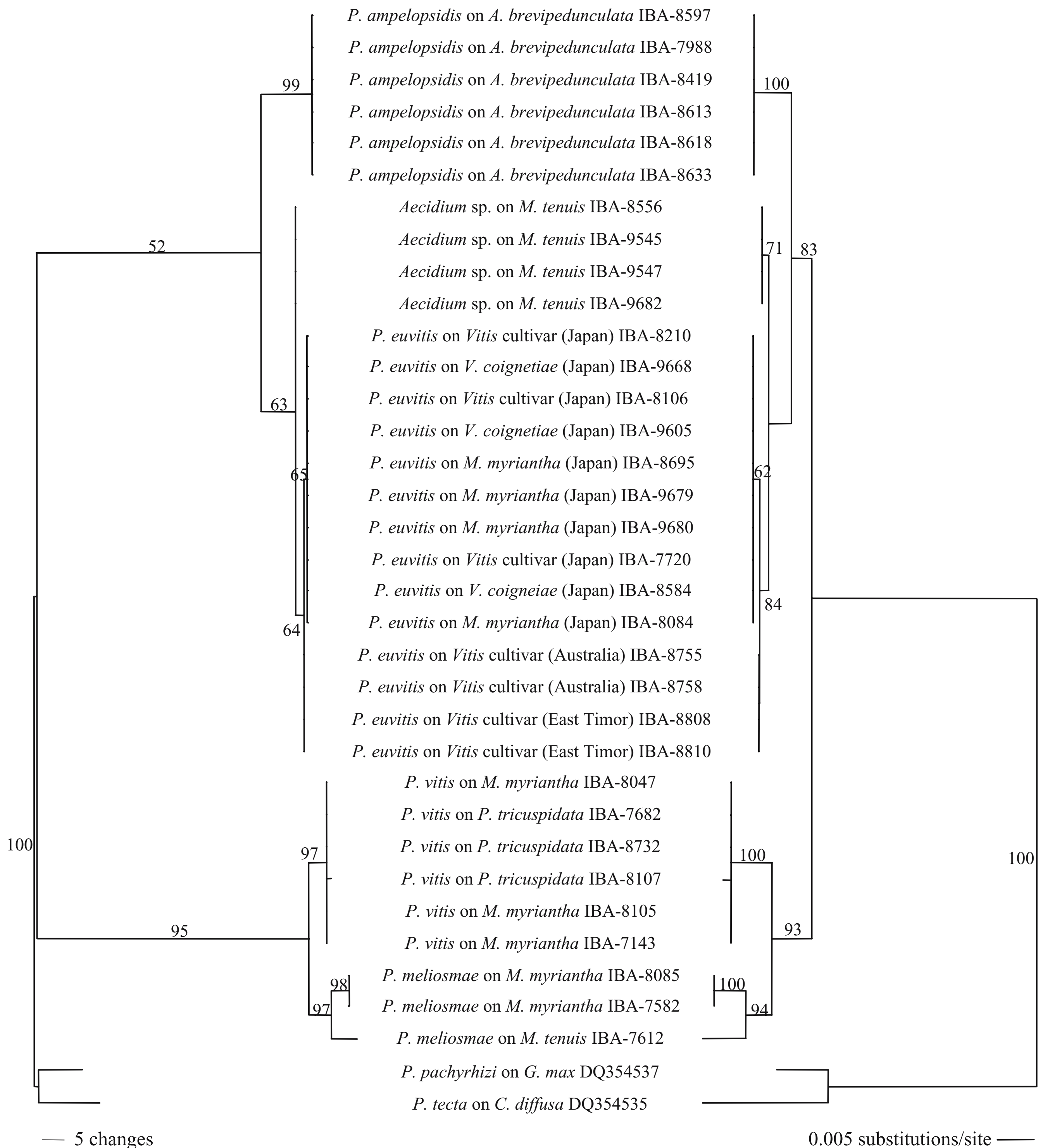

Fig. 1. A parsimony tree (*left:* tree length 123, CI 0.902, RI 0.955, and RC 0.862; the bootstrap values based on 1000 replications are given *above branches*) and a neighbor-joining tree (*right:* the bootstrap values based on 1000 replications are given *above branches*; length of branches is proportional to number of base changes as indicated by *bar at bottom*) constructed from sequences of the D1/D2 region

sive clade and another inclusive Vitis-Myriantha-Tenuis clade were in sister relationship. One of the major differences exhibited between the D1/D2 and ITS2 trees was the inferred relationships among the Aecidium, Japanese Euvitis, and Australian-E. Timor Euvitis clades. The Aecidium clade was a sister-group to the Japanese Euvitis clade, and the Australian-E. Timor Euvitis clade became sister to the clade inclusive of Aecidium and Japanese Euvitis in the ITS2 trees, the branching patterns being supported by high bootstrap values (Fig. 2). However, the relationships among the three

