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Differentiation of *Melampsora* rust species on willows in Japan using PCR-RFLP analysis of ITS regions of ribosomal DNA*

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To develop a reliable method for identifying *Melampsora* species parasitic on willows in Japan, we differentiated 10 *Melampsora* species by PCR-RFLP analysis. Internal transcribed spacer (ITS) regions, including 5.8S ribosomal DNA, of 63 collections of 10 *Melampsora* species and 4 collections of unidentified species were amplified by PCR. The fragments from the 67 collections varied in size (approximately 880 bp, 860 bp and 840 bp). The restriction sites in the amplified DNA fragments were mapped after the RFLP analysis using four restriction enzymes, *Dra* I, *Eco*R I, *Ssp* I and *Taq* I. All the collections were divided into 11 RFLP types. In the 6 species, *M. capraearum*, *M. epiphylla*, *M. kamikotica*, *M. larici-urbaniana*, *M. microsora* and *M. yezoensis*, the RFLP type was species-specific. The RFLP type of *M. chelidonii-pierotii* and *M. coleosporioides* was identical. The collections of *M. epitea* were separated into three RFLP types. One of these three types was identical with the type of *M. humilis*. It is suggested that the PCR-RFLP analysis of ITS regions is a useful and reliable method for species identification of *Melampsora*.

Key Words——internal transcribed spacer; *Melampsora*; PCR-RFLP; rust fungi; willow.

Fourteen *Melampsora* species have been reported to produce uredinia and telia on willows, *Salix*, *Toisusu* and *Chosenia* spp., in Japan (Hiratsuka and Kaneko, 1982). Among them, seven have heteroecious life cycles and produce spermogonia and aecia on larches, *Larix* spp., or several herbaceous plants, *Chelidonium* and *Corydalis* spp., but life cycles of the other species are unknown (Hiratsuka and Kaneko, 1982).

Melampsora spp. have been identified based on morphology of both uredinial and telial states and/or host species. However, identification of the species is very difficult since morphological delimitation of the species is not distinct. Furthermore, identification of the willow species is problematic because their morphological characteristics are highly variable and they easily intercross with one another naturally, producing many hybrids (Newsholme, 1992). Additionally, it is difficult to identify the rust species based on aecial morphology or by host species because the rusts are morphologically quite similar to each other in their aecial states and different rust species may occur on the same aecial host.

In recent years, polymerase chain reaction (PCR)-based methods have been developed and used as new tools for identification and phylogenetic studies of many fungi, e.g., PCR using species- or genus-specific primers, random amplified polymorphic DNA (RAPD) analysis, and PCR-restriction fragment length polymorphism (RFLP) analysis (Bruns et al., 1991; Hibbet, 1992; Foster et al., 1993; Hensen and French, 1993). For the identification and phylogenetic studies of genera or species, the ribosomal DNA (rDNA) is known to be useful because it is highly conserved genetically and has large copy numbers in genomes (Bruns et al., 1991; Hibbet, 1992; Hensen and French, 1993). Furthermore, internal transcribed spacer (ITS) regions, including 5.8S rDNA, which have high genetic variation, have been utilized for phylogenetic studies and for identification of many species (Bruns et al., 1991; Hibbet, 1992; Hensen and French, 1993).

Only a few studies using molecular techniques for identifying species of rust fungi have been made so far, e.g., in *Puccinia* spp. (Zambino and Szabo, 1993; Kropp et al., 1997) and in *Cronartium* spp. (Vogler, 1995). These studies showed that nucleotide sequences of ITS regions were useful for identifying the rust species. PCR-RFLP analysis is useful for detecting DNA polymorphism in the objective sequences more easily than through sequencing.

We report here the classification of 10 species of *Melampsora* on willows from Japan by using PCR-RFLP analysis of ITS regions of rDNA to develop a reliable method for identification.

Materials and Methods

Rust fungi used Fifty collections of *Melampsora* spp. producing uredinia and/or telia were collected with host

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Table 1. Collections of *Melampsora* species tested.

