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Identification of a rust fungus on *Pinus pumila* collected in the North Kurils, Russia

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Morphological examination and PCR-RFLP analysis of the D1/D2 region in nuclear LSU rDNA were carried out to identify a rust fungus on *Pinus pumila* collected in the North Kurils, Russia. Morphologically, the rust from the North Kurils was similar to *Peridermium kurilense* and *Endocronartium sahoanum* var. *hokkaidoense* in the spore surface structure, dimension and shape. In the RFLPs of the D1/D2 region it was identical to *E. sahoanum* var. *hokkaidoense* and *E. sahoanum* var. *sahoanum*, but distinct from *Cronartium ribicola*. Therefore, the rust fungus on *P. pumila* collected in the North Kurils was identified as *E. sahoanum* var. *hokkaidoense*. It was also clarified that a rust reported previously as *P. kurilense* was also identical with *E. sahoanum* var. *hokkaidoense*.

Key Words——*Cronartium ribicola*; *Endocronartium sahoanum* var. *hokkaidoense*; *Peridermium kurilense*; *Pinus pumila*; the North Kurils.

In the North Kuril islands, which lie south-east of the Kamchatka peninsula, a blister-causing rust was recorded on *Pinus pumila* (Pall.) Regel and described as *Peridermium kurilense* Dietel (Dietel, 1905). Since then, some authors (Jørstad, 1934; Hiratsuka, 1944; Hiratsuka et al., 1992) have considered the fungus to be synonymous with *Cronartium kamtschaticum* Jørstad (now treated as *C. ribicola* J. C. Fischer; Yokota and Uozumi, 1976), which is distributed widely in the Kamchatka region. However, others have doubted this taxonomic connection (Kuprevicz and Tranzschel, 1957; Azbukina, 1974; 1984). Because little information about *P. kurilense* was available from the single type collection, the biological and taxonomic relationships between the two fungi have remained uncertain.

In Japan, the blister-causing rust on *P. pumila* has been considered as *C. ribicola* (Hiratsuka, 1944; Yokota and Uozumi, 1976). In addition, new endocyclic rusts on *P. pumila*, *Endocronartium yamabense* (Saho et I. Takahashi) Pacit, *E. sahoanum* Imazu et Kakishima var. *sahoanum*, and *E. sahoanum* var. *hokkaidoense* Imazu et Kakishima, have been reported from Hokkaido and northern Honshu (Saho, 1981; Imazu et al., 1989; Imazu and Kakishima, 1992). Imazu and Kakishima (1992) and Imazu (1995) examined the type specimen of *P. kurilense* and found that the spores were morphologically similar to those of *C. ribicola* and the two varieties of *E. sahoanum*.

From 1996 to 1997, field surveys of the peridermioid, blister-causing rusts on *P. pumila* were conducted in the Kamchatka region and the North Kurils, Russia. Blister-causing rusts comparable to *P. kurilense* were found in Paramushir and Shumshu in the North Kurils. In both islands, the rust was commonly found on *P. pumila*, which predominated from seashore to mountain slopes. *Pedicularis* spp. and *Castilleja pallida* (L.) Spreng., potential uredinial-telial hosts of *C. ribicola* (=*C. kamtschaticum*), were also commonly growing around *P. pumila*. Nevertheless, no uredinial-telial fungus was found on these plants. Therefore, the rust fungus on *P. pumila* was suspected to be endocyclic.

To identify the rust fungi on *P. pumila* collected in the North Kurils, morphological examination and molecular analysis were carried out in comparison with many herbarium specimens including collections in Kamchatka and Japan.

Materials and Methods

Morphological observations The blister-causing rusts on *P. pumila* collected from several locations in Paramushir and Shumshu of the North Kurils, Sakhalin region, Russia were used for morphological observations (Table 1). The type specimen of *P. kurilense* (July 1904, K. Yendo, Swedish Museum of Natural History, Sweden, No. 549) was also used for comparative morphology. The spore morphology of these specimens was examined by light and scanning electron microscopy. Two hundred randomly selected spores were

Table 1. Specimens used for the study.

