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SHORT COMMUNICATION

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Phylogenetic relationships of four *Puccinia* species parasitic on *Artemisia* in Japan

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Abstract *Puccinia dioicae* var. *micropuncta* and *P. caricis-stipatae* complete their life cycle by host-alternating between *Artemisia* (spermogonial-aecial stage) and *Carex* (uredinial-telial stage). These species are suggested to be biologically distinct by inoculation experiments and field observations. Two additional *Puccinia ferruginosa* and *P. artemisiae-keiskeanae* produce only telial stage on *Artemisia*. Similarities in the teliospore morphology and host relationship of the four *Puccinia* species suggest their close phylogenetic relationship. Nucleotide sequences of D1/D2 region and ITS2 regions with partial 5.8S rDNA were analyzed to depict possible phylogenetic relationships among the four *Puccinia* species. In D1/D2 analysis, both macrocyclic and microcyclic species were closely positioned in one clade, not permitting resolution of the phylogenetic relationship between the species. The DNA sequence of ITS2 including partial 5.8S rDNA was sufficiently variable to separate two macrocyclic species and *P. artemisiae-keiskeanae*; however, confident resolution of phylogenetic relationships of the three species was not possible. Nevertheless, the analysis suggested the derivation of *P. artemisiae-keiskeanae* from a macrocyclic, heteroecious ancestor that is most likely to be an ancestor of both *P. caricis-stipatae* and *P. dioicae* var. *micropuncta.* In contrast, three isolates of morphologically identifiable *P. ferruginosa* were variously positioned in the phylogenetic tree, suggesting that *P. ferruginosa* is not monophyletic.

Key words Asteraceae · Cyperaceae · D1/D2 region · ITS regions · Tranzschel's law · Uredinales

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Six *Puccinia* species have been reported on *Artemisia* (Asteraceae), on which either spermogonial-aecial, uredinial-telial stage or only telial stage is produced depending on the rusts' life cycle. *Puccinia dioicae* P. Magnus var. *micropuncta* Y. Ono (Ono 1983), *P. caricis-stipatae* Y. Harada (Harada 1986), and *P. caricis-macrocephalae* Dietel are heteroecious macrocyclic in their life cycle, alternating between *Artemisia* (spermogonial-aecial host) and *Carex* (Cyperaceae: uredinial-telial host). *Puccinia absinthii* DC, autoecious macrocyclic in the life cycle, produces all the spore stages on *Artemisia* species (Hiratsuka 1980; see Newcombe 2003 for negative observations, cf. Harada et al. 1996). *Puccinia ferruginosa* P. Syd. & Syd. (including *P. artemisiicola* P. Syd. & Syd. as a synonym) and *P. artemisiae-keiskeanae* Miura are microcyclic in the life cycle, producing only telial stage on *Artemisia* species (Hiratsuka 1980). *Puccinia dioicae* var. *micropuncta, P. caricis-stipatae*, *P. ferruginosa*, and *P. artemisiae-keiskeanae* have been assumed to be biologically distinct by inoculation experiments (Harada 1986; cf. Ono 1983) and field observations. However, their similarities in host relationship and teliospore morphology suggest their close phylogenetic relationship, which may conform the empirically known "Tranzschel's law." This "law" describes that, in the course of rust fungus evolution, a microcyclic progeny might have derived from a macrocyclic, heteroecious parental species and that the derived species with reduced life cycle inhabits and produces teliospores on the spermogonial-aecial host of the parental species (Shattock and Preece 2000).

Development and sophistication of molecular phylogenetic methods have aided at tracing back the evolutionary history and resolving phylogenetic relationships among rust fungi that are morphologically similar and thus have been assumed to be closely related; further, application of the molecular techniques has even succeeded to detect "cryptic species" in a morphologically circumscribed species complex (Pfunder et al. 2001; Roy et al. 1998; Zambino and Szabo 1993; Weber et al. 2003; Vogler and Bruns 1998).