Species and collection No.	Host plant (Japanese common name)	Locality	Specimen No.
M. capraearum			
CAb-1	*Salix bakko* (Yamanekoyanagi)	Gumma	TSH-R9828
CAb-2	*S. bakko*	Nagano	TSH-R9829
CAb-3	*S. bakko*	Nagano	TSH-R9830
CAb-4	*S. bakko*	Miyagi	TSH-R9831
CAb-5	*S. bakko*	Fukushima	TSH-R9832
CAb-6	*S. bakko*	Tokyo	TSH-R9833
CAh-1	*S. hultenii* (Marubanobakkoyanagi)	Hokkaido	TSH-R9834
CAh-1-S	*S. hultenii*	Hokkaido	TSH-R9835
CAl-1	*S.* x *leucopithecia* (Furisodeyanagi)	Miyagi	TSH-R9836
Mca1Ac[b]	*S.* x *leucopithecia*	Ibaraki	
M. chelidonii-pierotii			
CHc-1	*S. chaenomeloides* (Marubayanagi)	Ibaraki	TSH-R9837
CHc-2	*S. chaenomeloides*	Hiroshima	TSH-R9838
CHc-3	*S. chaenomeloides*	Ibaraki	TSH-R9839
CHp-1	*S. pierotii* (Otachiyanagi)	Ibaraki	TSH-R9840
CHp-1-S[a]	*S. pierotii*	Ibaraki	TSH-R9841
CHp-2	*S. pierotii*	Shizuoka	TSH-R9842
Mch1B[b]	*S. pierotii*	Nagano	
M. coleosporioides			
COb-1	*S. babylonica* (Shidareyanagi)	Iwate	TSH-R9843
COb-2	*S. babylonica*	Ibaraki	TSH-R9844
CObr-1	*S. babylonica* (Rokkakuyanagi) var. *lavallei* forma *rokkaku*	Miyagi	TSH-R9845
COm-1	*S. matsudana* (Unryuyanagi) var. *tortuosa*	Tokyo	TSH-R9846
COm-2	*S. matsudana* var. *tortuosa*	Nagano	TSH-R9847
COm-2-S[a]	*S. matsudana* var. *tortuosa*	Nagano	TSH-R9848
Mco1Ab[b]	*S. babylonica*	Ibaraki	
M. epiphylla			
EPs-1	*S. sachalinensis* (Onoeyanagi)	Gumma	TSH-R9849
EPs-2	*S. sachalinensis*	Ibaraki	TSH-R9850
EPs-3	*S. sachalinensis*	Nagano	TSH-R9851
EPs-3-S[a]	*S. sachalinensis*	Nagano	TSH-R9852
EPs-4	*S. sachalinensis*	Nagano	TSH-R9853
EPs-5	*S. sachalinensis*	Niigata	TSH-R9854
EPs-6	*S. sachalinensis*	Fukushima	TSH-R9855
Mep1Ab[b]	*S. sachalinensis*	Tochigi	
Mep3B[b]	*S. kinuyanagi* (Kinuyanagi)	Miyagi	

plant leaves at various localities in Japan from June 1995 to November 1996 (Table 1). For DNA extraction, the collections with uredinia or immature telia obtained from the field were stored at −80°C. For morphological observation, dry specimens from these collections were made and kept as vouchers in the Mycological Herbarium, Institute of Agriculture and Forestry, University of Tsukuba (TSH) (Table 1). These collections were identified by morphological characteristics of both uredinial and telial states according to Hiratsuka and Kaneko (1982). Ten species of *Melampsora* were identified among them and four collections remained unidentified because of the absence of telial state (Table 1).

Additionally, nine single uredinial cultures including eight species were established from nine collections and used for DNA extraction (Table 1). They were derived from a single uredinium on a leaf and have been maintained on detached host leaves on 1% (w/v) agar con-

Table 1. Continued.

Species and collection No.	Host plant (Japanese common name)	Locality	Specimen No.
M. epitea			
ETf-1	*S. futura* (Oonekoyanagi)	Niigata	TSH-R9856
ETg-1	*S. gilgiana* (Kawayanagi)	Nagano	TSH-R9857
ETg-1-S[a]	*S. gilgiana*	Nagano	TSH-R9858
ETg-2	*S. gilgiana*	Iwate	TSH-R9859
ETg-3	*S. gilgiana*	Fukushima	TSH-R9860
ETg-4	*S. gilgiana*	Ibaraki	TSH-R9861
ETj-1	*S. japonica* (Shibayanagi)	Yamanashi	TSH-R9862
ETj-1-S[a]	*S. japonica*	Yamanashi	TSH-R9863
ETm-1	*S. miyabeana* (Ezonokawayanagi)	Hokkaido	TSH-R9864
ETr-1	*S. reinii* (Miyamayanagi)	Iwate	TSH-R9865
ETr-2	*S. reinii*	Nagano	TSH-R9866
Met1Be[b]	*S. futura*	Ishikawa	
Met2Cb[b]	*S. gilgiana*	Miyagi	
M. humilis			
HUi-1	*S. integra* (Inukoriyanagi)	Nagano	TSH-R9867
HUi-1-S[a]	*S. integra*	Nagano	TSH-R9868
HUi-2	*S. integra*	Nagano	TSH-R9869
HUi-3	*S. integra*	Iwate	TSH-R9870
Mhu1B[b]	*S. integra*	Nagano	
M. kamikotica			
KAa-1	*Chosenia arbutifolia* (Keshoyanagi)	Nagano	TSH-R9871
M. larici-urbaniana			
LAu-1	*Toisusu urbaniana* (Obayanagi)	Iwate	TSH-R9872
LAu-2	*T. urbaniana*	Gumma	TSH-R9873
M. microsora			
MIs-1	*S. subfragilis* (Tachiyanagi)	Hokkaido	TSH-R9874
MIs-2	*S. subfragilis*	Ibaraki	TSH-R9875
MIs-2-S[a]	*S. subfragilis*	Ibaraki	TSH-R9876
MIs-3	*S. subfragilis*	Tokyo	TSH-R9877
M. yezoensis			
YEj-1	*S. jessoensis* (Shiroyanagi)	Iwate	TSH-R9878
YEj-2	*S. jessoensis*	Miyagi	TSH-R9879
YEs-1	*S. serissaefolia* (Kogomeyanagi)	Gumma	TSH-R9880
YEs-2	*S. serissaefolia*	Nagano	TSH-R9881
YEs-2-S[a]	*S. serissaefolia*	Nagano	TSH-R9882
Unidentified species			
ft-1[c]	*S. futura*	Nagano	TSH-R9883
gl-1[c]	*S. gilgiana*	Aomori	TSH-R9884
jp-1[c]	*S. japonica*	Miyagi	TSH-R9885
jp-2[c]	*S. japonica*	Chiba	TSH-R9886

