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FULL PAPER

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Morphological and molecular phylogenetic analysis of *Melampsora* species on poplars in China

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Abstract Many species of *Melampsora* on *Populus* have been reported in China, based on morphological characteristics of both uredial and telial states, and on host species, but their morphology and taxonomy are still poorly defined. In this study, 196 specimens representing *Melampsora* species on poplars and collected from various areas of China were used for morphological observations. The morphological characteristics of urediniospores and teliospores were examined with light and scanning electron microscopy. The specimens could be classified into five groups based on their morphology. For the sequencing of the nuclear large subunit rDNA (D1/D2), 5.8S rDNA and their internal transcribed spacers, ITS1 and ITS2 region, 54 specimens were selected from the specimens used in morphological observations. These specimens were separated into six clades by phylogenetic analyses of the D1/D2 and ITS regions. Correlations among morphological groups and phylogenetic clades based on these results suggest a revision of these species. In particular, no evidence to discriminate specimens of *M. acedioides*, *M. magnusiana*, and *M. rostrupii* was found from either morphological characteristics or sequence analysis.

Key words *Melampsora* · Phylogeney · *Populus* · Rust fungus · Taxonomy · Uredinales

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Introduction

Since the genus *Melampsora* was established by Castagne in 1843 based upon *M. euphorbiae* (Schub.) Cast., about 90 species, showing either an autoecious or a heteroecious life cycle pattern, have been described worldwide (Kirk et al. 2001). Most of these occur on poplars and willows. Shang et al. (1986b) examined 34 species of poplar rusts reported in the world by using the host and characteristics of uredinia and telia and recognized 12 species. Dai (1989) studied species of *Melampsora* on poplars using 24 characters from urediniospores and teliospores and the aecial and telial hosts by the numerical taxonomic method. He reported 14 species. Bagyanarayana (1998) studied the morphology of *Melampsora* species on *Populus* species and recognized 9 species and 5 f. sp. under *M. populnea* (Pers. ex Pers.) Karst. After this, Cellerino (1999) listed 14 species of *Melampsora* on poplars. However, they are not definitive studies, and the taxonomy of *Melampsora* on various poplar species is not clear at the present time.

Poplar rusts caused by *Melampsora* spp. is one of the most important tree diseases in China. Five species, *M. larici-populina* Kleb., *M. laricis* Hart., *M. magnusiana* Wagn., *M. rostrupii* Wagn., and *M. pruinosae* Tranz., have been reported by Tai (1979). Yuan (1984) reported another three species, *M. abietis-canadensis* (Farl.) Ludw., *M. allii-populina* Kleb., and *M. occidentalis* Jacks. Shang et al. (1986a) described a new species, *M. multa* Shang, Pei & Yuan on *P.* × *euramericana* Moench., and they recognized *M. magnusiana* and *M. rostrupii* as a synonym of *M. aecidioides* Plowr. (Shang et al. 1990). The species *M. abietis-populi* Imai occurring on *Populus wilsonii* Schneid. in Shaanxi and Hubei Provinces was reported recently (Guo

1989; Zhang et al. 1997; Cao and Li 1999). Previously, it was reported only from Japan (Hiratsuka et al. 1992; Bagyanarayana 1998). Zhuang and Wei (1994) reported *M. populnea* on *Populus pseudoglauca* Wang & Fu. and *P. szechuanica* Schneid. var. *tibetica* Schneid. for the first time in China. Although these species are classified mainly by morphology of urediniospores and teliospores and by host range, identification is very difficult for several reasons. Almost all morphological characteristics of teliospores are very similar, and teliospores of some species do not appear during the growing season. Most species infecting poplars are heteroecious, with species of *Abies*, *Allium*, *Arum*, *Chelidonium*, *Corydalis*, *Fumaria*, *Larix*, *Mercurialis*, *Papaver*, *Picea*, *Pinus*, and *Tsuga* as secondary hosts, but characteristics on these secondary hosts are not useful for identification; also, some species overwinter as mycelia in the dormant buds of *Populus* without going to the alternate hosts, and some rust species may occur on the same aecial hosts. Although telial host range often is used to distinguish the *Melampsora* species, the same species of *Populus* can be infected by different *Melampsora* species. Nonhost poplars can be infected when artificial inoculations by urediniospores. Therefore, the morphology and taxonomy of *Melampsora* species on poplars are still confused.

Recently, molecular methods have been developed to clarify genetic variation and phylogenetic relationships of rust fungi (Nakamura et al. 1998; Vogler and Bruns 1998; Edwards et al. 1999; Newcombe et al. 2000; Ayliffe et al. 2001; Virtudazo et al. 2001; Hantula et al. 2002; Maier et al. 2003; Weber et al. 2003). The purpose of this study is to analyze morphological characteristics and phylogenetic relationships of *Melampsora* species on poplars in China, including *M. larici-populina*, *M. laricis*, *M. magnusiana* (=*M. aecidioides*), *M. rostrupii*, *M. pruinosae*, *M. allii-populina*, *M. multa*, *M. abietis-populi*, and *M. populnea*.

Materials and methods

Morphological observations

One-hundred ninety-six *Melampsora* specimens from 14 provinces of China were used for morphological analyses (Table 1). These specimens have been kept in the following herbaria: the Mycological Herbarium of College of Forestry, Northwest Sci-Tech University of Agriculture and Forestry, China (HMNWFC); the Mycological Herbarium of Institute of Microbiology, Chinese Academy of Sciences (HMAS); the Herbarium of College of Forestry, Inner Mongolia Agricultural University, China (HIM); and the Mycological Herbarium of Institute of Agriculture and Forestry, University of Tsukuba, Japan (TSH).

