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Differences in molecular characteristics between *Cronartium quercuum* from Japan and fusiform rust from USA

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Summary

The molecular characteristics were compared among *Cronartium quercuum* f. sp. *densiflorae* and *C. quercuum* f. sp. *thunbergii* from Japan and *C. quercuum* f.sp. *fusiforme*, fusiform rust from the USA. We examined the PCR-amplifield internal transcribed spacer (ITS) regions of ribosomal DNA from Japanese and American sources of aeciospores by restriction fragment length polymorphism (RFLP) analysis and nucleotide sequences. The ITS regions of six collections from *Pinus densiflora* and four collections from *P. thunbergii* from Japan and five collections from the native American hosts *P. taeda* or *P. elliottii* were amplified by PCR. The RFLP patterns with the three enzymes *Dra* I, *Hinf* I and *Taq* I showed clear differences between the two Japanese *formae speciales* and the American *C. quercuum* f. sp. *fusiforme*. No differences were found in the RFLP patterns between ff. spp. *densiflorae* and *thunbergii*. Nucleotide sequences of the ITS2 region confirmed the distinction between ff. sp. *densiflorae* and *fusiforme*. Comparisons with sequence data obtained from GenBank database on the other *formae speciales* in *C. quercuum* also comfirmed the distinctiveness of f. sp. *densiflorae*. These molecular data supported the morphological differences reported earlier between the Asian and American forms in the *C. quercuum* complex.

Key words: *Pinus densiflora*, *Pinus elliottii*, *Pinus taeda*

1 Introduction

In the *Cronartium quercuum* (Berk.) Miy. ex Shirai complex, Kaneko *et al.* (1991) and Kuhlman and Kaneko (1991) found apparent distinctions in basidiospore morphology between Asian and American collections. They also reported slight morphological differences among the four North American *formae speciales*. Others have reported distinctions within the North American *formae speciales* by isozyme analysis (Powers *et al.* 1989, 1991) and nucleotide sequences (Vogler and Bruns 1998).

Within the Japanese sources, Kondo (1975) suggested the existence of two physiological strains of the rust, a *Pinus densiflora* Sieb. et Zucc. (Japanese red pine) – *Quercus serrata* Thunb. strain and a *P. thunbergii* Parl. (Japanese black pine) – *Q. acutissima* Carr. strain, based on inoculation experiments.

Francisco, California. 573 p.
Suyama, Y., Kawamuro, K., Kinoshita, I., Yoshimura, K., Tsumura, Y. & Takahara, H. 1996. DNA sequence from a fossil pollen of *Abies* spp. from Pleistocene peat. Genes & Genetic Systems 71: 145–149.
Vogler, D.R. & Bruns, T.D. 1998. Phylogenetic relationships among the pine stem rust fungi (*Cronartium* and *Peridermium* spp.). Mycologia 90: 244–257.
White, T.J., Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfrand, D.H., Sninsky, J.J. & White, J. (eds.). PCR Protocols. Academic Press, San Diego. p. 315–322.

The two strains in Japan were designated as *C. quercuum* f. sp. *densiflorae* and f. sp. *thunbergii* by Kuhlman and Kaneko (1991).

To compare differences in the molecular characteristics between *C. quercuum* ff. spp. *densiflorae* and *thunbergii* from Japan, and also those between Japanese forms and the fusiform rust, *C. quercuum* f. sp. *fusiforme* in the USA, we examined the PCR-amplified internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) from aeciospores by restriction fragment length polymorphism (RFLP) analysis and nucleotide sequences.

2 Material and methods

2.1 Fungal collections

Ten aeciospore samples of *C. quercuum* were collected from various localities in Japan (Table 1). Six aeciospore collections were from *P. densiflora* galls and four aeciospore collections from *P. thunbergii* galls. The aeciospores of each collection were sampled from a single gall and were stored at –80° C without drying until use. Additionally, five vacuum-dried aeciospore isolates of *C. q. fusiforme* collected in Georgia and South Carolina, USA, were used.

2.2 DNA extraction and PCR amplification

Template DNA for PCR amplification was extracted from aeciospores following the procedures of Suyama *et al.* (1996). Two to three hundred aeciospores were crushed in a microtube with a pipette tip under an optical microscope. Twenty microliters of extraction buffer [10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; 50 mM KCl; 0.01% Proteinase-K; 0.01% (w/v) sodium dodecyl sulfate (SDS)] were added into the PCR tube, incubated for 60 min at 37° C, and heated for 10 min at 95° C. The extract was used directly as template.

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 200 nM concentrations of each primer, 200 µM concentrations of each deoxynucleotide triphosphate, two units of TaKaRa Ex Taq™ (Takara Biochemicals, Japan), and 1 X TaKaRa Ex Taq™ buffer. Two microliters of the above DNA extract was used as a template per reaction. The mixtures were covered with 40 µL of mineral oil to prevent evaporation. Reaction cycles were repeated 35 times with the following program: 95° C, 30 s; 55° C, 1 min; 72° C, 1 min. After amplification, aliquots (6 µL) of the reaction mixtures were subjected to electrophoresis 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).