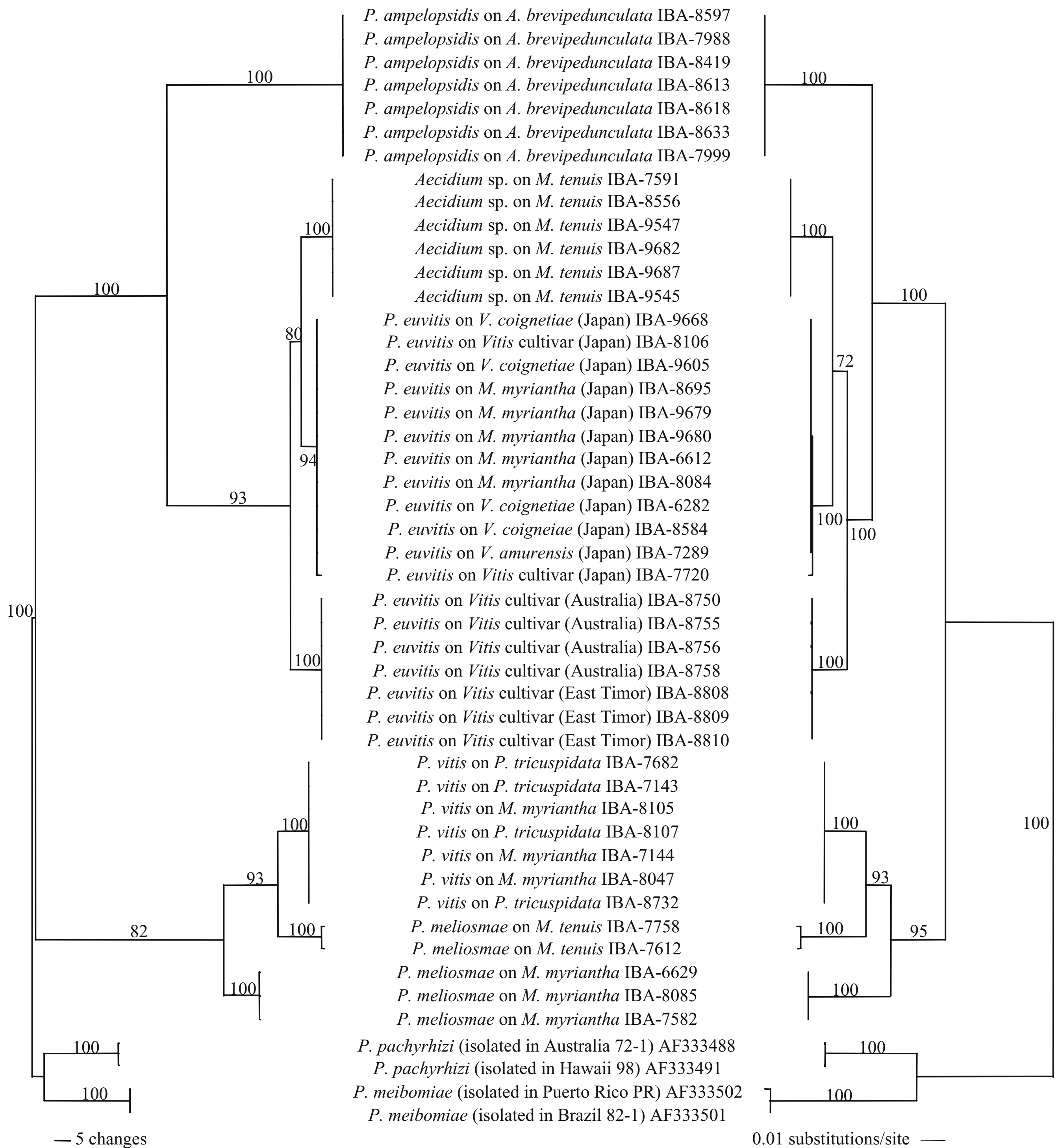

Fig. 2. The most parsimonious tree with the highest log-likelihood among two equally parsimonious trees [*left:* tree length 316, consistency index (CI) 0.854, retention index (RI) 0.964, rescaled consistency index (RC) 0.828; the bootstrap values based on 1000 replications are given *above branches*] and a neighbor-joining tree (*right:* the bootstrap values based on 1000 replications are given *above branches*; length of branches is proportional to number of base changes as indicated by *bar at bottom*) constructed from sequences of internal transcribed spacer (ITS)2 region including 5.8S rDNA

clades were ambiguous in the D1/D2 trees (Fig. 1). Another major difference was the branching patterns of the Vitis, Tenuis, and Myriantha clades between the D1/D2 and ITS2 trees, branching patterns of both trees being supported with high bootstrap values (see Figs. 1, 2). The Myriantha and Tenuis clades were in sister relationship; and the Vitis clade became sister to the Myriantha-Tenuis clade in D1/D2 tree (Fig. 1), while the Tenuis clade was a sister-group to the Vitis clade; and the Tenuis-Vitis clade then became sister to the Myriantha clade in the ITS2 tree (Fig. 2).

Discussion

Phakopsora euvitis, host-alternating between *M. myriantha* and *Vitis* spp., *P. vitis*, host-alternating between *M. myriantha* and *Parthenocissus* spp., and *P. ampelopsidis* s.s., occurring on *Ampelopsis* species with its heteroecious nature being unknown, have been assumed to be reproductively isolated, and thus genetically distinct, because of their distinct host specificity and consistent morphological differences (Ono 2000). Molecular phylogenetic analyses using the D1/D2 region of nuclear large subunit rDNA and the internal transcribed spacer 2 (ITS2) region including 5.8S rDNA support the biological and taxonomic distinctness of the three *Phakopsora* species and suggest the phylogenetic relationship of *P. euvitis* closer to *P. ampelopsidis* than to *P. vitis* (see Figs. 1, 2). This inferred phylogenetic relationships among the three rust species is not congruent with the relationships among *Vitis*, *Ampelopsis*, and *Parthenocissus* inferred from molecular phylogenetic analyses using three chloroplast markers (Soejima and Wen 2006), in which a *Parthenocissus-Yua* clade is sister to a *Vitis-Ampelocissus* clade, to which an inclusive clade of *Ampelopsis-Rhoicissus* is sister. The incongruence