a) Single uredinial culture.
b) Axenic culture of uredinial stage established by Yamaoka and Katsuya (1984a, b, c).
c) Lacking specimens of telial state.

taining 40 μg/ml benzimidazole in Petri dishes at 23$\pm$1°C with 16 h of fluorescent light (3,000 lx) per day.

Eight collections of axenic cultures of six species of *Melampsora* growing on an artificial medium were also used for DNA extraction (Table 1). These axenic cultures were established from uredinial states by Yamaoka and Katsuya (1984a, b, c) and have been maintained on QMS-7 medium (Yamaoka and Katsuya, 1984b) at 20°C in darkness.

DNA extraction Total DNA was extracted from uredinial and/or telial states on host tissue and from axenic cultures by a modified method of Dellaporta et al. (1983).

Leaves with uredinia and/or telia or axenic cultures (20 mg each) were frozen with liquid nitrogen, then ground with a mortar and pestle with 750 μl of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, and 2% (v/v) 2-mercaptoethanol). The ground material was transferred to Eppendorf tubes and supplemented with 50 μl of 20% (w/v) sodium dodecyl sulfate (SDS). These extracts were mixed thoroughly and incubated at 65°C for 20 min. After adding 250 ml of 5 M potassium acetate, they were incubated at 0°C for 20 min, then centrifuged for 10 min at 14,000 rpm. The supernatants were transferred into new Eppendorf tubes, supplemented with 600 μl of 2-propanol, and incubated at −80°C for 20 min. After centrifugation, the pellets in the tubes were dissolved in 200 μl of the buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0), then 200 μl of phenol/chloroform (1 : 1) mixture was added, and the whole mixture was shaken. After centrifugation for 10 min at 14,000 rpm, the aqueous phases were transferred to new tubes, and 20 μl of 3 M sodium acetate and 140 μl of 2-propanol were added. The precipitates, including nucleic acids, were centrifuged for 10 min at 14,000 rpm, washed with 70% (v/v) ethanol, dried, and suspended in 50 μl (100 μl in the case of axenic cultures) of RNase A solution (20 μg/ml in TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After incubation at 37°C for 30 min, the DNA preparations were stored at −20°C until use. Similarly, total DNA was extracted from rust-free plant leaves of 18 *Salix* spp., *Toisusu urbaniana* (Seemen) Kimura and *Chosenia arbutifolia* (Pall.) A. Skvortsov (Table 1).

PCR amplification The primer pair ITS1-F and ITS4-B designed by Gardes and Bruns (1993) was used for the amplification of ITS regions including 5.8S rDNA and for joining parts of the 3′ end of the small subunit rDNA (approximately 70 bp in size) and the 5′ end of the large subunit rDNA (approximately 200 bp in size). PCR amplification was performed in 100 μl of reaction mixture containing 1 μM concentrations of each primer, 1 mM concentrations of each dNTP, 2 units of *Tth* DNA polymerase (Toyobo, Japan), and reaction buffer (1 mM Tris-HCl, pH 8.9, 8 mM KCl, 0.15 mM $MgCl_2$, 50 μg/ml of bovine serum albumin, 0.01% (w/v) sodium cholate, and 0.01% (v/v) Triton X-100). Two microliters of DNA preparation (approximately 10–20 ng DNA) was used as a template per reaction. The mixtures were covered with 40 μl of mineral oil to prevent evaporation. Reaction cycles were repeated 40 times with the following program: 95°C, 30 s; 55°C, 1 min; 72°C, 1 min. After amplification, aliquots (8 μl) of the reaction mixtures were electrophoresed on 1% (w/v) agarose gels containing 0.5 μg/ml of ethidium bromide in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4).