Taking advantage of the molecular phylogenetic analysis methods and their preceding successful application on the rust fungi, we studied nucleotide sequence variations in the

D1/D2 region of 28S rDNA and internal transcribed spacer (ITS)2 regions with partial 5.8S rDNA of the four accepted species, *P. dioicae* var. *micropuncta*, *P. caricis-stipatae*, *P. ferruginosa*, and *P. artemisiae-keiskeanae.* Our attempts aimed at seeing whether *P. dioicae* var. *micropuncta* and *P. caricis-stipatae* share the most recent ancestor and whether the microcyclic *P. ferruginosa* and *P. artemisiae-keiskeanae* had originated, together or separately, from either *P. dioicae* var. *micropuncta* or *P. caricis-stipatae*.

Sixteen herbarium specimens from the Herbarium of Systematic Mycology, the College of Education, Ibaraki University (IBA), the Herbarium of the University of Tsukuba (TSH), and the Herbarium of Hirosaki University were selected for the DNA analyses (Table 1). DNA was extracted from about 100–200 urediniospores or teliospores in a single uredinium or telium, respectively. We followed DNA extraction methods described by Virtudazo et al. (2001). Polymerase chain reaction (PCR) was carried out using a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) under the conditions used by Virtudazo et al. (2001). The D1/D2 region was amplified using a new primer pair modified from NL1 and NL4 (O'Donnell 1993). The new primer pair, NL1-1 (5′-GGCGAATGAAGAGGGAAAAG) and NL4-1 (5′-GCTTACTGCATTCCTCAATC), was effective for most specimens for which the NL1 and NL4 primer pair did not work. The ITS2 region with partial 5.8S rDNA was amplified using primers ITS4 (Gardes and Bruns 1993) and ITS3r (Vogler and Bruns 1998). PCR products were then purified using MicroSpin S-400 HR columns (Amersham Pharmacia Biotech, Buckinghamshire, England) and directly sequenced using a Big Dye terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster, CA, USA) with the same primers used for PCR amplification. Sequencing was done using an ABI 377 Automated DNA Sequencer (Perkin-Elmer). Multiple alignments were performed by Clustal X version 1.8 (Thompson et al. 1997). *Puccinia recondita* Rob. f.sp. *tritici* (L08729) and *P. hordei* Otth. (L08725) were chosen as outgroups and were aligned together with all sequences of the D1/D2 region. DNA sequences of the ITS2 region with partial 5.8S rDNA were aligned and compared with the sequences of *P. obscura* J. Schröter (AF468042) and *P. distincta* MacAlpine (AF468040). Phylogenetic analyses of the data were done