Comparisons with the sequence data obtained from the GenBank database recorded by Vogler and Bruns (1998) for the other *formae speciales* in *C. quercuum* also indicated a separate position for f. sp. *densiflorae* from the other *formae speciales*. These molecular data support a previous report based on the morphological examinations (Kaneko 1992) suggesting that the Asian forms of *C. quercuum* may be different from the North American *C. quercuum*. In this paper, however, the nucleotide sequences of only the ITS2 region were examined. Comparisons of the whole ITS region between the Japanese forms and the North American forms are needed to resolve the phylogenetic position of the Japanese forms in the *C. quercuum* complex.

Acknowledgements

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Table 1. Fungal collections.

Forma specialis	Collection No.	Host pine species	Locality
densiflorae	MO1	*P. densiflora*	Iwate, JPN
	MO2	*P. densiflora*	Iwate, JPN
	MO3	*P. densiflora*	Iwate, JPN
	NB1	*P. densiflora*	Nagano, JPN
	TK1	*P. densiflora*	Ibaraki, JPN
	TK2	*P. densiflora*	Ibaraki, JPN
thunbergii	TK3	*P. thunbergii*	Ibaraki, JPN
	TK4	*P. thunbergii*	Ibaraki, JPN
	TT1	*P. thunbergii*	Tottori, JPN
	TT2	*P. thunbergii*	Tottori, JPN
fusiforme	BL2	*P. taeda*	Georgia, USA
	BL3	*P. taeda*	Georgia, USA
	I-83	*P. taeda*	Georgia, USA
	LOB88	*P. taeda*	Georgia, USA
	SCSC2	*P. elliottii*	South Carolina, USA

2.3 RFLP analysis, sequencing and sequence analysis

The amplified fragments were digested with 10 restriction enzymes, *Alw* 44 I, *Dra* I, *EcoR* I, *Hae* III, *Hha* I, *Hinf* I, *Msp* I, *Sau* 3A I, *Ssp* I, and *Taq* I, according to the manufacturer's instructions. The digested fragments were separated electrophoretically on 4% (w/v) agarose X (Nippon Gene, Japan) gel in TAE buffer and stained with ethidium bromide.

The amplified fragments and the ITS4 primer pair designed by White *et al.* (1990) was used for sequencing the ITS2 region using DyeDeoxy Terminator Cycle Sequencing Kit and 377 DNA Sequencing System (Perkin Elmer). The nucleotide sequence data were aligned and analyzed using GENETYX-MAC (Software Development, Japan). Dendrograms were constructed using the unweighted pair-group method based on arithmetic average (UPGMA) (Sneath and Sokal 1973) and the neighbor-joining method (Saitou and Nei 1987).

3 Results

3.1 RFLP analysis of amplified ITS regions

The primer pair ITS1-F and ITS4-B permitted the amplification of a single DNA fragment (approximately 900 bp in size) from all the collections tested. From the 10 enzymes used for digesting DNA fragments, the three enzymes *Hae* III, *Hha* I, and *Msp* I had no recognition site in the fragments. The RFLP patterns with the four enzymes *Alw* 44 I, *EcoR* I, *Sau* 3A I, and *Ssp* I were identical among all the collections. On the other hand, the number of recognition sites with the three enzymes *Dra* I, *Hinf* I, and *Taq* I differed from each other in the fragments, and DNA polymorphism in the amplified ITS regions was detected among the collections (Fig. 1).

The three enzymes *Dra* I, *Hinf* I, and *Taq* I produced the RFLP patterns for *C. q. densiflorae* that were identical with those of *C. q. thunbergii*. The RFLP patterns with the three enzymes for *Cronartium* collections from Japan were different from those of *C. q. fusiforme* collections from North America (Fig.

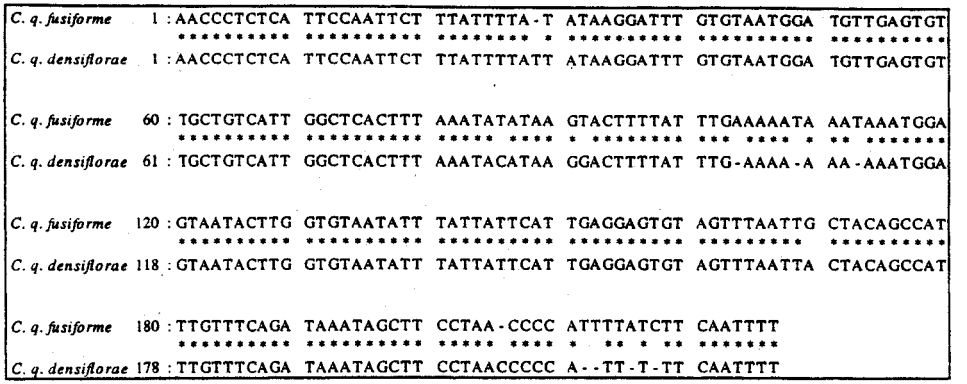

Figure 2. Nucleotide sequence alignment of ITS2 region in *Cronartium quercuum* f. sp. *densiflorae* (MO1) from Japan and f. sp. *fusiforme* (BL2) from the USA. The sequence of f. sp. *fusiforme* BL2 was identical with that of the same form from GenBank (No. L76494). Alignment gaps, insertations, or deletions are indicated by dashes.