Table 1. *Melampsora* specimens on *Populus* species used for morphological observations

Section of *Populus*	Species of host plants	Localities[a] (no. of specimens)
Leuce	*P. adenopoda*	Yunnan (3)
	P. alba	Xinjiang (10), Gansu (1)
	P. alba var. *pyramidalis*	Xinjiang (4), Gansu (2), Inner Mongolia (4), Shaanxi (2)
	P. davidiana	Shaanxi (2), Beijing (2), Heilongjiang (5), Tibet (1), Inner Mongolia (3), Jilin (2)
	P. hopeiensis	Inner Mongolia (1), Shaanxi (1)
	P. rotundifolia	Yunnan (1)
	Populus sp.	Shaanxi (1)
	P. tomentosa	Beijing (2), Jilin (4), Shaanxi (12), Henan (2)
	P. tremula	Xinjiang (4)
Leucoides	*P. pseudoglauca*	Tibet (4)
	P. wilsonii	Shaanxi (3)
Aigeiros	*P. berolinensis*	Inner Mongolia (2)
	P. × *beijingensis*	Liaoning (1), Jilin (2)
	P. × *canadensis*	Jilin (13), Liaoning (2), Hebei (1), Shaanxi (3)
	P. deltoides × *P. lasiocarp*	Shaanxi (1)
	P. nigra	Shaanxi (1), Heilongjiang (2)
	P. nigra var. *italica*	Jilin (13), Shaanxi (2), Qinghai (1)
	P. nigra var. *thevestina*	Shaanxi (1)
	P. nigra × *P. laurifolia*	Xinjiang (1)
Tacamahaca	*P. cathayana*	Gansu (2), Shaanxi (4), Inner Mongolia (2), Qinghai (1)
	P. laurifolia	Xinjiang (8)
	P. maximoniicizii	Inner Mongolia (1)
	P. opera	Inner Mongolia (2)
	P. purdomii	Shaanxi (4)
	P. pseudo-simonii × *P. deltoids*	Shaanxi (1)
	P. popularis	Shaanxi (1)
	P. simonii	Inner Mongolia (5), Jilin (9), Shaanxi (4)
	P. simonii var. *shomliftia*	Inner Mongolia (1)
	Populus sp.	Jilin (4), Xinjiang (1), Inner Mongolia (2), Shaanxi (4)
	P. szechuanica	Shaanxi (1)
	P. talassica	Xinjiang (1)
	P. yunnanensis	Yunnan (8)
Turanga	*P. euphratica*	Inner Mongolia (5), Xinjiang (2), Ningxia (1)

[a] Provinces of China

Urediniospores and teliospores from specimens were mounted in a drop of lactophenol solution. About 30–50 spores from each specimen were randomly chosen and observed under a BH 100 microscope (Olympus, Tokyo, Japan). Length, width, wall thickness of both apex and lateral, and distance between spines of urediniospores were measured with a Q-Win Image Analyzer (Leica, Tokyo, Japan). Statistics, including multivariate analyses of measured continuous numerical variables, were performed using the software package SPSS (SPSS Japan, Tokyo, Japan) run on Windows 2000 Professional. Discrete numerical or qualitative attributes or host species were superimposed on two- or three-dimensional scatter diagrams generated from the analyses to detect possible groups.

The surface features of urediniospores and teliospores were observed by scanning electron microscopy (SEM). For SEM, samples were coated with platinum-palladium and were observed with a S-4200 scanning electron microscope (Hitachi, Tokyo, Japan) operated at 15 kV.

Polymerase chain reaction amplification and sequencing of D1/D2 and internal transcribed spacer regions

Fifty-four specimens of *Melampsora* on poplars were selected from the specimens used in morphological observations and for molecular phylogenetic analysis (Table 2). DNA was extracted from about 100–200 urediniospores obtained from a single uredinium and teliospores obtained from a single telium. Spores were crushed between two sterile glass slides and suspended in 20 μl extraction buffer containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Proteinase K, and 0.01% sodium dodecyl sulfate (SDS), then incubated first at 37°C for 60 min and then at 95°C for 10 min (Suyama et al. 1996; Virtudazo et al. 1998). From these crude extracts, a 5 μl aliquot was used directly for PCR amplification.

Amplifications were done using 40 μl polymerase chain reactions (PCR) each containing 0.2 μM primer, 1 unit of TaKaRa Taq DNA polymerase, a commercial deoxynucleoside triphosphate (dNTP) mixture (containing 2.5 mM of each dNTP), and Taq reaction buffer (containing 2 mM Mg^{2+}). PCR was carried out using a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA) under the following conditions: 95°C for 3 min, then 35 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final step of 72°C for 10 min. PCR of the D1/D2 region of nuclear large subunit rDNA was done using the primer pair NL1 (5′-GCATATC AATAAGCGGAGGAAAAG) and NL4 (5′-GGTCCGT GTTTCAAGACGG) (O'Donnell 1993). The internal transcribed spacer (ITS) and 5.8S region of rDNA was amplified with primers ITS1F (5′-CTTGGTCATTTAGA GGAAGTAA) (Gardes and Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC) (White et al. 1990). After amplification, 3 μl aliquots of the reaction products were electrophoresed on 1% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide in TAE buffer [40 mM Tris-HCl, 20 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4].

PCR products were purified by using MicroSpin S-400 HR columns (Amersham Pharmacia Biotech, NJ, USA). The purified PCR products were sequenced directly using a Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) with the same primers used for PCR. The reaction was set up as 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. The resulting fragments were finally purified using Centri-Sep spin columns CS-901 (Princeton Separations, Adelphia, NJ, USA) and loaded onto the sequencing gel. Data were collected using an ABI 377 Automated DNA Sequencer (PE Applied Biosystems).

Phylogenetic analysis

DNA sequences alignment was performed using the CLUSTAL X multiple alignment program, version 1.8 (Thompson et al. 1997). Further manual alignment was done in Se-Al v2.07a (Rambaut 2001). Alignment gaps were treated as a "fifth characters" base in the analysis. Phylogenetic trees were constructed using PAUP* v4.0b10 (Swofford 2002) with the neighbor-joining method (Saitou and Nei 1987) from LSU rDNA and ITS1–5.8S–ITS2 (ITS region) sequences. Neighbor-joining (NJ) analysis of a distance matrix produced by the Kimura two-parameter model (Kimura 1980) with a transition:transversion rate of 2.0 was applied with the default parameters of the program. Bootstrap analysis was based on 1000 bootstrap replicates using the NJ option for NJ trees (Felsenstein 1985).

Results

Morphology

All specimens observed could be divided into five groups based on position of sori in leaves, shape, size, and wall thickness of urediniospores, and distance between spines on the surface of urediniospores (Table 3). Group I and II differ from other in the smooth apex and length of urediniospores (>25 μm; Fig. 1A). Group I has the greatest difference in the laterally thickened urediniospore walls (=5.1 μm) with group II (=2.8 μm) and other groups, and all specimens of group I were clearly separated from other groups, as seen in the scatter plot (Fig. 1B). There was greater divergence between group V (= 1.1 μm) and other groups (>1.5 μm) in distance between spines of urediniospores (Fig. 1C). In addition, group V differs from group III and IV in the amphigenous telia and uredinia, and this group only occurred on sections of *Turanga* (Table 3). Group IV is similar to group III in shape, size, and distance between spines of urediniospores, but differs in having thin urediniospore wall (up to 2.7 μm) and the host plants of group III in section *Leuce* of *Populus*. Morphological characteristics of these groups were as follows.

