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SHORT COMMUNICATION

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Variation of ITS sequences in a natural Japanese population of *Pleurocybella porrigens*

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Abstract Genetic relationships in a natural Japanese population of *Pleurocybella porrigens* were determined based on ITS sequence data. In a UPGMA similarity tree, all sequences of 23 specimens from 13 different geographic origins were grouped into two distinct clusters (groups A and B). Sequence variation of the ITS region between groups A and B consisted of 33–40 nucleotides, corresponding to 5%–6% of their total length, and specific nucleotide variations characterizing groups A and B were found. Although these results did not show correlation with differences of substrates for fruiting and geographic origins of the specimens, it was suggested that *P. porrigens* distributed in Japan include at least two genetically different populations.

Key words Genetic variation · Internal transcribed spacer (ITS) region · Large subunit rDNA · *Pleurocybella porrigens* · Sugihiratake

Pleurocybella porrigens (Pers.) Singer (Trichoromataceae) is a lignicolous agaric mushroom that produces pure white, relatively small pleurotoid fruiting bodies on rotting conifers, often in abundance. It is widely distributed in the north temperate regions, including Japan, and is known as edible (Breitenbach and Kränzlin 1991; Imazeki and Hongo 1986; Phillips 1991). In Japan, *P. porrigens* is fairly common in fall and has been well known as an excellent edible mushroom under the name "Sugihiratake," which means a Pleurotus-like mushroom ("Hiratake") growing on *Cryptomeria japonica* D. Don ("Sugi"). As suggested by the common name, it is most frequently encountered on the tree species, but also less frequently on other conifers, such as *Pinus* and *Picea*, and rarely on *Fagus crenata* Blume ("Buna").

In September and October 2004, an outbreak of acute encephalopathy causing death was reported among people who ate this mushroom in certain areas of Japan, mainly Yamagata, Akita, and Niigata prefectures (Kato et al. 2004; Kurokawa et al. 2005; Obara et al. 2005). The relationship of the consumption of the mushroom species to the outbreak of the disease still remains unclear and is under investigation by several scientists. This event prompted a serious question for us concerning the safety of *P. porrigens* as food. However, little has been known about the physiological and genetic characters of this fungus that might contribute to elucidating this question. Furthermore, there has been no research investigating the natural population structure of *P. porrigens*, which is necessary to understand the biology and ecology of this fungus in Japan.

Molecular approaches are considered to be powerful tools to investigate inter- and intragenetic relatedness on species of basidiomycetes, supporting results obtained from conventional methods such as morphological, physiological, and biochemical methods. Internal transcribed spacer (ITS) and intergenic spacer (IGS) regions of the ribosomal DNA (rDNA) are highly variable, thus permitting assessment of genetic variation at the intraspecific level (Hintz et al. 1989; Walsh et al. 1990; Bruns et al. 1991; Vilgalys and Sun 1994; Hibbett et al. 1995; Iraçabal et al. 1995).

In the present study, we investigated, by using the nucleotide sequences of ITS, the genetic relationships within a natural population of *P. porrigens* across its geographical range in Japan.

Twenty-eight specimens, including 1 derived isolate of *P. porrigens*, collected from different regions in Japan and from different kinds of wood substrata (Table 1) were used in this study. All specimens used in this study were identified as *P. porrigens* following Imazeki and Hongo (1986), based on morphological characters. Specimen no. 22 (TMIC-30952) was a culture from fruiting body tissue of no. 21 (TMI-14025) and was used to check the identity between rDNA sequences from fruiting body and cultured mycelia

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Table 1. List of specimens of *Pleurocybella porrigens* and their ribosomal DNA group