Sample No.	Species	Host	Locality	Date collected	Voucher specimen[a)]	Study method[b)]
1	Rusts collected in the North Kurils	*Pinus pumila*	Paramushir Isl., Sakhalin region, Russia.	26 Jul. 1997	TSH-R1651	M, R
2		*P. pumila*	Paramushir Isl., Sakhalin region, Russia.	26 Jul. 1997	TSH-R1677	M, R
3		*P. pumila*	Paramushir Isl., Sakhalin region, Russia.	28 Jul. 1997	TSH-R1656	M, R, S
4		*P. pumila*	Paramushir Isl., Sakhalin region, Russia.	30 Jul. 1997	TSH-R1678	M, R
5		*P. pumila*	Shumshu Isl., Sakhalin region, Russia.	21 Jul. 1997	VLA-6974	M, R
6	*Cronartium ribicola*	*Pedicularis resupinata*	Elizovsky, Kamchatka region, Russia.	4 Aug. 1996	TSH-R10004	R
7		*Pe. resupinata*	Mt. Avachinskaya, Kamchatka region, Russia.	19 Aug. 1997	TSH-R1668	R
8		*Pe. resupinata*	Mt. Karymskaya, Kamchatka region, Russia.	31 Aug. 1996	TSH-R10007	R
9		*Ribes triste*	Mt. Klyuchevskaya, Kamchatka region, Russia.	16 Aug. 1996	TSH-R10009	R
10		*P. pumila*	Mt. Kisokomagatake, Nagano Pref., Japan.	23 Jul. 1989	TSH-R1679	R
11		*P. pumila*	Mt. Norikuradake, Nagano Pref., Japan.	22 Jul. 1989	TSH-R1680	R, S
12		*P. strobus*	Tomakomai city, Hokkaido, Japan.	7 Jun. 1989	TSH-R1681	R
13	*Endocronartium sahoanum* var. *hokkaidoense*	*P. pumila*	Mt. Atosanupuri, Hokkaido, Japan.	15 Jun. 1989	TSH-R1682	R
14		*P. pumila*	Mt. Meakandake, Hokkaido, Japan.	10 Jun. 1989	TSH-R620	R, S
15		*P. pumila*	Mt. Rishirisan, Hokkaido, Japan.	14 Jun. 1989	TSH-R1684	R
16	*E. sahoanum* var. *sahoanum*	*P. pumila*	Mt. Iwakisan, Aomori Pref., Japan.	28 Jun. 1988	TSH-R530	R, S
17		*P. pumila*	Hakkoda Mts., Aomori Pref., Japan.	29 Jun. 1988	TSH-R1686	R
18		*P. pumila*	Mt. Hachimantai, Iwate Pref., Japan.	16 Jun. 1987	TSH-R527	R

a) TSH: the Mycological Herbarium of the Institute of University of Tsukuba, Japan, VLA: the Mycological Herbarium of Institute of Biology and Soil Science, Russian Academy of Sciences, Russia.

b) M: Morphological observation, R: RFLP analysis, S: Sequencing.

measured in each specimen.

Molecular analysis Five specimens of the rust fungi collected in the North Kurils, seven of *C. ribicola* collected in Kamchatka or Japan, three of *E. sahoanum* var. *hokkaidoense*, and three of *E. sahoanum* var. *sahoanum* were used for PCR-RFLP analysis of the D1/D2 region in nuclear large subunit ribosomal DNA (Table 1). To confirm RFLPs, one specimen of each rust fungus was also used for sequencing of this region (Table 1).

Total DNA was extracted from dried specimens using the procedures described by Lee and Taylor (1990) and Zambino and Szabo (1993). The crushed spores were suspended in 20 μl of extraction buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Tween 20, 0.2 mg/ml Proteinase K), incubated at 55°C for 3 h, and heated at 95°C for 10 min. The supernatant was diluted with 20 μl of distilled water and used as template for the polymerase chain reaction (PCR).

The D1/D2 region in nuclear LSU rDNA was amplified with the primers NL1 and NL4 (O'Donnell, 1993). The reaction mixture (50 μl) contained 5 μl of DNA solution, 200 μM dATP, dCTP, dGTP, and dTTP, 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, 50 mM KCl, 0.2 μM of primers, and 1.25 units of Ampli*Taq* Gold DNA polymerase (Perkin Elmer Cetus). PCR was performed for 45 cycles with an initial 12 min at 95°C for denaturation. Each cycle consisted of 1 min at 60°C, 1 min at 72°C, and 30 s at 95°C. A final extension at 72°C for 10 min was added.