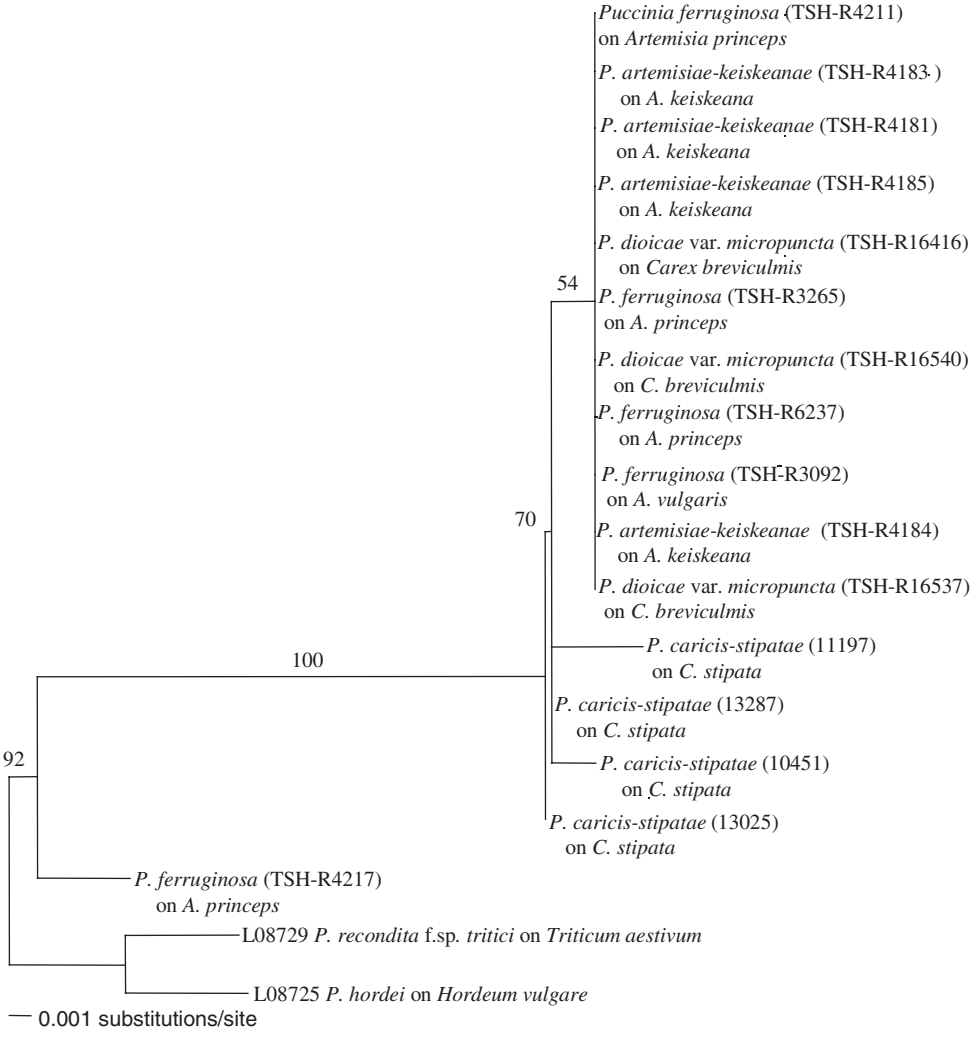

Fig. 1. Neighbor-joining tree inferred from sequences of D1/D2 region of 28S rDNA using clustal X; *values above the branches* indicate percentage bootstrap for 1000 replications. Length of branches is proportional to number of base changes, indicated by the *scale* at bottom

Table 1. The specimens used in phylogenetic analysis of D1/D2 and ITS2 regions with partial 5.8S rDNA

Species	Voucher specimens	Host plants	Locality in Japan	GenBank accession no.	
				D1/D2	5.8S + ITS2
Puccinia dioicae var. *micropuncta*	TSH-R16540 (IBA8667)	*Carex breviculmis*	Ibaraki	AB190889	AB188129
	TSH-R16537 (IBA2404)	*C. breviculmis*	Ibaraki	AB190890	AB188130
	TSH-R16416	*C. breviculmis*	Ibaraki	AB190891	AB188131
P. caricis-stipatae	11197 (Hirosaki University)	*C. stipata*	Aomori	AB190888	AB188134
	13287 (Hirosaki University)	*C. stipata*	Aomori	AB190886	AB188136
	10451 (Hirosaki University)	*C. stipata*	Aomori	AB190887	AB188137
	13025 (Hirosaki University)	*C. stipata*	Aomori	AB190885	AB188135
P. ferruginosa	TSH-R4217 (IBA8315)	*Artemisia princeps*	Tochigi	AB190900	AB188128
	TSH-R6237	*A. princeps*	Nagano	AB190897	AB188126
	TSH-R3092	*A. princeps*	Nagano	AB190898	AB188127
	TSH-R4211 (IBA2069)	*A. princeps*	Ibaraki	AB190899	NA[a]
	TSH-R3265	*A. princeps*	Nagano	AB190896	NA
P. artemisiae-keiskeanae	TSH-R4181 (IBA2422)	*A. keiskeana*	Ibaraki	AB190892	NA
	TSH-R4183 (IBA3253)	*A. keiskeana*	Ibaraki	AB190893	AB188133
	TSH-R4184 (IBA3256)	*A. keiskeana*	Ibaraki	AB190894	AB188132
	TSH-R4185 (IBA3259)	*A. keiskeana*	Ibaraki	AB190895	NA

TSH, Mycological Herbarium, University of Tsukuba, Japan; IBA, Herbarium of Systematic Mycology, Ibaraki University, Japan; ITS, internal transcribed spacer