No.	Materials used for DNA isolation	Geographical origin	Year/month	Substratum or habitat	Collection number[a]	rDNA group[c]	
						Sequence	RFLP
1	Heat-dried fruiting body	Furano, Hokkaido	1984/Sept.	*Picea jezoensis* (Sieb. & Zucc.) Carriere	TMI-9613	A	A
2	Heat-dried fruiting body	Kamikawa, Hokkaido	1995/Sept.	coniferous tree	TMI-20160	A	A
3	Heat-dried fruiting body	Aomori, Aomori	1998/Sept.	*Fagus crenata* Blume	TMI-26082	A	A
4	Heat-dried fruiting body	Aomori, Aomori	2004/Sept.	*Fagus crenata* Blume	TMI-26092	A	A
5	Heat-dried fruiting body	Senboku, Akita	1976/Oct.	*Cryptomeria japonica* D. Don	TMI-3998	B	B
6	Heat-dried fruiting body	Senboku, Akita	1976/Oct.	*Cryptomeria japonica* D. Don	TMI-4007	A	A
7	Heat-dried fruiting body	Niigata	2003/Sept.	*Cryptomeria japonica* D. Don	TMI-26089	B	B
8	Heat-dried fruiting body	Enuma, Ishikawa	1980/Sept.	unknown	TMI-7975	B	B
9	Heat-dried fruiting body	Enuma, Ishikawa	1980/Sept.	*Cryptomeria japonica* D. Don	TMI-7976	B	B
10	Heat-dried fruiting body	Kayano, Nagano	2004/Sept.	coniferous tree	CBM-FB-34468	–	A
11	Freeze-dried fruiting body	Kuwata, Kyoto	2004/Dec.	unknown	TMI-26097	–	A
12	Freeze-dried fruiting body	Yoshino, Nara	2004/Nov.	*Cryptomeria japonica* forest	TMI-26094	–	B
13	Freeze-dried fruiting body	Yoshino, Nara	2004/Nov.	*Cryptomeria japonica* forest	TMI-26095	A	A
14	Heat-dried fruiting body	Hino, Tottori	1977/Oct.	*Cryptomeria japonica* D. Don	TMI-3994	B	B
15	Heat-dried fruiting body	Tottori, Tottori	1977/Oct.	*Cryptomeria japonica* D. Don	TMI-3987	B	B
16	Heat-dried fruiting body	Hino, Tottori	1987/Oct.	*Pinus densiflora* Sieb. & Zucc.	TMI-13514	B	B
17	Heat-dried fruiting body	Maniwa, Okayama	1983/Sept.	coniferous tree	TMI-7978	B	B
18	Heat-dried fruiting body	Iishi, Shimane	1986/Oct.	coniferous tree	TMI-13290	B	B
19	Heat-dried fruiting body	Saeki, Hiroshima	1991/Oct.	coniferous tree	TMI-17136	B	B
20	Heat-dried fruiting body	Kuka, Yamaguchi	2004/Nov.	*Abies firma* Sieb. & Zucc.	TMI-26096	B	B
21	Heat-dried fruiting body	Kamiukena, Ehime	1989/Oct.	*Pinus densiflora* Sieb. & Zucc.	TMI-14025	B	B
22	Freeze-dried mycelium	Kamiukena, Ehime	1989/Oct.	*Pinus densiflora* Sieb. & Zucc.	TMIC-30952[b]	B	B
23	Freeze-dried fruiting body	Touon, Ehime	2004/Oct.	*Cryptomeria japonica* D. Don	TMI-26093	–	B
24	Freeze-dried fruiting body	Nishiusuki, Miyazaki	2000/Sept.	*Cryptomeria japonica* D. Don	B-E-581	B	B
25	Freeze-dried fruiting body	Ebino, Miyazaki	2002/Sept.	*Pinus densiflora* Sieb. & Zucc.	B-E-1722	B	B
26	Freeze-dried mycelium	Kumage, Kagoshima	1997/Dec.	*Cryptomeria japonica* D. Don	TMIC-31107	B	B
27	Heat-dried fruiting body	Kumage, Kagoshima	1995/Oct.	*Cryptomeria japonica* D. Don	TMI-19818	B	B
28	Heat-dried fruiting body	Kumage, Kagoshima	1995/Oct.	*Cryptomeria japonica* D. Don	TMI-19804	–	B

–, No sequence analysis
[a]TMI, Herbarium of Tottori Mycological Institute; TMIC, Culture Collection of Tottori Mycological Institute; CBM, Natural History Museum and Institute, Chiba; B–E, Miyazaki Prefectural Museum of Nature and History
[b]Tissue culture of TMI-14025
[c]Groups A and B correspond to those in Fig. 1
RFLP, restriction fragment length polymorphism

(Table 1). Fruiting body samples for analysis were prepared by air-drying at 40°–45°C or lyophilizing and stored under low-moisture conditions at room temperature, and mycelia were prepared as described previously (Matsumoto et al. 2003).