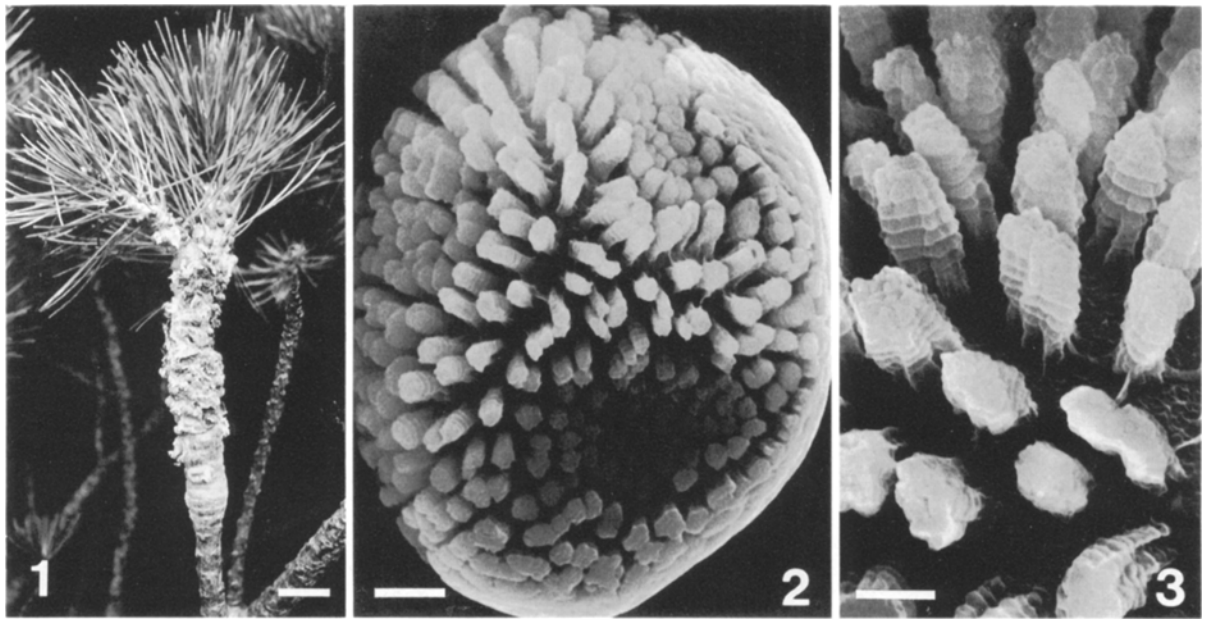
For RFLP analysis, the PCR products were purified by chloroform extraction and sodium acetate/ethanol precipitation. The amplified DNA was digested for 3 h with *Alu*I, *Dra*I, *Hin*fI, and *Taq*I. Digested DNA was electrophoresed on 3% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA).

For sequencing analysis, the PCR products were purified with SUPREC-02 (TaKaRa) and both strands were sequenced with the Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Pharmasia Biotec). The external primers NL1 and NL4 and the internal primers NL2A and NL3A (Kurtzman and Robnett, 1997) were used. Sequencing reactions were run on an ABI 377 DNA sequencer.

Results

Morphological observation A blister-causing rust fungus collected in the North Kurils produced pale-yellow sori on the surfaces of twigs and branches of *P. pumila* (Fig. 1). The spores were catenulate, broadly ellipsoidal, subglobose or obovoid, and 23–39 × 15–27 μm. The wall was hyaline and 2–3 μm thick. Scanning electron microscopic observations showed that the spores had annulated warts (Figs. 2, 3). All the specimens from the North Kurils were morphologically identical. The surface structure observed by SEM was similar to those of *C. ribicola*, *P. kurilense*, *E. sahoanum* var. *sahoanum*, and *E. sahoanum* var. *hokkaidoense* reported previously (Imazu and Kakishima, 1992; Imazu, 1995).

The average dimensions of 200 spores of the rust fungus from the North Kurils were compared with those of *C. ribicola*, *P. kurilense*, and two varieties of *E. sahoanum*, which were reported previously (Imazu and Kakishima, 1992; Imazu, 1995) (Fig. 4). The spores of the rusts from the North Kurils were larger than those of *C. ribicola* and narrower than those of *E. sahoanum* var.



Figs. 1–3. The rust fungus collected in the North Kurils.
1. Symptom and sori on *Pinus pumila*. 2. A spore observed by SEM. 3. Surface structure of a spore observed by SEM. (Scales: 1 = 1 cm, 2 = 3 μm, 3 = 1 μm.)