RFLP analysis and mapping of restriction sites Amplified DNA fragments were partially purified as follows: After the reaction mixture containing amplified DNA fragments was electrophoresed on a 1.5% (w/v) low temperature-melting agarose gel, then a piece of gel containing the objective fragment was cut off, transferred to an Eppendorf tube, and TE buffer was added to the tube. After heating the tube at 65°C, TE-saturated phenol in the same volume as the piece of gel and the TE buffer was added, and the tube was shaken. After centrifugation for 10 min at 14,000 rpm, the aqueous phase was transferred to a new tube, an equal volume of chloroform was added, and the tube was shaken. The aqueous phase was transferred to a new tube, and 1/10 volume of 3 M sodium acetate and 7/10 volume of 2-propanol were added. After centrifugation, the precipitates were washed with 70% (v/v) ethanol, dried, and dissolved in TE buffer. The purified fragments (approximately 50 ng) were digested with four restriction enzymes, *Dra* I, *Eco*R I, *Ssp* I, and *Taq* I, according to the manufacturer's instructions. The digested fragments were separated by 8% (w/v) polyacrylamide gel electrophoresis in TAE buffer and stained with ethidium bromide. To determine restriction maps, restriction patterns of DNA fragments amplified by PCR using two primer pairs (ITS1-F and ITS2, ITS3 and ITS4-B) and fragment patterns of the digestion by double enzymes were used. The primers ITS2 and ITS3 were designed by White et al. (1990) and located in 5.8S rDNA.

Results

Amplification of ITS regions The primer pair ITS1-F and ITS4-B permitted the amplification of a single DNA fragment (approximately 850 bp in size) from uredinia and/or telia on host plant tissue and axenic cultures among 10 *Melampsora* species. Amplification of DNA fragments from rust-free host plants was not recognized. We show the results of the amplification from the samples of

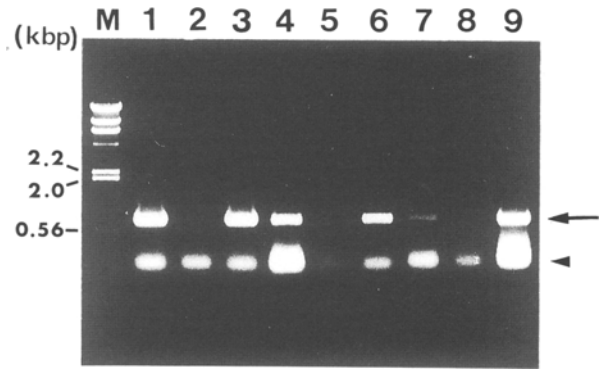

Fig. 1. Amplification by polymerase chain reaction (PCR) using primer pair ITS1-F and ITS4-B from DNA preparations extracted from uredinial states on host plant tissue and axenic cultures of three *Melampsora* species, and rust-free host plants.
Lane 1, *M. caprаearum* collection CAb-1; lane 2, *Salix bakko*; lane 3, *M. caprаearum* collection Mca1Ac; lane 4, *M. coleosporioides* collection COb-1; lane 5, *S. babylonica*; lane 6, *M. coleosporioides* collection Mco1Ab; lane 7, *M. epiphylla* collection EPs-1; lane 8, *S. sachalinensis*; lane 9, *M. epiphylla* collection Mep1Ab; M, fragment size markers (*Hin*d III-digested λ phage DNA). Rust-specific PCR products (approximately 850 bp in size) are marked with an arrow. The arrowhead indicates non-specific artifacts.

the 3 species and the respective host plants (Fig. 1). When electrophoresis on 8% polyacrylamide gel was performed to estimate the size of the DNA fragment precisely, variation in the size of the fragments was observed among the 10 species. The fragment sizes varied from approximately 840 to 880 bp (data not shown), but was uniform within each species except for *M. epitea* (Kunze et Schm.) Thümen. It was approximately 880 bp in all the collections of the 4 species *M. capraearum* (DC.) Thümen, *M. epiphylla* Dietel, *M. humilis* Dietel, and *M. yezoensis* Miyabe et Matsumoto; approximately 860 bp in all the collections of *M. kamikotica* Kaneko et Hiratsuka and *M. larici-urbaniana* Matsumoto; and approximately 840 bp in all the collections of *M. chelidonii-pierotii* Matsumoto, *M. coleosporioides* Dietel, and *M. microsora* Dietel. On the other hand, the fragments from eight collections of *M. epitea* were approximately 880 bp in size and the fragments from three collections were 840 bp in size.

