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SHORT COMMUNICATION

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# Correlation between pathogenicity and molecular characteristics in the willow leaf rusts *Melampsora epitea* and *M. humilis* in Japan

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**Abstract** We studied the correlation between pathogenicity and restriction fragment length polymorphism (RFLP) type, which was determined by polymerase chain reaction-based RFLP analysis of the internal transcribed spacer regions of ribosomal DNA, in the willow leaf rust fungi *Melampsora epitea* and *M. humilis*. Eighteen clones of eight *Salix* species were inoculated with urediniospores from seven collections of the two rust species. *M. epitea* and *M. humilis* (RFLP type-5 collections) were pathogenic to six to eight *Salix* species. RFLP type-7 collections of *M. epitea* were pathogenic to only two *Salix* species. The taxonomic relationships of the two rust species are discussed.

**Key words** Inoculation · *Melampsora epitea* · *Melampsora humilis* · Pathogenicity · Willow leaf rust

Willows, which belong to the Salicaceae, are a valuable source of biomass because of their fast growth and easy propagation (Newsholme 1992). Willow leaf rust diseases, caused by *Melampsora* species, often occur in willow plantations and occasionally result in serious damage owing to premature defoliation. *Melampsora epitea* (Kunze et Schmidt) Thümen is one of the most destructive rust species to cultivated willow and is distributed in Europe, North America, New Zealand, and central and eastern Asia (Wilson and Henderson 1966; Ziller 1974; Hiratsuka et al. 1992; Spiers and Hopcraft 1996). This fungus has a heteroecious life cycle and produces uredinia and telia on a large number of *Salix* species and spermogonia and aecia on various woody and herbaceous plants.

European *M. epitea* were separated into six races based on the aecial hosts (Wilson and Henderson 1966). In addition, one of the six races, *M. larici-epitea* Klebahn., whose alternative hosts are larches, was separated into seven formae speciales based on its uredinial and telial hosts (Gaümann 1959). Consequently, *M. epitea* has been treated as a species complex in Europe and North America (Gaümann 1959; Wilson and Henderson 1966; Ziller 1974).

In *M. epitea* in Japan, the spermogonial and aecial hosts are species of *Larix*, and the uredinial and telial hosts are seven species of *Salix* (Hiratsuka et al. 1992; Kondo et al. 1994). However, races or formae speciales within the species were unrecognized because of few inoculation experiments with basidiospores and urediniospores. Recently, Nakamura et al. (1998) separated *M. epitea* collected from three *Salix* species into three groups based on their restriction fragment length polymorphism (RFLP) types (types 5, 6, and 7), which were determined by polymerase chain reaction (PCR)-based RFLP analysis of the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). They suggested a positive correlation between the RFLP type of *M. epitea* and its uredinial and telial hosts because each of the three groups of *M. epitea* was collected from the different uredinial host plants. They also found that the RFLP type 5 of *M. epitea* was identical to that of *M. humilis* Dietel in RFLP band patterns. *M. humilis* is distributed in a limited area of eastern Asia, including Japan (Hiratsuka and Kaneko 1982). The pathogenicity of the uredinial state of *M. humilis* to the host plants of *M. epitea* was not reported precisely.

We investigated the pathogenicity of the uredinial state of RFLP types 5 and 7 of *M. epitea*, as well as that of *M. humilis* (RFLP type 5), to clarify the correlation between pathogenicity and the RFLP types, and discussed the taxonomic considerations on these species.

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**Table 1.** Collections and isolates of *Melampsora epitea* and *M. humilis*

| Species and collection no. | Host | Locality | RFLP type[a] | Isolates used in inoculation experiments[b] |
|---|---|---|---|---|
| *M. epitea* | | | | |
| ETf-1 | *Salix futura* | Niigata | Type 7 | ETf-1-m |
| ETg-1 | *S. gilgiana* | Nagano | Type 5 | ETg-1-S, ETg-1-S2, ETg-1-S3 |
| ETg-3 | *S. gilgiana* | Fukushima | Type 5 | ETg-3-S, ETg-3-S2, ETg-3-S3 |
| ETj-1 | *S. japonica* | Yamanashi | Type 7 | ETj-1-S, ETj-1-S2, ETj-1-S3 |
| *M. humilis* | | | | |
| HUi-2 | *S. integra* | Nagano | Type 5 | HUi-2-S, HUi-2-S2, HUi-2-S3 |
| HUi-3 | *S. integra* | Iwate | Type 5 | HUi-3-S, HUi-3-S2, HUi-3-S3 |
| RS1 | *S. integra* | Gunma | Type 5 | HU1[c] |