between the inferred phylogenies of the three *Phakopsora* species and the three vitaceous host genera and the limited geographic distribution of the three fungi to temperate/tropical Asia (despite the broad geographic distribution of the three plant genera in temperate/tropical Asia and southern North and South America; Soejima and Wen 2006) indicate a possible origin of the fungal complex in Asia and that their speciation is not necessarily tracking the host evolution (Roy 2001).

It has not been conclusively determined whether *P. euvitis* is indigenous in the Americas, although Ono (2000) determined that *Uredo vilalae* Lagerheim (holotype on a *V. vinifera* cultivar in Jamaica) and *U. vitis* Thümen (holotype on *Vitis* sp. in South Carolina, USA) were conspecific with *P. euvitis* because of the similarity in the uredinial morphology. According to Buriticá (1994, 1999), however, *P. uva* Buriticá & Hennen, with *U. vilalae* being an anamorph, occurs on *Cissus* sp., four indigenous *Vitis* species, and *Vitis vinifera* cultivars in southern North America and South America. Furthermore, Buriticá (1999) published another grapevine leaf rust fungus, *P. muscadiniae* Buriticá with *Uredo vitis* Thümen being an anamorph, which occurs on two indigenous *Vitis* species in the southern United States and Mexico. It is likely that *P. euvitis* is not indigenous in the Americas and that the American grapevine leaf rust is caused either by *P. uva* or *P. muscadiniae* or both at any given locality in the Americas. Any incidence of *P. euvitis* in the Americas, as detected in Brazil in 2001, could be of recent introduction. This situation is comparable with the recent invasion of the Asian soybean rust fungus, *P. pachyrhizi* Sydow & P. Sydow, to the Americas (where the American soybean rust fungus, *P. meibomiae* (Arthur) Arthur, is indigenous): the fungus was first found in Paraguay in 2001, spread to Brazil (Morel and Yorinori 2002), and to the southern United States in 2004 (Anonymous 2004; Dorrance 2004) and is now posing a threat to soybean production there. Thus, the introduction and establishment of *P. euvitis* could be dangerous to viticulture in North America, particularly in the states of California, Washington, New York, and Michigan, where the cultivation of European grape (*V. vinifera* cultivars) prevails, particularly if genetic recombination takes place between the American and the Asian grapevine leaf rust fungi.