The total DNA was extracted using a Genelute Plant Genomic DNA Kit (Sigma, Toronto, Canada) according to the manufacturer's instructions. The 5.8S rDNA intervening ITS region, ITS-1 and ITS-2, of the nuclear genome was amplified using the primer pairs ITS-4 (5′-TCCTCCGCTT ATTGATATGC-3′) and ITS-5 (5′-GGAAGTAAAAGTC GTAACAAGG-3′) (White et al. 1990). The 50-μl reaction mixture for PCR amplification contained the following: 0.1 μg total DNA, 1× amplification buffer for Ex Taq DNA polymerase (Takara Bio, Otsu, Japan), 200 μM each deoxynucleoside triphosphate (dNTP: dATP, dCTP, dGTP, and dTTP), 0.4 μM each primer, and 0.5 U Ex Taq DNA polymerase. Amplifications were performed in a Mp 100 Thermal Cycler (Takara Bio), with an initial denaturation step 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. The polymerase chain reaction (PCR) products were electrophoresed through 2.0% (w/v) agarose gel to confirm whether a single product was obtained, and following that were purified by ultrafiltration (Microcon-100; Millipore, Bedford, MA, USA). If a single PCR product was not obtained, the corresponding product in fragment size was excised from the gel and extracted by using the Wizard SV Gel and PCR Clean-UP system (Promega, Madison, WI, USA) according to the manufacturer's instruction.

These amplification products were subjected to restriction analysis or sequencing. Restriction fragment length polymorphism (RFLP) analysis of digested amplification DNA with two endonucleases, *Pvu*II and *Sau*3AI (Nippon Gene, Tokyo, Japan) was carried out with a 2% agarose gel according to the manufacturer's instruction. Electrophoresis and detection of DNA fragments were done in the same manner as that described in Matsumoto et al. (2003). All sequencing was carried out on an ABI Prism 310 Genetic Analyzer with the BigDye Terminator Cycle Sequencing Ready Reaction Kit ver. 1.1 according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). Four sequencing primers, ITS-2 (5′-GCTGCG TTCTTCATCGATGC-3′, 5.8S rDNA–5′-end), ITS-3 (5′-GCATCGATGAAGAACGCAGC-3′, 5.8S rDNA–3′-end), ITS-4 (28S rDNA–5′-end), and ITS-5 (18S rDNA–3′-end), described by White et al. (1990), were used. Confirming sequence analysis for large subunit rDNA (LrDNA) regions was done as described above using the amplification primers LR7 (5′-TACTACCACCAAGAT CT-3′) and LR0R (5′-ACCCGCTGAACTTAAGC-3′) (Georgiev et al. 1981), and sequencing for products was done by the primer walking method using LR7 and LR0R as the start primers. Amplification and sequence analysis were conducted at least twice for each sample.

Sequences were aligned with the CLUSTAL V multiple alignment program in a computer application software of GeneWorks (version 2.5; IntelliGenetics, Mountain View, CA, USA). A phylogenetic tree based on ITS rDNA sequences was constructed by using the unweighted pair-group method with arithmetic averages (UPGMA; Sokal and Michener 1958) and drawn via the computer software GeneWorks (version 2.5, IntelliGenetics) (Fig. 1).