Group I. Telia mainly epiphyllous (Fig. 2-1), small, single or coalescing in groups, light brown. Uredinia hypophyllous or rarely epiphyllous, especially in heavy infections.

Table 2. Specimens of *Melampsora* species on *Populus* species and their GenBank accession numbers used for phylogenetic analysis

Host plants	Localities[a]	Year and collector	Voucher specimens[b]	GenBank accession no.	
				D1/D2	ITS
P. adenopoda	Yunnan	1985, J.Y. Zhuang	TSH-R04133 (HMAS50159)	AB116807	AB116865
P. adenopoda	Yunnan	1985, J.Y. Zhuang	TSH-R04134 (HMAS50160)	AB116808	AB116866
P. adenopoda	Yunnan	1998, M. Kakishima	TSH-R20045	AB116819	AB116825
P. alba	Xinjiang	1966, Z.K. Liu	TSH-R04125 (HMAS37769)	AB116811	AB116854
P. alba	Xinjiang	1984, C.L. Wang	TSH-R04126 (HMAS58560)	AB116810	AB116855
P. alba	Xinjiang	1982, Z.K. Liu	HMNWFC-T041	AB116814	AB116850
P. alba var. *pyramidalis*	Xinjiang	1986, J.Y. Zhuang	TSH-R04136 (HMAS52886)	AB116813	AB116857
P. alba var. *pyramidalis*	Gansu	2001, C.M. Tian and Y.M. Liang	HMNWFC-T021 (TSH-R16945)	AB116815	AB116843
P. alba var. *pyramidalis*	Inner Mongolia	2001, C.M. Tian and Y.Z. Shang	HMNWFC-T022 (TSH-R16946)	AB116818	AB116849
P. alba var. *pyramidalis*	Xinjiang	1974, Z.Y. Zhao	TSH-R04129 (HMAS58565)	AB116817	AB116844
P. alba var. *pyramidalis*	Xinjiang	1983, C.L. Wang	TSH-R04130 (HMAS58578)	AB116812	AB116856
P. berolinensis	Inner Mongolia	1993, Z.S. Hou	HMNWFC-T008	AB116786	AB116830
P. cathayana	Gansu	2000, C.M. Tian	HMNWFC-T003 (TSH-R16927)	AB116769	AB116828
P. × *canadensis*	Jilin	2001, Q. Wang	TSH-R16983	AB116778	AB116840
P. davidiana	Tibet	1983, J.Y. Zhuang	TSH-R4149 (HMAS46905)	AB116809	AB116867
P. davidiana	Inner Mongolia	1991, Y.Z. Shang	HMNWFC-T033	AB116804	—
P. davidiana	Inner Mongolia	1994, Y.Z. Shang	HMNWFC-T038	AB116805	AB116863
P. euphratica	Ningxia	1980, N.X. Tian	HMAS49649	AB116793	AB116862
P. euphratica	Inner Mongolia	1992, Y.Z. Shang	HMNWFC-T036	AB116795	AB116858
P. euphratica	Inner Mongolia	1992, Y.Z. Shang	HMNWFC-T073	AB116794	AB116861
P. euphratica	Inner Mongolia	1992, Y.Z. Shang	HMNWFC-T045	AB116792	AB116859
P. euphratica	Inner Mongolia	1993, Y.Z. Shang	HMNWFC-T046	AB116796	AB116860
P. hopeiensis	Inner Mongolia	1992, Y.Z. Shang	HMNWFC-T031	AB116816	AB116846
P. laurifolia	Xinjiang	1984, Z.K. Liu	HMNWFC-T040	AB116788	AB116835
P. laurifolia	Xinjiang	1986, J.Y. Zhuang	TSH-R04141 (HMAS52890)	AB116801	AB116875
P. laurifolia	Xinjiang	1986, J.Y. Zhuang	TSH-R04139 (HMAS52888)	—	AB116873
P. laurifolia	Xinjiang	1986, J.Y. Zhuang	TSH-R04140 (HMAS52889)	AB116800	AB116874
P. laurifolia	Xinjiang	1986, J.Y. Zhuang	TSH-R04138 (HMAS52892)	AB116803	AB116872
P. maximoniicizii	Inner Mongolia	1993, Z.S. Hou	HMNWFC-T013	AB116776	AB116832
P. nigra var. *italica*	Jilin	2001, Q. Wang	TSH-R16975	AB116784	AB116836
P. nigra var. *italica*	Jilin	2001, Q. Wang	TSH-R16978	AB116771	AB116837
P. nigra var. *italica*	Jilin	2001, Q. Wang	TSH-R16980	AB116783	AB116842
P. opera	Inner Mongolia	2001, C.M. Tian and Y.Z. Shang	HMNWFC-T002 (TSH-R16926)	AB116774	AB116827
P. opera	Inner Mongolia	1992, Y.Z. Shang	HMNWFC-T015	AB116787	AB116833
P. pseudoglauca	Tibet	1990, J.Y. Zhuang	HMAS67387	AB116798	—
P. pseudoglauca	Tibet	1990, J.Y. Zhuang	HMAS67388	AB116797	AB116868
P. purdomii	Shaanxi	1999, C.M. Tian	HMNWFC-T004 (TSH-R16928)	AB116779	AB116829
P. popularis	Shaanxi	2000, C.M. Tian and Y.M. Liang	HMNWFC-T001 (TSH-R16925)	AB116770	AB116826
P. simonii	Inner Mongolia	1994, Z.S. Hou	HMNWFC-T017	AB116785	AB116834
P. simonii	Jilin	2001, Q. Wang	TSH-R16977	AB116781	AB116838
P. simonii	Jilin	2001, Q. Wang	TSH-R16979	AB116782	AB116839
P. simonii	Jilin	2001, Q. Wang	TSH-R16981	AB116775	AB116841
P. simonii var. *shomliflia*	Inner Mongolia	1994, Y.Z. Ren	HMNWFC-T011	Ab116772	AB116831
P. tomentosa	Shaanxi	1973, J. Xu and T.Z. Wang	HMAS56276	AB116822	AB116847
P. tomentosa	Shaanxi	2003, C.M. Tian	HMNWFC-T075	AB116791	AB116851
P. tomentosa	Shaanxi	2000, C.M. Tian	HMNWFC-T023 (TSH-R16947)	AB116780	AB116848
P. tomentosa	Jilin	2001, Q. Wang	TSH-R16987	AB116806	AB116864
P. tomentosa	Shaanxi	1978, Y. Jing	HMNWFC-T025	AB116777	AB116845
P. talassica	Xinjiang	1981, Z.K. Liu	HMNWFC-T035	AB116802	AB116871
P. tremula	Xinjiang	1981, M. Shi	HMNWFC-T043	AB116789	AB116852
P. tremula	Xinjiang	1981, J. Lan	HMNWFC-T044	AB116790	AB116853
P. wilsonii	Shaanxi	1994, N. Zhang	HMAS55410	AB116799	AB116870
P. wilsonii	Shaanxi	1996, Z.M. Cao	HMNWFC-TR0009	—	AB116869
P. yunnanensis	Yunnan	1998, M. Kakishima	TSH-R20046	AB116821	AB116823
P. yunnanensis	Yunnan	1998, M. Kakishima	TSH-R20042	AB116820	AB116824