RFLP, restriction fragment length polymorphism
[a] Determined according to Nakamura et al. (1998)
[b] Isolate ETf-1-m is a mass-uredinial isolate; the other isolates are single-uredinial isolates
[c] Provided by R. Suzuki, University of Tsukuba

The uredinial and telial states of four rust materials (collections ETg-1, ETg-3, ETj-1, and ETf-1) were collected along with host-plant leaves from three *Salix* species (*S. futura* Seemen, *S. gilgiana* Seemen, and *S. japonica* Thunb.), and of two rust materials (collections HUi-2 and HUi-3) from *S. integra* Thunb., from June 1995 to November 1996 in Japan (Table 1). The four "ET" collections were identified as *M. epitea*, and the two "HU" collections as *M. humilis*, on the basis of the morphology of the uredinial and telial states, according to the criteria of Hiratsuka and Kaneko (1982). Voucher specimens of our collections were deposited in the Mycological Herbarium, Institute of Agriculture and Forestry, University of Tsukuba (Nakamura et al. 1998).

Isolates that were derived from several uredinia, which we call a mass-uredinial isolate, from each collection were maintained separately on detached host leaves on 1% (w/v) agar plates containing 40 μg/ml benzimidazole in Petri dishes (9 cm diameter) at 23°C under fluorescent lighting (16 h light/day). Inoculations with the isolate to new leaves were repeated at intervals of 2–3 weeks. Three single-uredinial isolates were also obtained from each mass-uredinial isolate from three collections of *M. epitea* (ETg-1, ETg-3, and ETj-1) and from two collections of *M. humilis* (HUi-2 and HUi-3) (Table 1). These isolates were derived from a single uredinium produced after inoculation with a mass-uredinial isolate to a new host plant leaf and were maintained described as above. A single-uredinial isolate of another *M. humilis* collection (RS1) was also used. RFLP types in the collections were determined as in our previous report (Nakamura et al. 1998) (see Table 1).

Eighteen clones from eight *Salix* species, which were reported to be uredinial host plants for *M. epitea* or *M. humilis*, were obtained from various sites in Japan and maintained in growth chambers (Table 2). Two *Salix* species (*S. integra* and *S. koriyanagi* Kimura) are known to be host plants for *M. humilis* and seven *Salix* species (all except *S. koriyanagi*) for *M. epitea* (Hiratsuka et al. 1992; Kondo et al. 1994). The scientific names of the *Salix* species used in this study follow the nomenclature of A. Kimura (Iizumi et al. 1980).

**Table 2.** *Salix* species used for inoculation experiments with urediniospores of *Melampsora epitea* and *M. humilis*

| *Salix* species | Clone no. | Origin in Japan |
|---|---|---|
| *S. futura*[a] | SF-1 | Nagano |
| | SF-A1 | Niigata |
| *S. gilgiana*[a] | HB-101 | BG1 |
| | HB-500 | BG1 |
| | SGL-1 | Nagano |
| *S. gracilistyla*[a] | SGR-B1 | BG2 |
| var. *pendula* f. *pendula* | HB-60 | BG2 |
| var. *adscendens* f. *latifolia* | HB-528 | BG1 |
| *S. integra*[a,b] | SI-1 | Nagano |
| | SI-B1 | BG2 |
| f. *pendula* | HB-602 | BG1 |
| *S. japonica*[a] | HB-606 | BG1 |
| | SJ-1 | Chiba |
| *S. koriyanagi*[b] | 1H | BG1 |
| | HB508 | BG1 |
| *S. miyabeana*[a] | SM-S1 | Hokkaido |
| *S. reinii*[a] | SR-1 | Nagano |
| | SR-2 | Iwate |

BG1, Botanical Garden, Tohoku University, Sendai, Miyagi; BG2, Botanical Garden, Tsukuba University, Ibaraki
[a] Uredinial hosts of *M. epitea* reported by Hiratsuka et al. (1992) and Kondo et al. (1994)
[b] Uredinial hosts of *M. humilis* reported by Hiratsuka et al. (1992)

Inoculation experiments were conducted with detached leaves from these clones, as follows. Fully expanded young leaves detached from potted plants were placed topside downward onto a 1% agar plate containing 40 μg/ml benzimidazole in a glass Petri dish. Urediniospores were put onto small pieces (3 × 3 mm) of filter paper wet with sterilized water, and the pieces were placed upside down on the lower surface of the detached leaves. The Petri dishes containing the inoculants and leaves were kept in a dark moist chamber at 20°C for 2 days and then were transferred to a growth chamber at 23°C under fluorescent lights (16 h/day). The inoculated leaves were kept under these conditions for a month to observe uredinia produced on them.