RFLP analysis and mapping of restriction sites Preliminary experiments were conducted to select restriction enzymes suitable for detection of DNA polymorphism in the amplified fragments from 10 species. The DNA fragments from each representative collection were digested with 12 enzymes, *Alw*44 I, *Dra* I, *Eco*R I, *Hae* III, *Hha* I, *Hinc* II, *Hinf* I, *Hind* III, *Msp* I, *Rsa* I, *Ssp* I, and *Taq* I, and electrophoresed on 8% (w/v) polyacrylamide gels. Five enzymes, *Alw*44 I, *Hha* I, *Hinc* II, *Hind* III, and *Msp* I, had no recognition site, but *Hae* III, *Hinf* I, and *Rsa* I had one, four and three sites, respectively. On the other hand, the numbers of the recognition sites with 4 enzymes, *Dra* I, *Eco*R I, *Ssp* I, and *Taq* I, were not identical among the 10 species. As a result, these 4 enzymes were used for subsequent RFLP analysis (data not shown).

Each of the amplified DNA fragments from all the collections was digested with the four restriction enzymes, *Dra* I, *Eco*R I, *Ssp* I, and *Taq* I, and restriction maps were made. The three enzymes *Dra* I, *Eco*R I, and *Ssp* I had one or no recognition site in the fragments, while *Taq* I had two or three sites (Fig. 2). All the recognition sites with the four enzymes used for mapping were located in either 5.8S rDNA or the ITS 2 region, which flanks the large subunit rDNA (Fig. 3). One out of two *Taq* I sites in the fragments of all the collections was located in nucleotide sequences of primers ITS2 and ITS3 (Fig. 3).

Consequently, all the collections of 10 species were divided into 11 RFLP types according to RFLP pattern using four enzymes (Table 2, Fig. 3). In all the collections

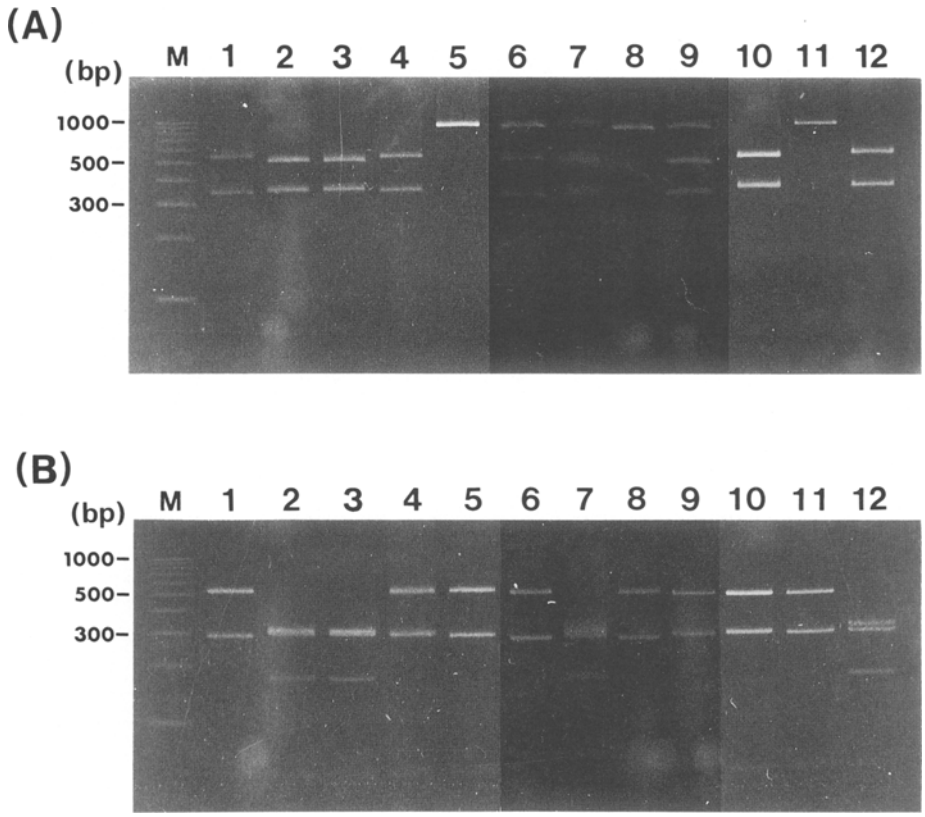

Fig. 2. Restriction patterns of internal transcribed spacer regions of ribosomal DNA amplified from 10 *Melampsora* species with *Eco*R I (A) and *Taq* I (B).
Lane 1, *M. capraearum* collection CAb-1; lane 2, *M. chelidonii-pierotii* CHc-1; lane 3, *M. coleosporioides* COb-1; lane 4, *M. epiphylla* EPs-1; lane 5, *M. epitea* ETg-1; lane 6, *M. epitea* Met1Be; lane 7, *M. epitea* ETj-1; lane 8, *M. humilis* HUi-1; lane 9, *M. kamikotica* KAa-1; lane 10, *M. larici-urbaniana* LAu-1; lane 11, *M. microsora* MIs-1; lane 12, *M. yezoensis* YEj-1; M, fragment size markers (100 bp DNA ladder).