[a]No analyses

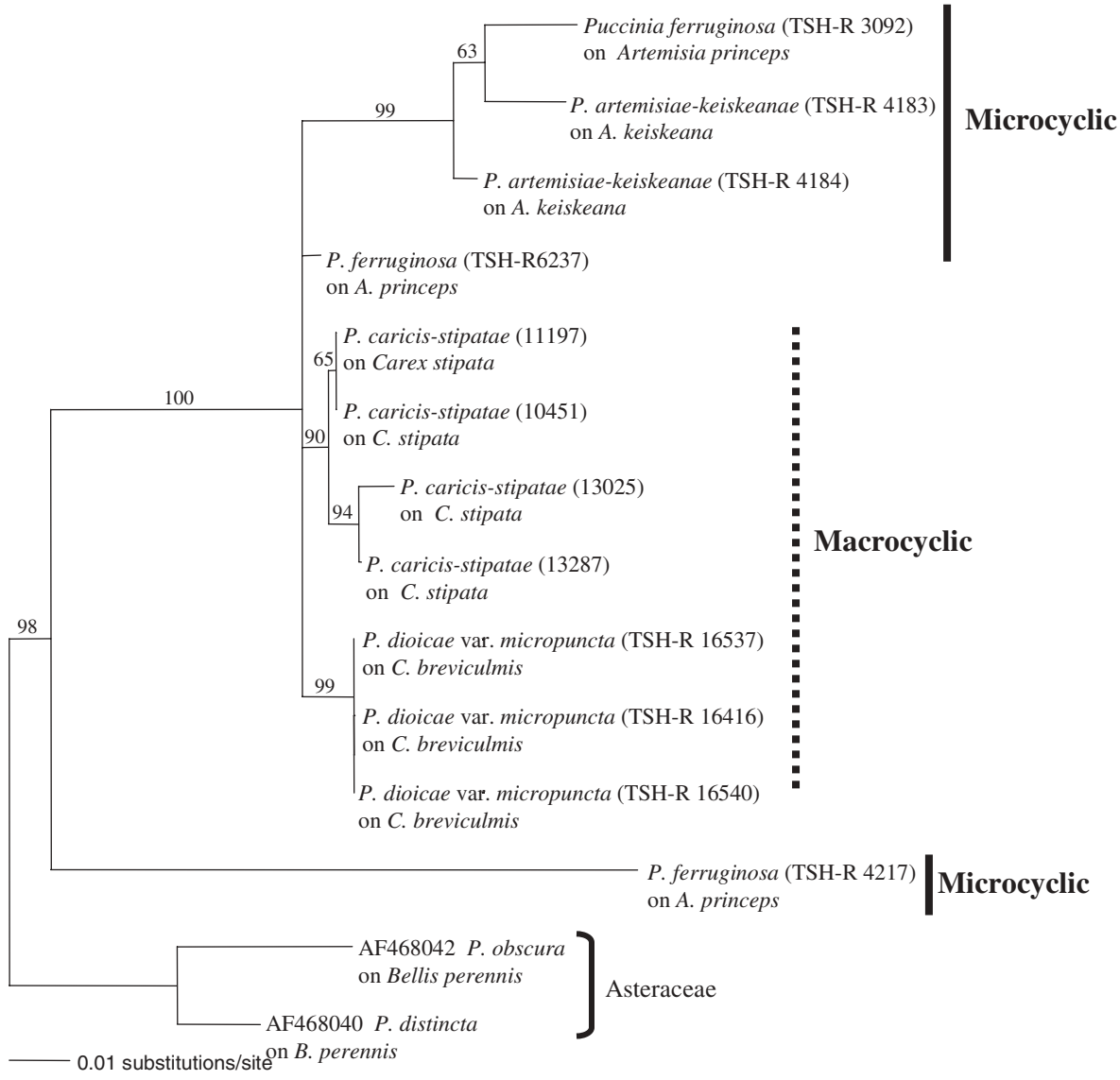

Fig. 2. Neighbor-joining tree inferred from sequences of internal transcribed spacer (ITS)2 region with partial 5.8S rDNA using clustal X, *values above the branches* indicate percentage bootstrap for 1000 replications. Length of branches is proportional to number of base changes, indicated by the *scale* at bottom

by the distance method. The most appropriate evolutionary model was determined for a given data set using PAUP version 4.0b (Swofford 1999) and Modeltest 3.06 (Posada and Crandall 1998). The distance matrix for the aligned sequences was calculated using the Tamura–Nei method and analyzed with the neighbor-joining (NJ) method (Saitou and Nei 1987) using the program PAPU version 4.0b. Reliability of the inferred trees was estimated by 1000 bootstrap resamplings using the same program.