Phylogenetic relationships between the Japanese collections and the Australian-East Timor collections of the grapevine leaf rust fungus are puzzling (Fig. 2). No morphological difference is detected in the uredinial stage between the collections; however, no other aspects of their biological nature has been surveyed for the Australian-East Timor grapevine leaf rust fungus, except for an inoculation study, which proved the infectivity of the Australian fungus to *Ampelocissus* species native to Australia (Daly et al. 2005). The Japanese grapevine leaf rust fungus has also been proven to infect *Ampelocissus* species introduced from Australia (Ono, unpublished data). Despite the assumed conspecificity of the Japanese and Australian-East Timor grapevine leaf rust fungi, ITS2 sequence differences are found at 20 base positions between the Japanese and Australian-East Timor fungal collections, each geographic collection being uniform for the 20 positions except for one Japanese collection (IBA-7720), which differs from other Japanese collections at sites 170 and 367 (Table 4). The grapevine leaf rust was first detected in Darwin, Northern Territory, Australia in 2001 (Weinert et al. 2003) and has been considered to be of recent introduction from adjacent regions. The situation is similar to the assumed recent introduction of a daylily rust fungus, *Puc. hemerocallidis* Thümen, into North America (William-Woodward et al. 2001). Peculiarly, ITS sequence differences at 13 base positions were detected between the Japanese and North American daylily rust collections (Hernándes et al. 2002; Chatasiri et al. 2006). No pertinent interpretation was possible for the genetic differences between them because neither spermogonial-aecial nor uredinial-telial hosts of the fungus are indigenous in North America.

It is likely that the grapevine leaf rust fungus in Darwin has recently been introduced from adjacent regions where *P. euvitis* has become established. Alternatively, a question might be raised as to if the grapevine leaf rust fungus might have established itself in Northern Territory for a long time on native *Ampelocissus* species with cryptic infection. However, the long dormancy of defoliated *Ampelocissus* plants during the dry season in northern Australia would not permit persistent occurrence of the grapevine leaf rust fungus (Daly and Hennessy 2006).

If the grapevine leaf rust fungus has recently been introduced from nearby East Timor and/or other tropical Asian regions, the grapevine leaf rust fungus distributed in tropical Asia should have genetically differentiated from, and may comprise a species distinct from, the grapevine leaf rust fungus distributed in temperate Asia. It has been pointed out that the grapevine leaf rust is quiescent during hot summers and becomes epidemic just before harvest in autumn in cool temperate regions, whereas it is

Table 4. Sequence differences in ITS2 region among *Phakopsora euvitis* collections examined in this study

Herbarium accession no.	Base position																				
	170	183	191	195	201	202	203	227	232	256	277	278	279	324	331	332	367	386	392	393	395
IBA-9688	C	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	C	C	T	C	T
IBA-8106	C	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	C	C	T	C	T
IBA-9605	C	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	C	C	T	C	T
IBA-8695	C	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	C	C	T	C	T
IBA-9679	C	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	C	C	T	C	T
IBA-9680	C	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	C	C	T	C	T
IBA-6612	C	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	C	C	T	C	T
IBA-8084	C	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	C	C	T	C	T
IBA-7720	T	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	T	C	T	C	T
IBA-6282	C	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	C	C	T	C	T
IBA-8584	C	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	C	C	T	C	T
IBA-7289	C	T	C	A	G	G	A	T	C	G	A	C	G	C	C	A	C	C	T	C	T
IBA-8750	T	–	G	C	T	T	T	C	T	T	G	T	T	T	A	T	C	A	A	T	A
IBA-8755	T	–	G	C	T	T	T	C	T	T	G	T	T	T	A	T	C	A	A	T	A
IBA-8756	T	–	G	C	T	T	T	C	T	T	G	T	T	T	A	T	C	A	A	T	A
IBA-8758	T	–	G	C	T	T	T	C	T	T	G	T	T	T	A	T	C	A	A	T	A
IBA-8808	T	–	G	C	T	T	T	C	T	T	G	T	T	T	A	T	C	A	A	T	A
IBA-8809	T	–	G	C	T	T	T	C	T	T	G	T	T	T	A	T	C	A	A	T	A
IBA-8810	T	–	G	C	T	T	T	C	T	T	G	T	T	T	A	T	C	A	A	T	A