Inoculation experiments with urediniospores from each isolate were done one to four times, mostly twice, per plant clone. The 18 *Salix* clones were inoculated with

**Table 3.** Results of inoculation experiments with urediniospores in *Melampsora epitea* and *M. humilis* collections

| Plants inoculated | | Collection no. (RFLP type[a]) and isolate no.[b] | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Salix* species | Clone no. | *M. epitea* | | | | | | | | | | *M. humilis* | | | | | | |
| | | ETg-1 (type 5) | | | ETg-3 (type 5) | | | ETf-1 (type 7) | ETj-1 (type 7) | | | HUi-2 (type 5) | | | HUi-3 (type 5) | | | RS1 (type 5) |
| | | ETg-1-S | ETg-1-S2 | ETg-1-S3 | ETg-3-S | ETg-3-S2 | ETg-3-S3 | ETf-1-m | ETj-1-S | ETj-1-S2 | ETj-1-S3 | HUi-2-S | HUi-2-S2 | HUi-2-S3 | HUi-3-S | HUi-3-S2 | HUi-3-S3 | HU1 |
| *S. futura* | SF-1 | − | nt | −[c] | + | + | + | + | − | + | − | − | (+) | − | − | − | −*[d] | − |
| | SF-A1 | − | − | − | − | − | − | + | − | − | − | − | − | − | nt | − | − | − |
| *S. gilgiana* | HB-101 | + | − | + | + | + | + | − | − | − | − | − | − | + | − | − | −* | + |
| | HB-500 | + | − | − | + | + | + | − | − | − | − | − | + | + | − | − | −* | + |
| | SGL-1 | + | + | + | + | + | + | − | − | − | − | + | + | + | − | − | − | + |
| *S. gracilistyla* | SGR-B1 | − | − | − | − | − | − | nt | − | − | − | − | + | − | − | − | − | − |
| | HB-60 | − | − | − | + | − | − | nt | − | − | − | − | + | − | nt | − | − | + |
| | HB-528 | − | nt | − | + | + | − | − | − | − | − | + | + | + | − | nt | − | + |
| *S. integra* | SI-1 | + | + | + | + | + | + | nt | − | − | nt | + | + | + | − | + | − | + |
| | SI-B1 | + | + | + | + | + | + | − | − | − | − | − | − | −* | + | + | + | + |
| | HB-602 | + | + | + | + | + | + | − | − | − | − | + | + | + | + | + | + | + |
| *S. japonica* | HB-606 | − | + | − | − | − | + | + | + | + | + | − | + | + | − | − | −* | + |
| | SJ-1 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | + | − | − |
| *S. koriyanagi* | 1H | − | − | + | + | + | + | − | − | − | − | + | − | + | − | − | −* | + |
| | HB508 | − | + | − | − | + | + | nt | − | − | − | + | + | + | − | − | −* | − |
| *S. miyabeana* | SM-S1 | − | + | − | − | − | −* | nt | − | − | nt | − | − | − | − | − | − | − |
| *S. reinii* | SR-1 | + | + | + | + | + | + | − | − | − | − | + | + | + | nt | − | − | − |
| | SR-2 | − | nt | − | + | + | + | nt | − | nt | − | + | − | + | − | − | −* | + |

nt, not tested
[a] Determined according to Nakamura et al. (1998)
[b] Isolate ETf-1-m is a mass-uredinial isolate; the other isolates are single-uredinial isolates
[c] −, No uredinia appeared; +, uredinia appeared; (+), immature uredinia without urediniospores appeared
[d] A mass-uredinial isolate produced uredinia in preliminary experiments