ITS, internal transcribed spacer

[a] Provinces of China

[b] HMAS, the Mycological Herbarium of Institute of Microbiology, Chinese Academy of Sciences; HMNWFC, the Mycological Herbarium of College of Forestry, Northwest Sci-Tech University of Agriculture and Forestry, China; TSH, the Mycological Herbarium of Institute of Agriculture and Forestry, University of Tsukuba, Japan

Table 3. Morphological groups of *Melampsora* species on *Populus* species

Groups	Position of uredinia	Urediospores				Smooth parts	Position of telia	Sect. of host plants	No. of specimens
		Shape	Size (average) (μm)	Wall equatorial part thickness (average) (μm)	Distance between spines (average) (μm)				
I	Hypophyllous	Ellipsoid Oblong	20.5–54.5 × 11.3–29.8 (34.0) (18.4)	1.3–12.4 (5.1)	1.1–4.6 (2.3)	Apex	Epiphyllous	*Tacamahaca* *Aigeiros* *Leucoides*	103
II	Amphigenous	Clavoid Oblong	20.7–40.1 × 10.7–23.7 (29.4) (15.9)	1.3–6.8 (2.8)	1.3–3.5 (2.2)	Apex	Amphigenous	*Tacamahaca* *Leuce*	9
III	Hypophyllous	Globose Ovate	13.3–33.3 × 12.2–25.6 (21.8) (16.7)	1.1–5.0 (2.8)	1.1–4.4 (2.7)	Absent	Hypophyllous	*Leuce*	69
IV	Hypophyllous	Globose Oblong Ovate	17.7–27.9 × 9.2–23.4 (22.7) (16.0)	0.8–2.7 (1.8)	0.9–2.4 (1.6)	Absent	Hypophyllous	*Leucoides* *Tacamahaca*	7
V	Amphigenous	Globose Ellipsoid	19.2–32.1 × 14.8–24.6 (24.3) (19.9)	2.4–5.7 (3.5)	0.7–1.4 (1.1)	Absent	Amphigenous	*Turanga*	8

Urediniospores mostly ellipsoid or oblong, 20.2–54.5 × 11.3–29.8 μm (average, 34.0 × 18.4 μm); walls strongly thickened laterally (average, 5.1 μm, up to 12.4 μm; Fig. 2-2), echinulate except smooth at apex (Fig. 2-3), distance between spines 1.1–4.6 μm (average, 2.3 μm). Host plants in sect. *Tacamahaca*, sect. *Leucoides*, and sect. *Aigeiros* of *Populus* (Table 3). This group differs from other groups in the laterally thickened urediniospore walls (Fig. 1B), longer urediniospores (average length, >30 μm), and the epiphyllous telia.

Group II. Telia amphigenous (Fig. 2-4), small, 0.5–1 mm, single, red-brown. Uredinia amphigenous, orange-yellow. Urediniospores mostly clavoid or oblong, 20.7–40.1 × 10.7–23.7 μm (average, 29.4 × 15.9 μm); walls usually uniformly thick or rarely irregularly thick, 1.3–6.8 μm (average, 2.8 μm; Fig. 2-5). echinulate, except smooth at apex (Fig. 2-6), distance between spines 1.3–3.5 μm (average, 2.2 μm). Host plants in sect. *Tacamahaca* and sect. *Leuce* of *Populus*. This group differs from others in the uniformly thick walls and smooth apex of urediniospores (Table 3).

Group III. Telia mainly hypophyllous (Fig. 2-7), golden to light brown initially, dark reddish-brown to black when mature. Uredinia mostly hypophyllous or rarely epiphyllous. Urediniospores globose, ovate, or elongate and 13.3–33.3 × 12.2–25.6 μm (average, 21.8 × 16.7 μm); walls uniformly thick, 1.1–5.0 μm (average, 2.8 μm; Fig. 2-8), distance between spines 1.1–4.4 μm (average 2.7 μm; Table 3). Host plants of this group are in sect. *Leuce* of *Populus*. This group differs from other groups in urediniospore shape and size (Fig. 2-8; Table 3).

Group IV. Telia hypophyllous (Fig. 2-10), single, red-brown. Uredinia hypophyllous, scattered, small, 0.1–0.5 mm, light yellow. Urediniospores globoid, ellipsoid, or oblong, 17.7–27.9 × 9.2–23.4 μm (average, 22.7 × 16.0 μm); walls uniformly thick, 0.8–2.7 μm (average, 1.8 μm; Table 3, Fig. 2-11), echinulate, distance between spines 0.9–2.4 μm (average, 1.6 μm). Host plants *P. wilsonii* Schneid and *P. pseudoglauca* Wang et Fu of sect. *Leucoides* and *P. yunnanensis* Dode of sect. *Tacamahaca.* This group differs from others in having a thin urediniospore wall.

Group V. Telia amphigenous (Fig. 2-13), single, reddish-brown. Uredinia amphigenous (Fig. 2-13), scattered or coalescing in groups, orange-yellow. Urediniospores globoid or ellipsoid, 19.2–32.1 × 14.8–24.6 μm (average, 24.3 × 19.9 μm); walls uniformly thick (Fig. 2-14), 2.4–5.7 μm (average, 3.5 μm), echinulate, distance between spines 0.7–1.4 μm (average, 1.1 μm). Host plant *P. euphratica* Oliv of sect. *Turanga*. This group differed from other groups in having a small echinulate (Fig. 2-15) and short distance between spines (see Table 3).