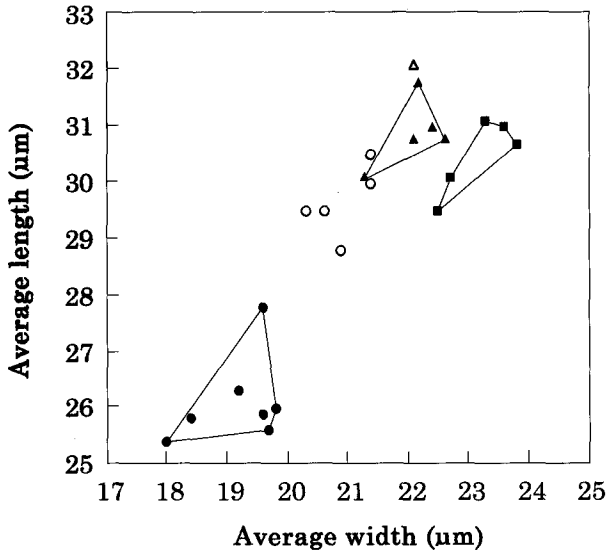


Fig. 4. The average dimensions of spores of the rust fungus collected in the North Kurils and morphologically related rust fungi. ○, the rust fungus from the North Kurils; △, *P. kurilense*; ●, *C. ribicola*; ▲, *E. sahoanum* var. *hokkaidoense*; ■, *E. sahoanum* var. *sahoanum*. Data on *P. kurilense*, *C. ribicola*, and two varieties of *E. sahoanum* are from previous studies (Imazu and Kakishima, 1992; Imazu, 1995).

sahoanum, while they were similar to those of *E. sahoanum* var. *hokkaidoense*. Although the type specimen of *P. kurilense* bore larger spores than those of rusts from the North Kurils, the average spore dimensions were continuous among *P. kurilense*, *E. sahoanum* var. *hokkaidoense* and the rusts from the North Kurils. The spore shapes (ratio of length to width) were also similar.

Molecular analysis The PCR products of the D1/D2 region in nuclear LSU rDNA for each rust sample were about 650 bp long. Digestion of this product with *Alu*I, *Hin*fI, and *Taq*I resulted in the same banding patterns for all samples in each case, while digestion with *Dra*I exhibited two different banding patterns (Fig. 5). In the *Dra*I digestions the banding pattern of the rusts from the North Kurils was identical with that of *E. sahoanum* var. *hokkaidoense* and *E. sahoanum* var. *sahoanum* but distinct from that of *C. ribicola* because of the additional restriction site.

The nucleotide sequences of this region were identical in the 607 bp stretch between the primers NL1 and NL4 among the rust from the North Kurils, *E. sahoanum* var. *hokkaidoense*, and *E. sahoanum* var. *sahoanum*. On the other hand, in *C. ribicola* one nucleotide deletion and three nucleotide substitutions, one of them at the *Dra*I restriction site of the other species, were recognized (Fig. 6). Accordingly, the result of sequencing analysis was consistent with that of RFLP analysis.

Discussion

The blister-causing rust fungi collected in the North Kurils were found to be morphologically identical. Their surface structure was similar to those of *C. ribicola*, *P. kurilense*, and two varieties of *E. sahoanum*, but their spore dimensions were different from those of *C. ribicola* and *E. sahoanum* var. *sahoanum*. Accordingly, the rusts from the North Kurils are morphologically identical with *P. kurilense* and *E. sahoanum* var. *hokkaidoense*. The results of molecular analysis also demonstrated that the rust fungi from the North Kurils were closely related to two varieties of *E. sahoanum* but distinct from *C. ribicola*. Consequently, morphological and molecular data showed that the rusts from the North Kurils are apparently different from *C. ribicola* but not distinguishable from *P. kurilense* and *E. sahoanum* var. *hokkaidoense*. In addition, field observations strongly suggested that the rusts from the North Kurils were endocyclic in life cycle. Therefore, we concluded that the rusts collected in