urediniospores from each of 3 single-uredinial isolates from our five collections (ETg-1, ETg-3, and ETj-1 from *M. epitea*; HUi-2 and HUi-3 from *M. humilis*), a single-uredinial isolate from the RS1 collection of *M. humilis*, and a mass-uredinial isolate from the ETf-1 collection of *M. epitea*. Pathogenicity of the single-uredinial isolates in the five collections of the two rust species did not necessarily correspond within a species and a collection. Also, differences in the pathogenicity of a rust isolate to the clones of each *Salix* species were recognized in most. These differences were conspicuous in the inoculations with ETg-1 and HUi-2 to *S. gilgiana* clones and collection ETg-3 to *S. gracilistyla* Miquel clones (Table 3). The results of inoculations can be summarized as follows (Table 3). Two type-5 collections of *M. epitea* (ETg-1 and ETg-3, which were collected on *S. gilgiana*) produced uredinia on six and seven *Salix* species, respectively. A mass-uredinial isolate from collection ETg-3 was pathogenic to *S. miyabeana* Seemen in preliminary experiments, although its single-uredinial isolates did not show pathogenicity. Two type-7 collections of *M. epitea* (ETj-1 from *S. japonica* and ETf-1 from *S. futura*) were each pathogenic to both these *Salix* species. Two type-5 collections of *M. humilis* (HUi-2 and RS1, from *S. integra*) produced uredinia on six *Salix* species. Single-uredinial isolates of collection HUi-3 were pathogenic to only two *Salix* species (*S. integra* and *S. japonica*), although in preliminary experiments a mass-uredinial isolate from the same collection was pathogenic on six *Salix* species, including these two species.

**Table 4.** Summary of results of inoculation experiments with urediniospores of *Melampsora epitea* and *M. humilis*[a]

| *Salix* species inoculated | *M. epitea* | | *M. humilis* |
|---|---|---|---|
| | RFLP type 5[b] | RFLP type 7[b] | RFLP type 5[b] |
| *S. futura* | (+) | + | (+) |
| *S. gilgiana* | + | − | + |
| *S. gracilistyla* | (+) | − | (+) |
| *S. integra* | + | − | + |
| *S. japonica* | + | + | + |
| *S. koriyanagi* | + | − | + |
| *S. miyabeana* | + | − | − |
| *S. reinii* | + | − | + |

[a] +, uredinia were produced by all collections; (+), uredinia were produced by one of two collections in *M. epitea* and one or two of three collections in *M. humilis*; −, no uredinia was produced in any collection
[b] According to Nakamura et al. (1998)

To facilitate a comparison of the host ranges of *M. humilis* and the two types of *M. epitea*, the results of the inoculation experiments are summarized in Table 4. Type-5 *M. epitea* could infect all eight host *Salix* species tested. The host range of *M. humilis* (all collections were type 5) was the same except that it did not infect *S. miyabeana*. Type-7 *M. epitea* infected only two *Salix* species. These results show

a strong correlation between pathogenicity and RFLP type in these fungi.

Gaümann (1959) separated *M. larici-epitea* into seven formae speciales based on their uredinial and telial hosts. Pei et al. (1999) reported, on the basis of crossing experiments, that three formae speciales of *M. epitea* infecting larches (i.e., *M. larici-epitea*) were genetically different populations. The ITS regions of rDNA, which we used to determine the RFLP types of our fungal collections, are considered very suitable for identification and phylogenetic studies of fungal species because they show high genetic variation (Bruns et al. 1991; Hibbett 1992). Therefore, each of the two RFLP types, types 5 and 7, in *M. epitea* could be considered to be an independent species. In addition, RFLP type 5 of *M. epitea* probably has an extremely close genetic relationship to *M. humilis*. However, precise morphological comparisons among these fungal groups were not made in this study.

Hiratsuka and Kaneko (1982) provisionally treated *M. humilis* as an independent species because *M. humilis* was distinguished from *M. epitea* by only a slight difference in teliospore length. More precise morphological examinations are required for taxonomic reevaluation of *M. humilis* and the two groups of *M. epitea*. A group of *M. epitea* showing RFLP type 6 (Nakamura et al. 1998), although not used in this study, should also be reevaluated. In addition, the alternative hosts of *M. humilis* and three groups of *M. epitea* need to be confirmed. It will be especially important to examine the teliospore size produced on different host species by inoculation with a single rust collection. Comparative investigations of type specimens or specimens collected from type localities of *M. epitea sensu stricto* (Europe) and *M. humilis* (Asia) will be necessary to clarify the taxonomic situation of this group of rust.

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