Phylogeny

The PCR products of the D1/D2 regions of the LSU rDNA of specimens on *Populus* ranged from 605 to 610 bp in

length. The alignment data matrix consists of 55 taxa and 614 characters, of which 563 were constant and 12 variable characters were parsimony uninformative, leaving 39 informative characters in the analyses. The specimens were separated into six clades (clades A, B, C, D, E, and F) based on the phylogenetic tree using the NJ method when *M. lini* (L20283) and *M. helioscopiae* (AF426197) were used as outgroups (Fig. 3). The consistency index (CI) was 0.791, retention index (RI) was 0.953, and rescaled consistency index (RC) was 0.754.

Clade A, supported by 89% of the bootstrap replicates, included 17 specimens on sections *Tacamahaca* and *Aigeiros* of *Populus*. Specimens belonging to morphological group I were included in this clade. Clade B, supported by 100% of the bootstrap replicates, included 6 specimens on *P. davidiana*, *P. adenopada* and *P. tomentosa* of sect. *Leuce*. Specimens belonging to this clade were included in morphological group III together with clade F. Clade C, supported by 98% of the bootstrap replicates, included 5 specimens on *P. wilsonii*, *P. pseudoglauca* of sect. *Leucoides*, and *P. yunnanensis* of sect. *Tacamahaca* of *Populus* (morphological group IV). Specimens on sections *Tacamahaca* and *Leuce* of *Populus* were included in clade D. Specimens belonging to morphological group II were included in this clade. Clade E, supported by 94% of the bootstrap replicates, included 5 specimens on *P. euphratica* (morphological group V). Specimens on *P. alba*, *P. alba* var. *pyramidalis*, *P. hopeiensis*, *P. tremula*, and *P. tomentosa* of sect. *Leuce* were included in clade F, and they belong to morphological group III.

The ITS rDNA amplification products of specimens on *Populus* ranged from 659 to 679 bp in length, included ITS1–5.8S–ITS2 regions. The alignment data matrix consists of 690 characters, of which 558 were constant and 9 variable characters were parsimony uninformative, leaving 123 informative characters in the analyses. The CI was 0.791, RI was 0.968, and RC was 0.766. The NJ tree constructed from ITS and 5.8 S rDNA regions also separated the specimens into six clades (clades A, B, C, D, E, and F) with high bootstrap support when *M. occidentalis*