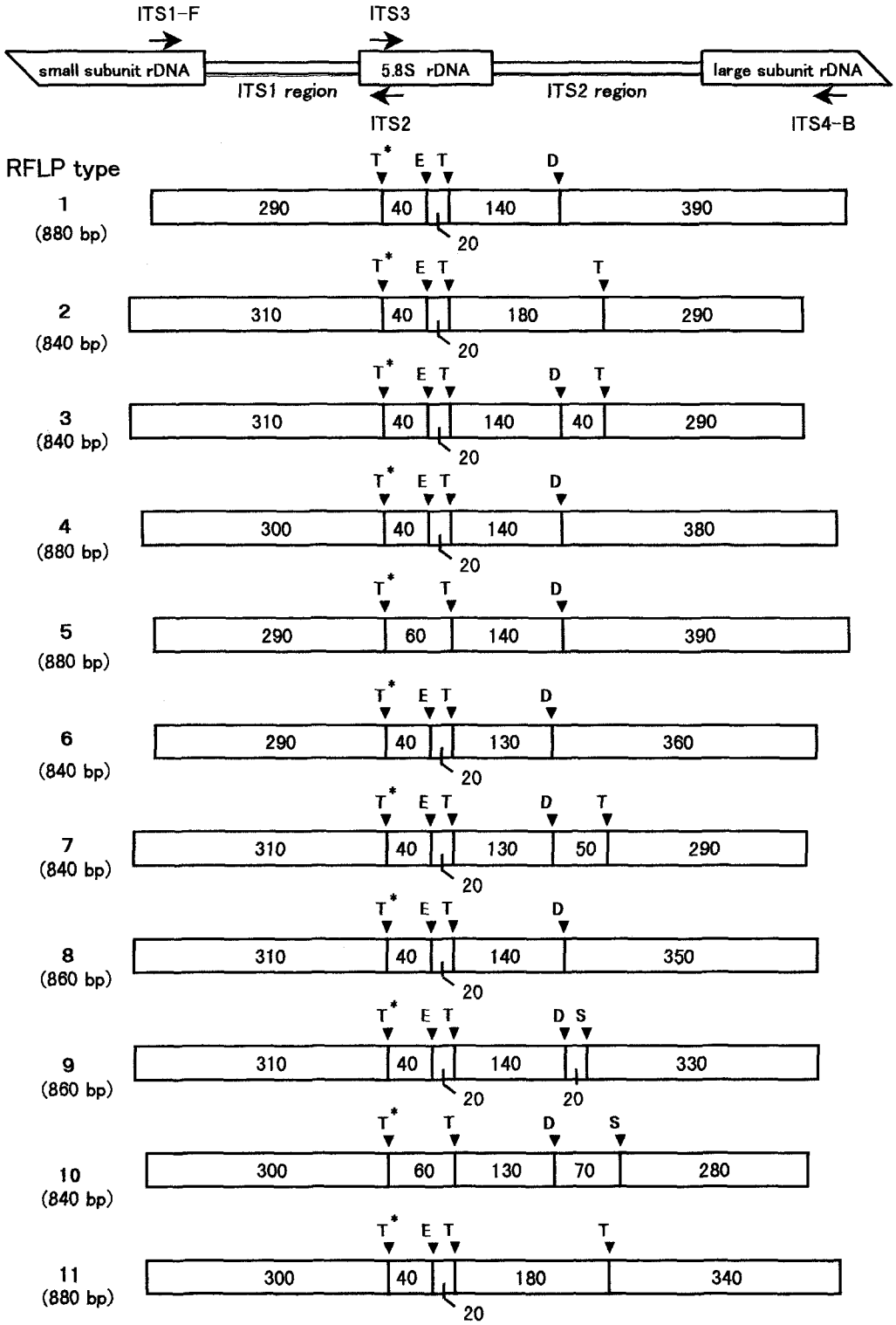

Fig. 3. Representation of the ribosomal DNA (rDNA) repeat showing internal transcribed spacer (ITS) regions, including 5.8S rDNA, and restriction maps of ITS regions amplified by using the primer pair ITS1-F and ITS4-B.
Locations of primers used for amplification are delineated as arrows. Estimated sizes of restriction fragments are indicated in base pairs. ▼ indicates recognition sites for four enzymes: D, *Dra* I; E, *Eco* R I; S, *Ssp* I; T, *Taq* I. The asterisked *Taq* I site is located in the sequences of primers ITS2 and ITS3. Sizes of intact DNA fragments amplified from *Melampsora* collections are parenthesized under RFLP type number.