Only positions where differences occurred are shown; all other positions are identical
Dashes represent alignment gaps

destructive throughout the year in warm temperate and subtropical regions (Ono 2000). This assumed physiological difference indicates the biological differentiation between the tropical/subtropical and cool temperate populations of the Asian grapevine leaf rust fungus. The grapevine leaf rust collections we studied were limited to Japan, Australia, and East Timor, however, and thus appropriate geographic collections representing all of Australasia should be studied as to the degree and extent of genetic and physiological differentiation, including host range and life cycle, to solve the species problem in grapevine leaf rust fungus in Australasia and some other possible locations.

The life cycle connection between uredinial/telial *P. ampelopsidis* and *Aecidium* on *M. tenuis* was easily presumed; however, no positive results had been obtained from repeated cross-inoculations with basidiospores of *P. ampelopsidis* and aeciospores of the *Aecidium* fungus. Our study shows that the unconnected *Aecidium* comprises a distinct clade and is more closely related to *P. euvitis* than to *P. ampelopsidis* (Figs. 1, 2). Undoubtedly, the *Aecidium* fungus is a spermogonial/aecial state of a *Phakopsora* species, as suggested by Ono (2000).

Autoecious *Phakopsora* fungus populations on *M. myriantha* and *M. tenuis* have been considered as conspecific (Hiratsuka et al. 1992; Ono 2000). The estimated phylogenetic patterns derived from nine collection data (see Table 2, Figs. 1, 2) show, however, that the two fungi as collected on the different host species have genetically diverged: two groups of collections examined differ at 11 base positions in D1/D2 and 71 base positions in ITS2 sequences, respectively (data not shown). Additional host-specificity study together with detailed morphological comparisons between the two fungal populations is needed to confirm their taxonomic status.

Genetic diversion is undoubtedly a common phenomenon among morphologically circumscribed, pleophagous rust species with wide geographic distribution, and two or more genealogically determined "phylogenetic species" may be recognized within a "morphological species," as in other groups of fungi (Taylor et al. 2000). No rust fungi have been analyzed for multiple gene genealogy at a species level, however. A few studies with heteroecious rust fungi show that well-resolved clades in molecular phylogenetic trees generated from ITS sequences correspond with species circumscribed by life cycle and host specificity (e.g., *Uromyces pisi* complex, Pfunder et al. 2001; *Puc. hemerocallidis* complex, Chatasiri et al. 2006; *Puc. andropogonis* complex and *Puc. coronata* complex, Szabo 2006; *P. ampelopsidis* complex, in this study) and even by morphology (in this study; Ono 2000). In contrast, molecular phylogenetic analyses of *Puc. monoica* complex resulted in distinct clades that are circumscribed neither by life cycle nor by spermogonial/aecial host specificity (Roy et al. 1998; Roy 2001). It is of great interest to see how each phylogenetic group in the *Puc. monoica* complex corresponds with uredinial/telial host species. It seems likely that, in sexually reproducing rust fungi, "biological species" circumscribed by life cycle and host specificity correspond with "phylogenetic species" determined by multiple gene genealogy, because the speciation in sexually reproducing rust fungi is likely to take place by genetic diversions to jump or shift to new host species that are ecogeographically available for new genetic variants (Roy 2001).

Acknowledgments We thank Mathew Weinert, Northern Australian Quarantine Strategy, Australia for providing us with the grapevine leaf rust collections from East Timor. This study was supported, in part, by a Grant-in-Aid for Scientific Research No. 07640921 from the Ministry of Education, Science and Culture, Japan and No. 18570081 from the Japan Society for the Promotion of Sciences.

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