For the UPGMA analysis, the ITS2 region sequences of 10 *Cronartium* species were obtained from the GenBank database (Vogler and Bruns 1998): *C. coleosporioides* Arth. (GenBank accession No. L76500), *C. comandrae* Peck (L76485), *C. conigenum* Hedgc. et N. Hunt (L76486), *C. flaccidum* (Alb. et Schw.) Wint. (X83901), *C. q. banksianae* (L76491), *C. q. echinatae* (L76493), *C. q. fusiforme* (L76494), *C. q. virginianae* (L76495), *C. ribicola* J.C. Fisch. (L76496), *C. strobilinum* Hedgc. et Hahn (L76482). In these alignments, the sequence of *Chrysomyxa arctostaphyli* Diet. (GenBank accession No. L76488) was used as outgroup.

The sequence of *C. q. fusiforme* collection BL2 by us was identical with that of *C. q. fusiforme* from the GenBank database. In the UPGMA similarity dendrogram derived from the sequence data of the ITS2 region, the *Cronartium* species formed a single cluster. The collection MO1 of *C. q. densiflorae* from Japan was placed in a different lineage from the other *formae speciales* in *C. quercuum* and was placed between *C. conigenum* and *C. ribicola*.

In the dendrogram produced by the neighbor-joining method, the *Cronartium* species consisted of three main clusters. Three species, *C. conigenum*, *C. quercuum* including the five *formae speciales*, and *C. strobilinum*, were included in one cluster. In this cluster, *C. q. densiflorae* from Japan and *C. conigenum* were included in a distinct lineage from the other four *formae speciales* in the USA.

4 Discussion

Clear distinctions were found in RFLP patterns between the two *formae speciales* of *C. quercuum* from Japan and *C. q. fusiforme* from the USA. No distinctions were found in the patterns among the collections from Japan, consisting of the two *formae speciales* f. sp. *densiflorae* and f. sp. *thunbergii*. The analysis of the nucleotide sequences of the ITS2 region confirmed the distinction between f. sp. *densiflorae* and f. sp. *fusiforme*.

1). No differences were found in the RFLP patterns among the five collections of *C. q. fusiforme*.

3.2 Sequencing and sequence analysis

Nucleotide sequences of the ITS2 region were determined for one representative collection of each of *C. q. densiflorae* (MO1) from Japan and *C. q. fusiforme* (BL2) from the USA. The size of the ITS2 region in *C. q. densiflorae* (MO1) was 220 bp pairs and that in *C. q. fusiforme* (BL2) was 225 bp pairs (Fig. 2).

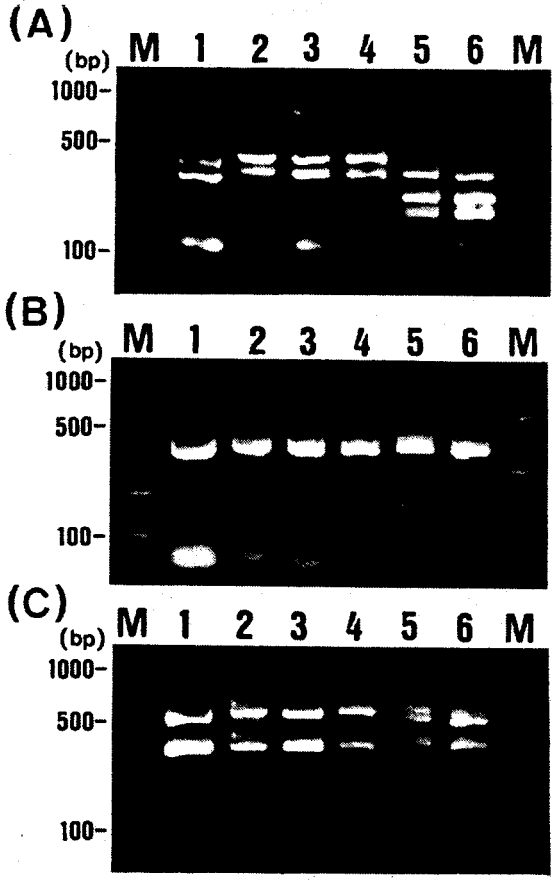

Figure 1. Restriction patterns of internal transcribed spacer regions of ribosomal DNA amplified from six collections in three *formae speciales* of *Cronartium quercuum* with *Dra* I (A), *Hinf* I (B), *Taq* I (C). Lane 1, *C. quercuum* f. sp. *densiflorae* (MO1); lane 2, *C. quercuum* f. sp. *densiflorae* (TK1); lane 3, *C. quercuum* f. sp. *thunbergii* (TK4); lane 4, *C. quercuum* f. sp. *thunbergii* (TK3); lane 5, *C. quercuum* f. sp. *fusiforme* (BL2); lane 6, *C. quercuum* f. sp. *fusiforme* (LOB88); M, fragment size markers (100 bp DNA ladder).