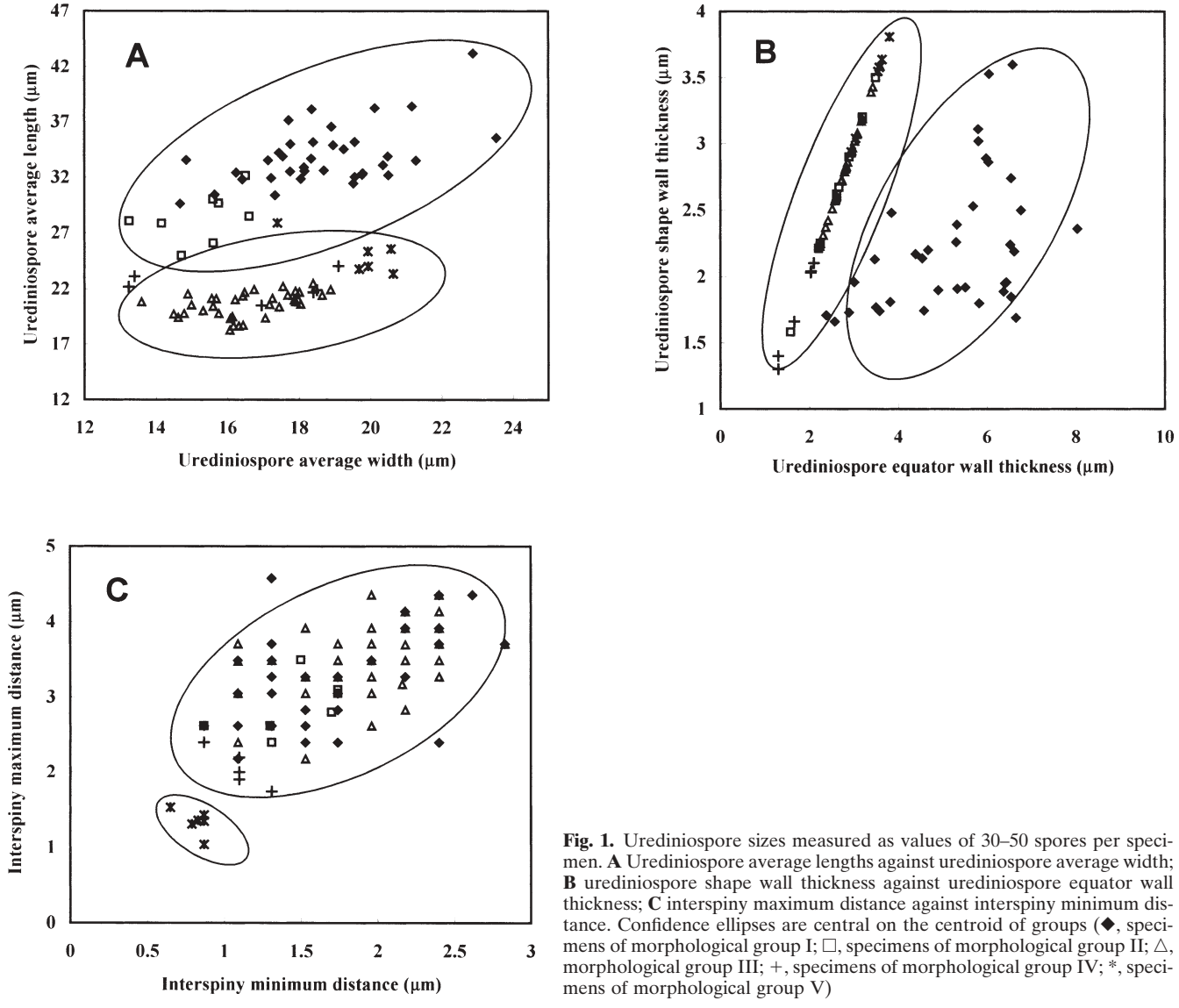
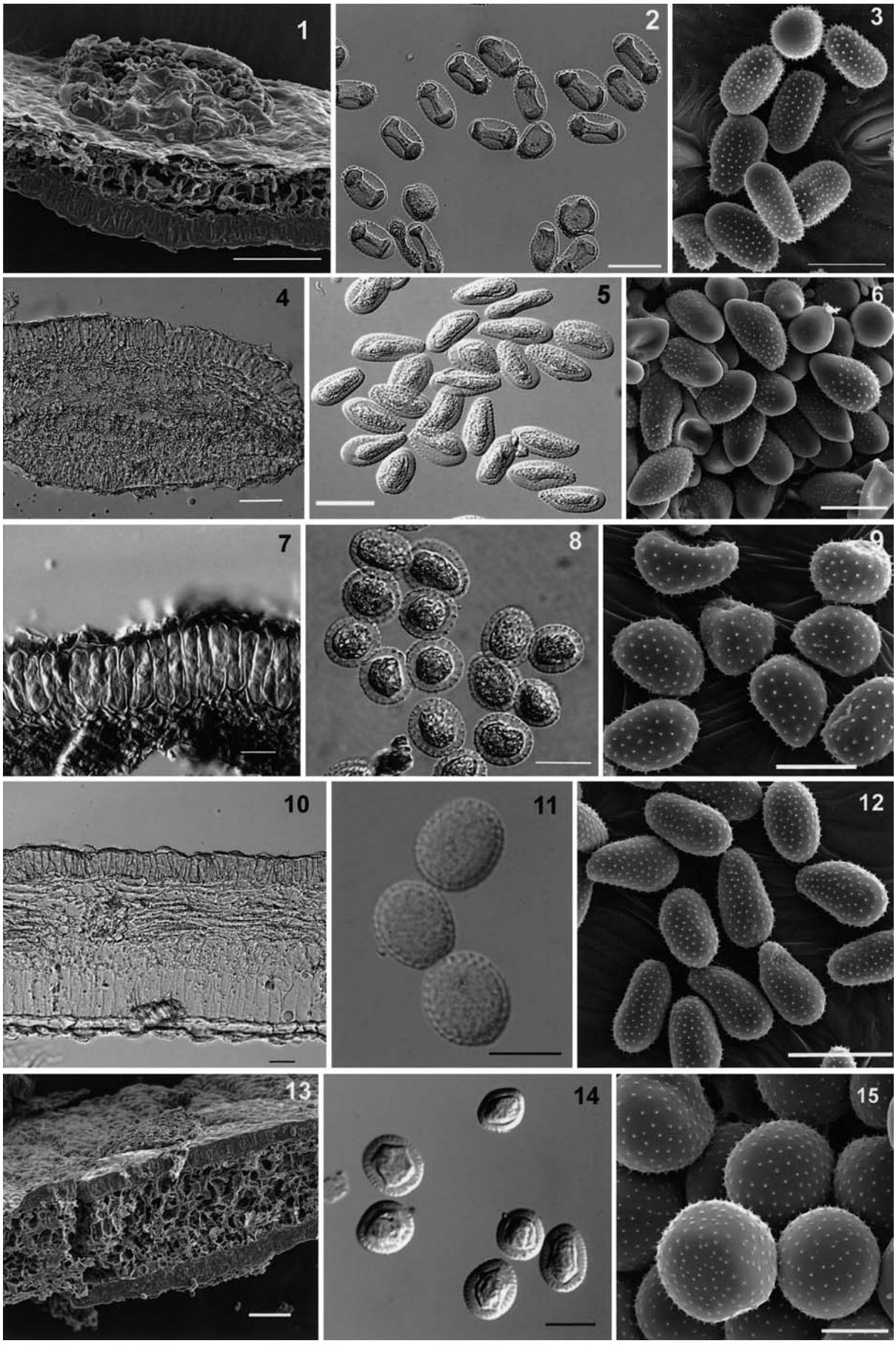


Fig. 1. Urediniospore sizes measured as values of 30–50 spores per specimen. **A** Urediniospore average lengths against urediniospore average width; **B** urediniospore shape wall thickness against urediniospore equator wall thickness; **C** interspiny maximum distance against interspiny minimum distance. Confidence ellipses are central on the centroid of groups (◆, specimens of morphological group I; □, specimens of morphological group II; △, morphological group III; +, specimens of morphological group IV; *, specimens of morphological group V)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(AF087711) and *M. medusae* (AF087710) were used as outgroups (Fig. 4).

Specimens belonging to morphological group I were included in clade E; morphological group II was included in clade C; specimens belonging to morphological group III were included in clade D and clade F; morphological group IV was included in clade A; and morphological group V was included in clade B. However, phylogenetic trees showed high similarity of genetic variation between ITS region and LSU rDNA regions from the specimens of *Melampsora* on *Populus*.

Discussion

In this study, *Melampsora* specimens on *Populus* species were divided into five different groups by morphological observations. These specimens were separated into six clades by phylogenetic analyses of the LSU rDNA (D1/D2) and ITS1–5.8S–ITS2 regions (Table 4).

Specimens on sections *Tacamahaca*, *Aigeiros*, and *Leucoides* belonging to morphological group I were included in the same phylogenetic groups as D1/D2 and ITS sequences. Specimens of morphological II were included in the same group (D1/D2 clade D and ITS clade C).

Specimens on sect. *Leuce* belonging to morphological group III were separated into two phylogenetic groups. Based on D1/D2 regions, these specimens can be separated into two clades (D1/D2 clade B and F), and the same specimens were separated into two clades (ITS clade D and F) based on sequence analysis of the ITS regions. Specimens of D1/D2 clade F contain 15 collections of *P. alba*, *P. alba* var. *pyramidalis*, *P. hopeiensis*, *P. tomentosa*, and *P. tremula*; these same specimens were separated into ITS clade D. Specimens belonging to D1/D2 clade B and ITS clade F are the same, and included specimens on *P. tomentosa*, *P. davidiana*, and *P. adenopoda* belonging to morphological group III. Sequence analyses confirmed that genetic relationship of specimens of D1/D2 clade B and ITS clade F are closer to morphological group IV (D1/D2 clade C and ITS clade A) than clade F (D1/D2) and clade D (ITS). Although there are no obvious morphological differences within group III, the phylogenetic analyses strongly suggest that there are two distinct taxa within the group.