of 8 species, *M. capraearum*, *M. coleosporioides*, *M. epiphylla*, *M. humilis*, *M. kamikotica*, *M. larici-urbaniana*, *M. microsora*, and *M. yezoensis*, the RFLP pattern was identical within species. The RFLP types of the collections obtained from the field corresponded with those of the single uredinial cultures (Table 2). RFLP type 1 (*M. capraearum*), type 4 (*M. epiphylla*), type 8 (*M. kamikotica*), type 9 (*M. larici-urbaniana*), type 10 (*M. microsora*) and type 11 (*M. yezoensis*) were species-specific. The collections of *M. epitea* from which 840 bp fragments were amplified were divided into two RFLP types, type 6 (the collection Met1Be) and type 7 (the collections ETf-1 and ETj-1). All the collections of *M. humilis* and 9 collections of *M. epitea* from which 880bp fragments were amplified showed the same RFLP type, type 5 (Table 2, Figs. 2, 3). Consequently, the collections of *M. epitea* were separated into 3 RFLP types, types 5, 6 and 7. All the collections, except for collection Mch1B (type 3), of *M. chelidonii-pierotii* and *M. coleosporioides* showed the same RFLP type, type 2.

The RFLP types of the collections among unidentified species were as follows: type 6 of the collection ft-1 on *Salix futura* Seemen, type 5 of gl-1 on *S. gilgiana* Seemen, type 1 of jp-1 on *S. japonica* Thunb. and type 7 of jp-2 on *S. japonica* (Table 2).

Discussion

Gardes and Bruns (1993) reported that the primer pair ITS1-F and ITS4-B was intended to be specific to basidiomycetes. In our experiments, this primer pair permitted the amplification of a single DNA fragment from both leaves with rust sori and axenic cultures, but no amplification of any DNA fragment from rust-free host plant leaves. Therefore, this primer pair is confirmed to be useful for the amplification of the DNA of rust fungi from

Table 2. RFLP Type of collections of *Melampsora* species.

Species	Collection No.	RFLP type
M. capraearum	CAb-1, CAb-2, CAb-2-S[a]	1
	CAb-3, CAb-4, CAb-5	
	CAb-6, CAh-1, CAl-1,	
	Mca1Ac[b]	
M. chelidonii-pierotii	CHc-1, CHc-2, CHc-3	2
	CHp-1, CHp-1-S[a], CHp-2	2
	Mch1B[b]	3
M. coleosporioides	COb-1, COb-2, CObr-1	2
	COm-1, COm-2, COm-2-S[a]	
	Mco1Ab[b]	
M. epiphylla	EPs-1,EPs-2, EPs-3	4
	EPs-3-S[a], EPs-4, EPs-5,	
	EPs-6, Mep1Ab[b], Mep3B[b]	
M. epitea	ETg-1, ETg-1-S[a], ETg-2	5
	ETg-3, ETg-4, ETm-1	
	ETr-1, ETr-2, Met2Cb[b]	
	Met1Be[b]	6
	ETf-1, ETj-1, ETj-1-S	7
M. humilis	HUi-1, HUi-1-S, HUi-2	5
	HUi-3, Mhu1B[b]	5
M. kamikotica	KAa-1	8
M. larici-urbaniana	LAu-1, LAu-2	9
M. microsora	MIs-1, MIs-2, MIs-2-S	10
	MIs-3	
M. yezoensis	YEj-1, YEj-2, YEj-2-S	11
	YEs-1, YEs-2	
Unidentified species	ft-1	6
	gl-1	5
	jp-1	1
	jp-2	7

a) Single uredinial culture.
b) Axenic culture of uredinial state established by Yamaoka and Katsuya (1984a, b, c).
c) Lacking specimens of telial state.

rust-infected plants, as reported previously (Vogler, 1995).

Sizes of the amplified DNA fragments were identical within each species except for *M. epitea*. They differed slightly among 10 species as follows: approximately 880 bp in 4 species, approximately 860 bp in 2 species, and approximately 840 bp in 3 species. A fragment of either approximately 880 bp or approximately 840 bp was amplified from the collections identified as *M. epitea* based on the morphological characteristics. Polymorphism of fragment size of ITS regions was also recognized as reported previously in other fungal groups (Gardes et al., 1991; Harlton et al., 1995; Crawford et al., 1996; Sreenivasaprasad et al., 1996) and it was thought to be variable in the sequences of ITS regions because of nucleotide deletions or insertions. However, we consider that it is not beneficial for the identification of species because no significant difference was detected electrophoretically.