Amplification of the ITS rDNA of 27 specimens resulted in a single product, and the sizes of their ITS regions, ITS-1–5.8S–ITS-2, were 654–657 bp in length from sequencing analysis for 23 specimens. Nucleotide sequences of ITS rDNA from fruiting body of no. 21 (Fig. 2) prepared with heat-dried specimens were identical to those from its mycelia (no. 22, data not shown), indicating that there were no artifact DNA sequences caused by errors by using heat-dried fruiting body specimens. The multiple sequence alignment showed that the dissimilarity among 23 specimens was 6.0% at maximum. This level of intraspecific variation in the ITS region was higher than that observed for several other edible mushroom species from Japan, for example, *Lentinula edodes* (Berk.) Sing. (Hibbett et al. 1995) and *Pleurotus pulmonarius* (Fr.) Quel. (Vilgalys and Sun 1994), which were less than 1% and 2%, respectively. The topology of the dendrogram based on sequence analysis, performed by UPGMA cluster analysis, indicated that the specimens were divided into two distinct groups, one of which included 6 specimens (group A) whereas the other group included 17 specimens (group B) (see Fig. 1). Results of the neighbor-joining cluster analysis were very similar to those of UPGMA analysis (data not shown). Sequence variation of the ITS region between groups A and B consisted of 33–40 nucleotides, corresponding to 5%–6% of their total length. In addition, specific nucleotide variations characterizing groups A and B were found for both ITS-1 and ITS-2 regions (Fig. 2).

Specific nucleotides T at position 246 characterized group A described above, which produced a restriction site of the endonuclease *Pvu*II (see Fig. 2). Other specific nucleotides A and T at positions 240 and 259 that characterized group A also produced *Sau*3AI sites in addition to the existing sites at positions 4 and 288 in both groups, respectively (Fig. 2). From these restriction sites of the ITS region, we could discriminate directly groups A and B of ITS rDNA by RFLP analysis of PCR products using two restriction endonucleases, *Pvu*II and *Sau*3AI. As a result, we determined the ITS sequence genotypes of all 27 specimens used in this study (Table 1). Hereafter, RFLP analysis with these two enzymes will be useful to estimate the molecular genotype of many samples of this fungus from various ecological niches and geographical origins.

Despite the high sequence variations in the both regions of ITS-1 and ITS-2, all nucleotide sequences of the 5.8S rDNA from 23 specimens were identical in both length (158 bp) and sequences (Fig. 2). In contrast with the 5.8S rDNA, 13 nucleotide differences (1.0%) were found between the partial sequences of LrDNA (approximately 1.3 kb) of specimen no. 4 of group A (DDBJ accession no. AB1213656) and no. 21 of group B (DDBJ accession no. AB1213656) (data not shown). Additionally, the LrDNA sequences of specimen no. 4 described above are identical with the LrDNA sequences of GenBank accession no.