Table 4. Relationship among morphological groups and phylogenetic clades

Morphological group[a]	D1/D2 clade[b]	ITS clade[c]
I	A	E
II	D	C
III	F	D
III	B	F
IV	C	A
V	E	B

[a] Table 4 and Fig. 1
[b] Fig. 3
[c] Fig. 4

The specimens on *P. tremula*, *P. tomentosa*, *P. adenopoda*, and *P. davidiana* were usually identified as *M. laricis* based on characters of urediniospores in China (Wang 1949; Tai 1979; Yuan 1984; Zhuang 1986; Shang et al. 1990; Guo 1989). Cao and Li (1999) described specimens on *P. adenopoda* and *P. davidiana* as *M. populnea*, and *M. laricis* was identified as a synonym of *M. populnea*, as there is no morphological difference. However, these specimens were separated into two different groups based on our molecular analyses. Specimens on *P. tremula*, along with other specimens on sect. *Leuce*, were included in D1/D2 clade F and ITS clade D. Specimens on *P. davidiana* and *P. adenopoda* were included in D1/D2 clade B and ITS clade F.

In former various reports, specimens on *P. tomentosa, P. alba, P. alba* var. *pyramidalis*, and *P. hopeiensis* were identified as *M. magnusiana* (Tai 1979; Yuan 1984; Guo 1989; Cao and Li 1999), *M. aecidioides* (Liu and Wang 1936; Shang et al. 1990), or *M. rostrupii* (Ge et al. 1964; Tai 1979) in China. Our morphological and phylogenetical analyses showed no morphological and genetic variation among specimens identified as *M. aecidioides*, *M. rostupii*, and *M. magnusiana* (group III, D1/D2 clade F and ITS clade D).

All specimens on *P. euphratica* were morphologically and phylogenetically included in the same group (morphological group V, D1/D2 clade E and ITS clade B) and were clearly separated from other groups with high bootstrap support. Therefore, we consider that this group represents a distinct taxon from other groups.

Specimens belonging to morphological group IV were clearly included in the same genetic group (D1/D2 clade C or ITS clade A). Some specimens on *Populus* species were identified as *M. populnea* or *M. laricis* in China (Guo 1989; Zhuang and Wei 1994); however, in this study, these specimens can be placed in morphological group IV based on

Fig. 2. Urediniospores and teliospores of *Melampsora* spp. on poplars observed by light microscopy and SEM. **1–3** Group I. **1** Hypophyllous urediniospores and epiphyllous teliospores on *Populus opera* (HMNWFC-T002). **2** Ellipsoid or oblong urediniospores with wall thickened equatorially on *P. laurifolia* (HMNWFC-T040). **3** Urediniospores echinulate except for smooth apex on *P. simonii* × *P. nigra* var. *italica* (TSH-R16925). **4–6** Group II. **4** Amphigenous telia on *P. laurifolia* (HMAS4138). **5** Oblong urediniospores with uniform cell wall on *P. laurifolia* (HMAS4138). **6** Urediniospores with smooth apex on *P. talassica* (HMNWFC-T035). **7–9** Group III. **7** Teliospores on *P. alba* var. *pyramidalis* (HMAS4132). **8** Globose, or ovate urediniospores with uniform cell wall on *P. tomentosa* (HMNWFC-T024). **9** Urediniospores with echinulate surface on *P. tomentosa* (HMNWFC-T026). **10–12** Group IV. **10** Hypophyllous teliospores on *P. wilsonii* (HMNWFC-TR0009). **11** Globose, or ellipsoid urediniospores with a thin cell wall on *P. yunnanensis* (TSH-R20042). **12** Urediniospores with echinulate surface on *P. yunnanensis* (TSH-R20046). **13–15** Group V. **13** Amphigenous uredinia and telia on *P. euphratica* (HMNWFC-T046). **14** Globose, or ovate urediniospores with uniform cell wall. **15** Urediniospores surface with small echinulate on *P. euphratica* (HMNWFC-T037). *Bars* **1** 120 μm; **2** 35 μm; **3** 21 μm; **4** 55 μm; **5** 35 μm; **6** 20 μm; **7** 35 μm; **8** 20 μm; **9** 14 μm; **10** 30 μm; **11** 18 μm; **12** 20 μm; **13** 100 μm; **14** 20 μm; **15** 8.4 μm