In making the restriction maps based on RFLP patterns using the four restriction enzymes, *Dra* I, *Eco*R I, *Ssp* I and *Taq* I, all collections of 10 species were divided into 11 RFLP types. The collections of 6 species, *M. capraearum*, *M. epiphylla*, *M. kamikotica*, *M. larici-urbaniana*, *M. microsora*, and *M. yezoensis* revealed species-specific RFLP types. The RFLP type was identical within a species in spite of differences in host plants and localities. Furthermore, because no difference was found in the RFLP type between the collections obtained from the field and collections of single uredinial cultures, genetic variation within collections was not found to be present in any of the collections tested. Therefore, the RFLP type is considered to be a useful character for distinguishing these species.

On the other hand, no difference was detected in the RFLP types between *M. chelidonii-pierotii* (except for one collection Mch1B) and *M. coleosporioides*. Also, no difference in the RFLP patterns was detected between the two species in preliminary experiments with three enzymes, *Hae* III, *Hin*f I, and *Rsa* I (data not shown). Uredinial and telial states of these two species are also morphologically similar. Therefore, we suspected a close genetic relationship between the two species. The RFLP type of *M. chelidonii-pierotii* Mch1B differed from other collections of the same species. Mch1B was identified based on the morphology of urediniospores and host species once. It will now be necessary to re-identify the collection by examining morphology of teliospores.

The RFLP type (type 5) was identical for all the collections of *M. humilis*. The RFLP type of nine collections of *M. epitea* from which the 880 bp fragment was amplified was the same as that of *M. humilis*. In the preliminary experiments with three enzymes, *Hae* III, *Hin*f I, and *Rsa* I, no distinction was found in the RFLP patterns between the two species (data not shown). Hiratsuka and Kaneko (1982) distinguished *M. humilis* from *M. epitea* by slight differences in morphology of teliospores and provisionally treated it as an independent species. Our results suggested a close genetic relationship between *M. humilis* and the collections of *M. epitea*.

Neither RFLP types 6 and 7 in the collections of *M. epitea* from which 840 bp fragments were amplified were identical with any other types found in the other *Melampsora* species used in the present study. The collections of *M. epitea* were separated into three groups based on RFLP types. Three RFLP types in *M. epitea* seemed to be correlated with the natural host species, i.e., the collections on *S. gilgiana*, *S. miyabeana* Seemen, and *S. reinii* Franch. et Savat. showed type 5; the collections on *S. futura* showed type 6; and the collections on *S. futura* and *S. japonica* showed type 7. In Europe and North America, *M. epitea* has been treated as a species complex because of different spermogonial and aecial hosts and/or uredinial and telial hosts (Hylander et al., 1953; Wilson and Henderson, 1966; Ziller, 1974).

The RFLP types distinguished in the present study might be regarded as markers for delimitation of species, because the types were species-specific for six species as described above. Consequently, *M. epitea* in Japan is suggested to be a species complex including at least three independent species. To solve these taxonomic problems involving the three groups in *M. epitea*, cross-inoculation experiments, the confirmation of alternate hosts, and more precise morphological observations are needed.

Among the species of *Melampsora* that were unidentified because of a lack of specimens of their telial states, gl-1, ft-1 and jp-2 were of RFLP type 5, type 6 and type 7, respectively. These three types were found in *M. epitea* used in the present study, and the RFLP types and host plants were well correlated as described above. Furthermore, as the morphological characteristics of urediniospores and host species in these collections were identical with the description of *M. epitea* reported by Hiratsuka and Kaneko (1982), the three collections were considered to belong to the *M. epitea* complex described above.

The RFLP type of the collection jp-1 on *S. japonica* was identical with that of *M. capraearum*, type 1. However, *M. capraearum* has not been reported to be parasitic on *S. japonica*. Although the morphology of urediniospores in the collection jp-1 is similar to that of *M. capraearum*, the telial state is necessary for correct identification of this collection. Confirmation of the species name by this process will show the RFLP type to be an important character for identifying species of *Melampsora*.

In making the restriction maps, two *Taq* I sites in all the fragments and the *Eco*R I site were reported to occur in nucleotide sequence of 5.8S ribosomal DNA judging from sequence data of *Puccinia* and *Cronartium* species (Zambino and Szabo, 1993; Morrica et al., 1996; White et al., 1996). All the restriction sites of the four enzymes used for mapping are located in either the 5.8S rDNA or the ITS 2 region, which flanks the large subunit rDNA. Therefore, the ITS 2 region was considered to be genetically more variable than the ITS 1 region and also to be very useful for identifying species of *Melampsora* by using RFLP analysis.

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