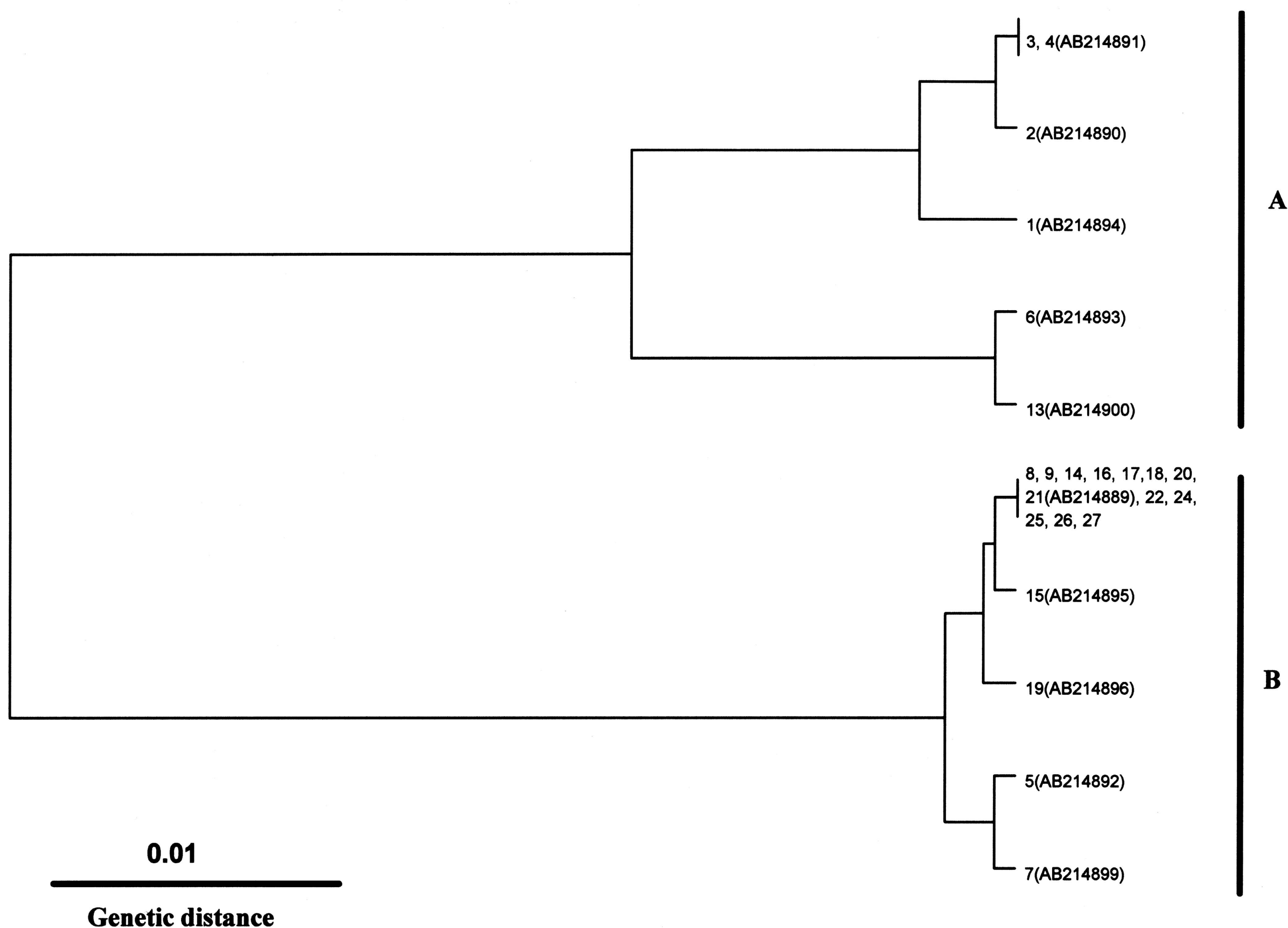

Fig. 1. Genetic relationships among 23 *Pleurocybella porrigens* specimens based on internal transcribed spacers (ITS) sequences. The dendrogram was constructed by the unweighted pair-group method with arithmetic averages (UPGMA). The accession numbers of ITS sequences in DDBJ are shown in *parentheses*

AF042594, which was reported by Moncalvo et al. (2000). It was speculated that sequence variations in LrDNAs between groups A and B may exist.

In assessment of the relationship between molecular genotypes and biological character, it is important to know that whether the variations of ITS sequences are associated with ecological niches where fruiting bodies occurred, and also with the geographic origins of specimens used in this study. As shown in Table 1, specific variation of ITS sequences, however, was not found in DNA of specimens from the five different wood substrates, *Abies firma* Sieb. et Zucc. ("Momi"), *F. crenata*, *C. japonica*, *Picea jezoensis* (Sieb. & Zucc.) Carriere ("Ezomatsu"), and *Pinus densiflora* Sieb. & Zucc. ("Akamatsu"). Furthermore, as shown in Fig. 1 and Table 1, group A of ITS rDNA consisted of specimens from Hokkaido, Aomori, Akita, Nagano, Kyoto, and Nara, whereas group B consisted of those from all regions examined in this study except for Hokkaido, Aomori, Nagano, and Kyoto, suggesting that there was no clear correlation between ITS sequences and geographic origin of specimens used in this study.

In conclusion, the Japanese *P. porrigens* could be divided into two groups based on the characteristics of ITS rDNA sequence, suggesting that the natural population in Japan includes high genetic variations and is composed of at least two genetically different groups. This finding was also supported by the sequence data of LrDNA. The present molecular data may prompt a question concerning the taxonomic identity of the two groups found in the morphological species *P. porrigens*, which caused the outbreak of the poisoning incidents in certain areas of Japan last year. To answer this question, we need more detailed morphological observations of fresh basidiocarps and mating tests between the two groups.