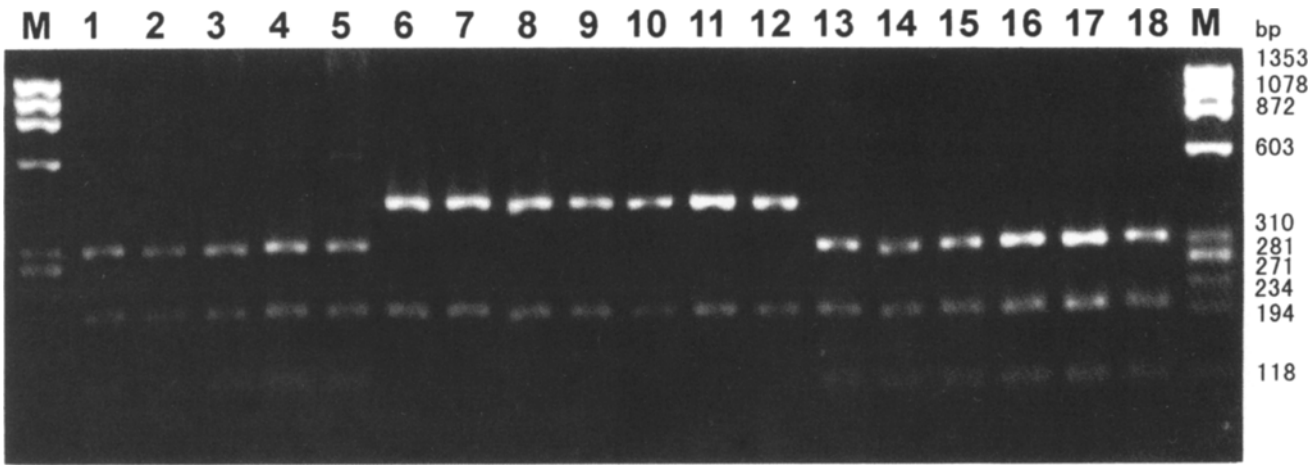


Fig. 5. Electrophoretic patterns of the amplified D1/D2 region in LSU rDNA of the rust fungus collected in the North Kurils and morphologically related rust fungi digested with *Dra*I. Lanes are designated with the sample numbers listed in Table 1. (1–5, the rust fungus from the North Kurils; 6–12, *C. ribicola*; 13–15, *E. sahoanum* var. *hokkaidoense*; 16–18, *E. sahoanum* var. *sahoanum*). Markers (M) are $\phi \times 174$ DNA digested with *Hae*III.