DNA sequences of the D1/D2 region of four *Puccinia* species ranged from 503 to 510 bp in length, whereas the sequences of ITS2 regions with partial 5.8S rDNA ranged from 307 to 327 bp in length. All sequences of both regions were aligned and analyzed with the corresponding region of selected outgroups. The aligned sequences of D1/D2 region, including the gap matrix in the analysis, led to a total of 519 characters. The aligned sequences ITS2 regions with partial 5.8S rDNA contained a total length (including gap) of 343 bp.

The DNA sequence of D1/D2 regions analyzed by the neighbor-joining method showed that all macrocyclic, *P. dioicae* var. *micropuncta* and *P. caricis-stipatae*, and microcyclic *P. artemisiae-keiskeanae* were grouped together in one clade. The result, therefore, did not permit resolution of phylogenetic relationships among the three species. D1/D2 regions of large subunit (LSU) rDNA were often said to be more conserved than ITS regions in rust fungi. Therefore, sequence variation in D1/D2 region is often insufficient to distinguish biological species (Maier et al. 2003), whereas sequence variation in the ITS region was commonly large enough to separate taxa at a species level (White et al. 1990). Recent studies on sugarcane rusts (Virtudazo et al. 2001) and bean and cowpea rusts (Chung et al. 2004) showed poor correlations between morphologically circumscribed species and molecular phylogenetic clades, while clades generated from ITS1 and ITS2 sequence analysis corresponded well with morphologically circumscribed species that permit precise recognition of the species and depict possible phylogenetic relationships. In this study, DNA sequence of ITS2 including partial 5.8S rDNA was sufficiently variable to separate the three biological species *P. caricis-stipatae*, *P. dioicae* var. *micropuncta*, and *P. artemisiae-keiskeanae* into three subclades within one clade, which suggested the monophyly of the three species. However, further confident resolution of phylogenetic relationships between *Puccinia caricis-stipatae*, *P. dioicae* var. *micropuncta*, and *P. artemisiae-keiskeanae* was not possible. Nevertheless, the phylogenetic trees suggested the derivation of microcyclic *P. artemisiae-keiskeanae* from a macrocyclic, heteroecious ancestor that is most likely to be an ancestor of both *P. caricis-stipatae* and *P. dioicae* var. *micropuncta*. This can be an additional case that agrees with Tranzschel's law, for which several examples have been documented based primarily on molecular phylogenetic studies (i.e., *Tranzschelia* species, Maier et al. 2003; the *P. monoica* species complex, Roy et al. 1998).

In contrast to the morphological and molecular distinction between the three species, three isolates of morphologically identifiable *P. ferruginosa* were variously positioned in the phylogenetic tree: the first isolate (TSH-R6237) was the sister-group of the two macrocyclic species and microcyclic *P. artemisiae-keiskeanae*, the second isolate (TSH-R3092) was positioned in the *P. artemisiae-keiskeanae* clade, and the third isolate (TSH-R4217) was the sister-group to all other rust isolates. This multiple positioning of *P. ferruginosa* isolates was also detected in D1/D2 analysis. The results suggested that *P. ferruginosa* circumscribed by morphology and putative host specificity might not be monophyletic and might have been originated from two or more parental species.

Our study revealed the evolutionary divergence in the four *Puccinia* species that share *Artemisia* as a common host. However, molecular information utilized and isolates examined in our study are limited and thus identification of speciation points in the evolutionary history of the four species is not definite. In addition, multiple placement of morphologically circumscribed *P. ferruginosa* cannot be fully discussed because the biological nature of the *P. cnici-oleracei* species complex, in which *P. ferruginosa* is currently included, has not been sufficiently studied in terms of morphology and host specificity. Our ongoing study, which includes more species with a reasonable number of isolates, will confirm the findings herein reported and reveal more precise phylogenetic relationships among the four species and their allies.

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