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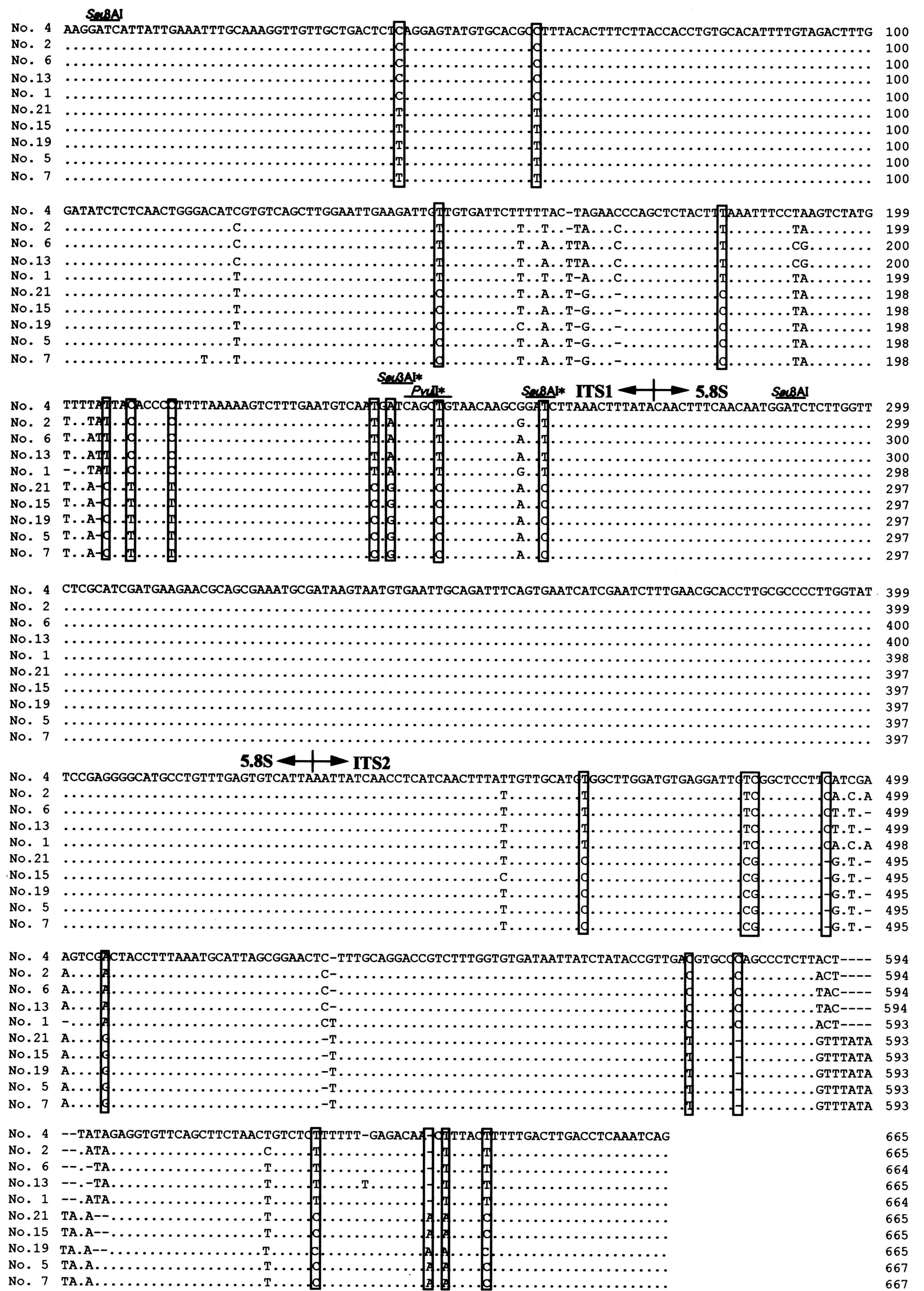

Fig. 2. Sequence alignment of specimen nos. 1, 2, 4, 6, and 13 belonging to group A and specimen nos. 5, 7, 15, 19, and 21 belonging to group B of *Pleurocybella porrigens* as shown in Table 1 and Fig. 1. Their sequences are referred to as that of specimen no. 4. Identical nucleotides between sequences are indicated by "." and gaps inserted for the alignment by "–". *Squares* show specific nucleotide variations characterizing groups A and B, without influence of the locations of gaps in the sequence alignments; *Pvu*II and *Sau*3AI, the restriction sites of endonucleases (* shows sites existed only in the sequences of group A)

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