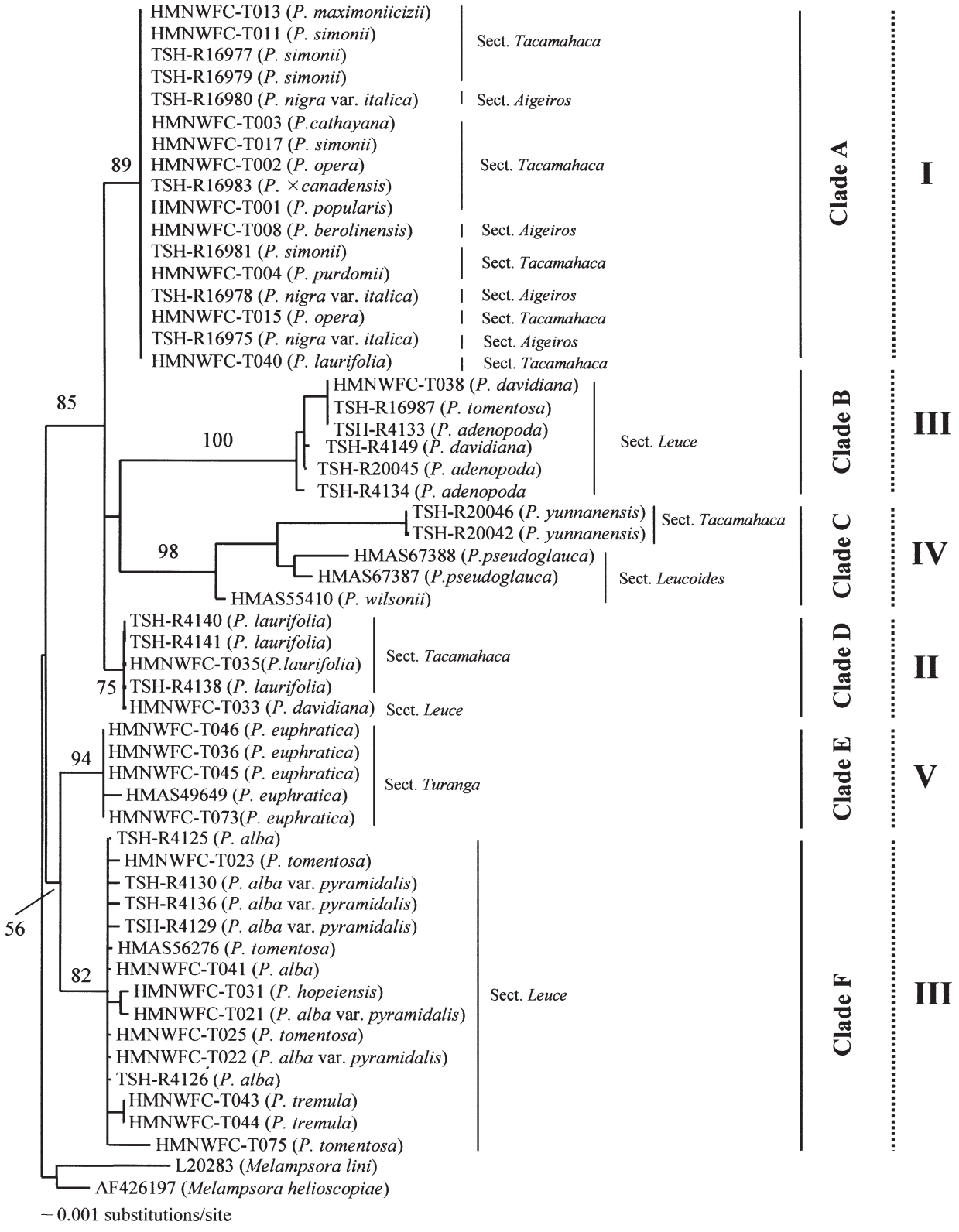

Fig. 3. Phylogenetic tree constructed by neighbor-joining method for 53 specimens of *Melampsora* on poplars based on nucleotide sequences of the LSU rDNA region. The values at the nodes are the confidence levels from 1000 replicate bootstrap samplings

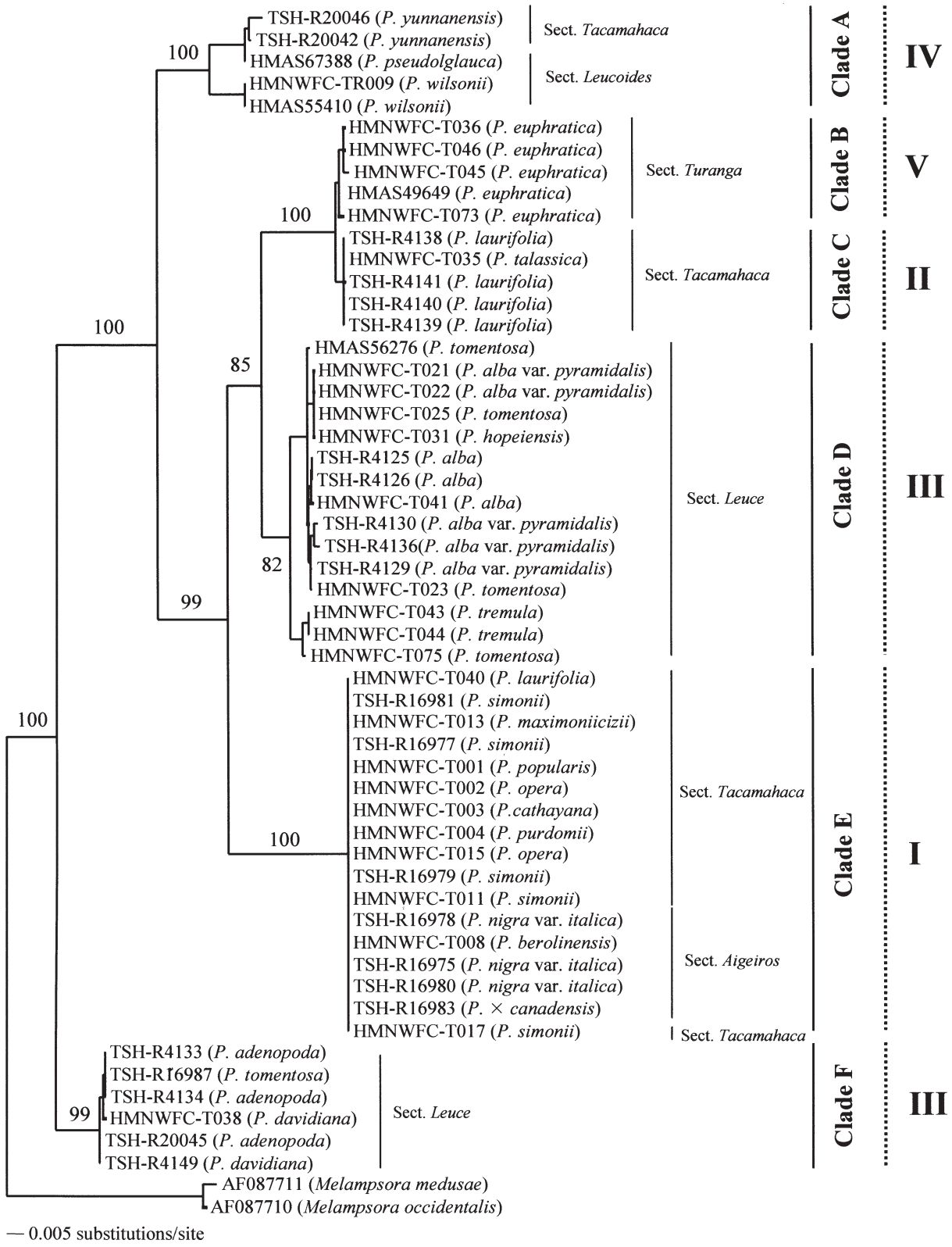

Fig. 4. Phylogenetic tree constructed by neighbor-joining method for 53 specimens of *Melampsora* on poplars based on nucleotide sequences of the ITS1–5.8S–ITS2 region of rDNA. The values at the nodes are the confidence levels from 1000 replicate bootstrap samplings

urediniospore wall thickenings and phylogenetical analyses. Therefore, we consider that group IV is a distinct taxon from others, and the thin urediniospore wall (average, $<2\,\mu m$) is a consistently dependable character for identification of this group from other groups. We will discuss the taxonomic treatment of these taxa including examinations of authentic specimens (including type specimens) in another paper.

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