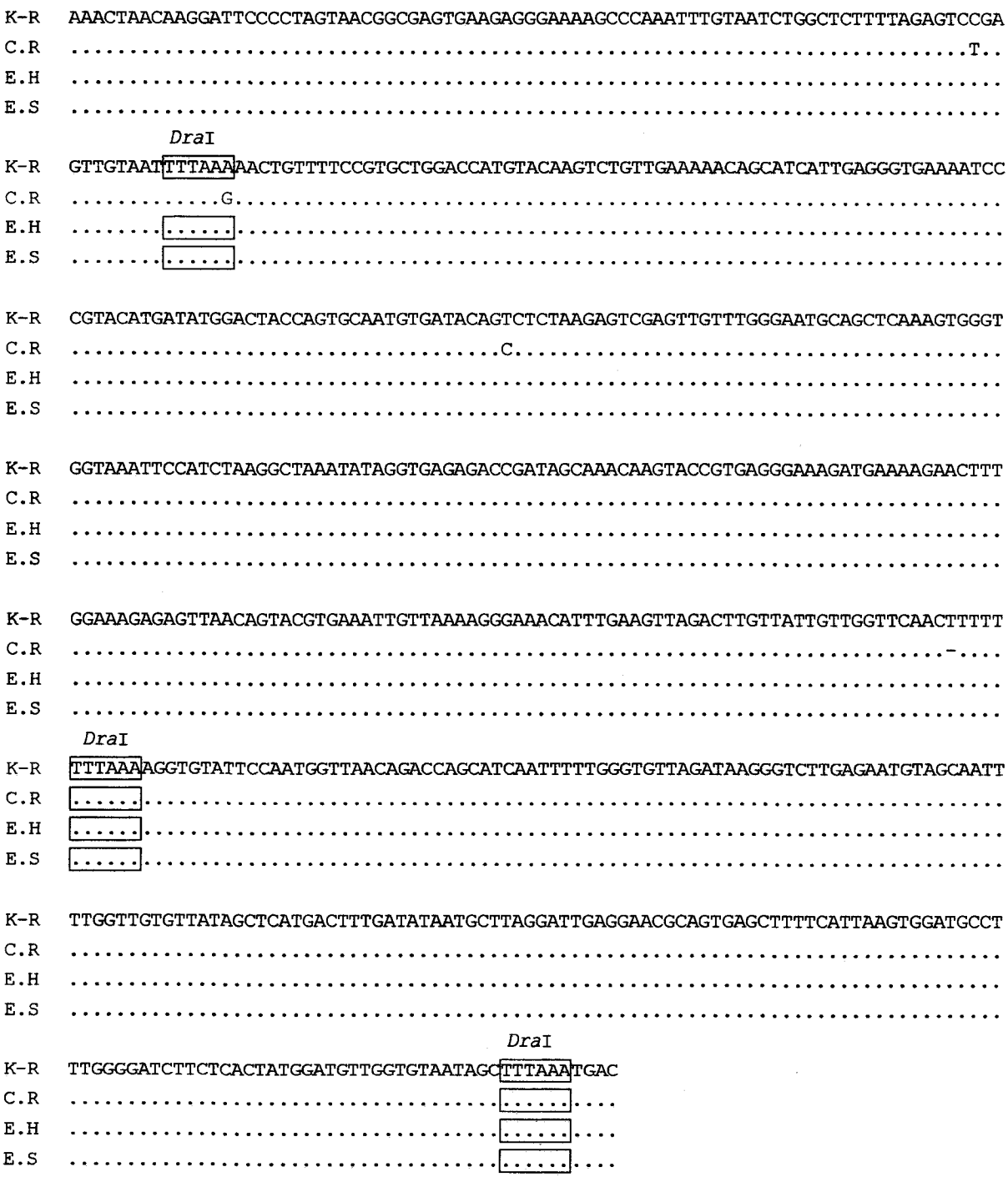


Fig. 6. Sequence alignment of the D1/D2 region in LSU rDNA of the rust fungus collected in the North Kurils and morphologically related rust fungi. The sequences are written 5′ to 3′. Identical nucleotides are indicated by dots, and deletion is indicated by a hyphen. The bases enclosed in a rectangle indicate the *Dra*I restriction site.
Abbreviations: K-R, the rust fungus from the North Kurils (TSH-R1656); C. R, *C. ribicola* (TSH-R1680); E. H, *E. sahoanum* var. *hokkaidoense* (TSH-R620); E. S, *E. sahoanum* var. *sahoanum* (TSH-R530).

the North Kurils were identical with *E. sahoanum* var. *hokkaidoense*, and that *P. kurilense* was also identical with *E. sahoanum* var. *hokkaidoense*.

The D1/D2 region in nuclear large subunit ribosomal DNA that was submitted to molecular analysis has been shown to be useful for a phylogenetic analysis among species and genera of fungi (Guadet et al., 1989; Kurtzman and Robnett, 1991, 1997; O'Donnell, 1993). The nucleotide sequences of *E. sahoanum* var. *hokkaidoense* and *E. sahoanum* var. *sahoanum* are identi-

cal, whereas that of *C. ribicola* differs in sevaral nucleotides. As reported in the previous study (Imazu and Kakishima, 1992), the two varieties of *E. sahoanum*, which have minor morphological differences, have geographic distributions in Hokkaido and northern Honshu, respectively. On the other hand, *C. ribicola* is known to occur in the Russian Far East (Azbukina, 1974, 1995; Kakishima et al., 1995) but not in the North Kurils, in spite of the abundance there of potential aecial and telial host plants. Furthermore, in Japan, the natural geographic distribution of *C. ribicola* is restricted to *P. pumila* in mountainous areas of central Honshu; and it is geographically separated from two varieties of *E. sahoanum* (Imazu, 1995). Accordingly, their genetic variation as revealed by the analysis of this region in rDNA corresponds with the morphological and biogeographical differences among them.

Recent studies of rDNA variation in the pine stem rust fungi (Vogler and Bruns, 1998; Moricca et al., 1996; Moricca and Ragazzi, 1998) revealed that autoecious species are closely related to the putative parental heteroecious species (e.g., *P. harknessii* with *C. quercuum* f. sp. *bankusianae*, *P. pini* with *C. flaccidum*) and suggested that the former arose from the latter. Further studies with molecular analyses, together with morphological and ecological studies are required to confirm the taxonomic positon and phylogenetic relationships of the blister-causing rust fungi on *P. pumila*.

We will discuss the taxonomic and nomenclatural treatments of *P. kurilense*, *E. sahoanum* var. *hokkaidoense*, and the related species in another paper.

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