

## Sequence differences in the internal transcribed spacer 1 and 5.8S ribosomal RNA among three *Moniezia* species isolated from ruminants in Japan

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**ABSTRACT.** This study was designed to clarify the differences in the internal transcribed spacer (ITS) 1 and 5.8S nucleotide sequences of *Moniezia expansa*, *M. benedeni* and *M. monardi* isolated from ruminants in Japan and to determine their phylogenetic relationships. A 98% similarity in the 5.8S sequences was observed among the 3 *Moniezia* species, whereas many nucleotide indels and substitutions were observed in the ITS1 sequences among the three *Moniezia* species. These results suggest that the ITS1 region could serve as a potential marker for discriminating the 3 *Moniezia* species. In the phylogenetic tree based on the ITS1 sequences, *M. monardi* and *M. benedeni* showed genetically closer relationship to each other than to *M. expansa*.

**KEY WORDS:** 5.8S rRNA, ITS1, *Moniezia benedeni*, *Moniezia expansa*, *Moniezia monardi*, phylogeny

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*Moniezia* cestodes of the family Anoplocephalidae are intestinal parasites of animals belonging to the orders Perissodactyla, Artiodactyla and Primates. At least 12 *Moniezia* species have been identified in domestic and wild ruminants [8], and 3 of these, namely, *M. expansa*, *M. benedeni* and *M. monardi* have been detected in sheep, cattle and Japanese serows (*Capricornis crispus*), respectively, in Japan [2, 5, 6]. The 3 cestode species have been differentiated based on morphological differences, such as their interproglottidal glands and eggs; however, it is common to find individuals that lack the interproglottidal glands, and the proglottids excreted into the feces sometimes have no interproglottidal glands. Moreover, the excreted eggs are sometimes deformed altering their diagnostic outer shell characteristics, preventing morphological differentiation up to the species level [10, 12].

Recently, molecular identification of parasitic helminthes has been developed using nucleotide sequences of the nuclear ribosomal RNA (rRNA) genes [1, 7, 13]. The internal transcribed spacer (ITS) regions within the rRNA gene have been used as molecular markers of species identification, because of the unequivocal differences in the sequence of closely related species and minimal intraspecific sequence variations. The 5.8S rRNA gene shows conserved sequences; therefore, its sequence has been used for phylogenetic analysis among distantly related organisms belonging to higher taxa and for designing their common PCR primers. The present study was designed to identify the differences in the ITS1 and 5.8S nucleotide sequences and thus establish the phylogenetic relationships among *M. expansa*, *M. benedeni*

and *M. monardi*.

We used 13 *Moniezia* cestodes (7 *M. monardi*, 3 *M. benedeni* and 3 *M. expansa*) isolated from the intestines of 13 distinct animals (7 Japanese serows, 3 cattle and 3 sheep, respectively) in Iwate and Akita Prefectures, Japan, during 1993 and 2006. The proglottides of these cestodes that were fixed with 70% ethanol were stained with Hematoxylin-Carmine solution for morphological observation, and the species were precisely identified based on Schmidt [8], Spassky [10] and Machida *et al.* [5], especially depending on the shape of their interproglottid gland.

Total DNA was extracted from the proglottides of individual cestodes by using E.Z.N.A Mollusc DNA kits (Omega Bio-tek, Doraville, GA, U.S.A.) according to the manufacturer's instruction. DNA fragments (approximately 1,700 bp) were amplified by using the polymerase chain reaction (PCR) using ITS1-F and ITS2-R primers [3]. PCR was performed in a 25- $\mu$ l reaction volume containing 2  $\mu$ l of DNA template, 0.2 mM of each dNTP, 0.1  $\mu$ M of each primer, 1.25 U of GoTaq DNA polymerase (Promega, Madison, WI, U.S.A.) and the manufacturer-supplied reaction buffer. Reaction cycles consisted of an initial denaturation step at 94°C for 90 sec, followed by 30 cycles at 94°C for 90 sec, 53°C for 90 sec and 72°C for 120 sec, with a final extension at 72°C for 10 min using the GeneAmp PCR Systems 2700 (Applied Biosystems, Tokyo, Japan). PCR amplicons were precipitated with ethanol / sodium acetate, dissolved in MilliQ water and directly sequenced in both directions by using two forward primers, MO6 (5'-GATGAAGAGTGCAGCCAAC-3') and MO12 (5'-GCAAGGCATAAGACGTTTGG-3') and a reverse primer, MO11 (5'-TGATCCACCGCACACAGT-3'), as well as the same primer set used for PCR and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.). The sequencing reactions were run on a 3500 Genetic Analyzer (Applied Biosystems). The ITS1 and 5.8S sequences were aligned using MAFFT software ver. 7 [4]. A neighbor-joining (NJ) phylogram was

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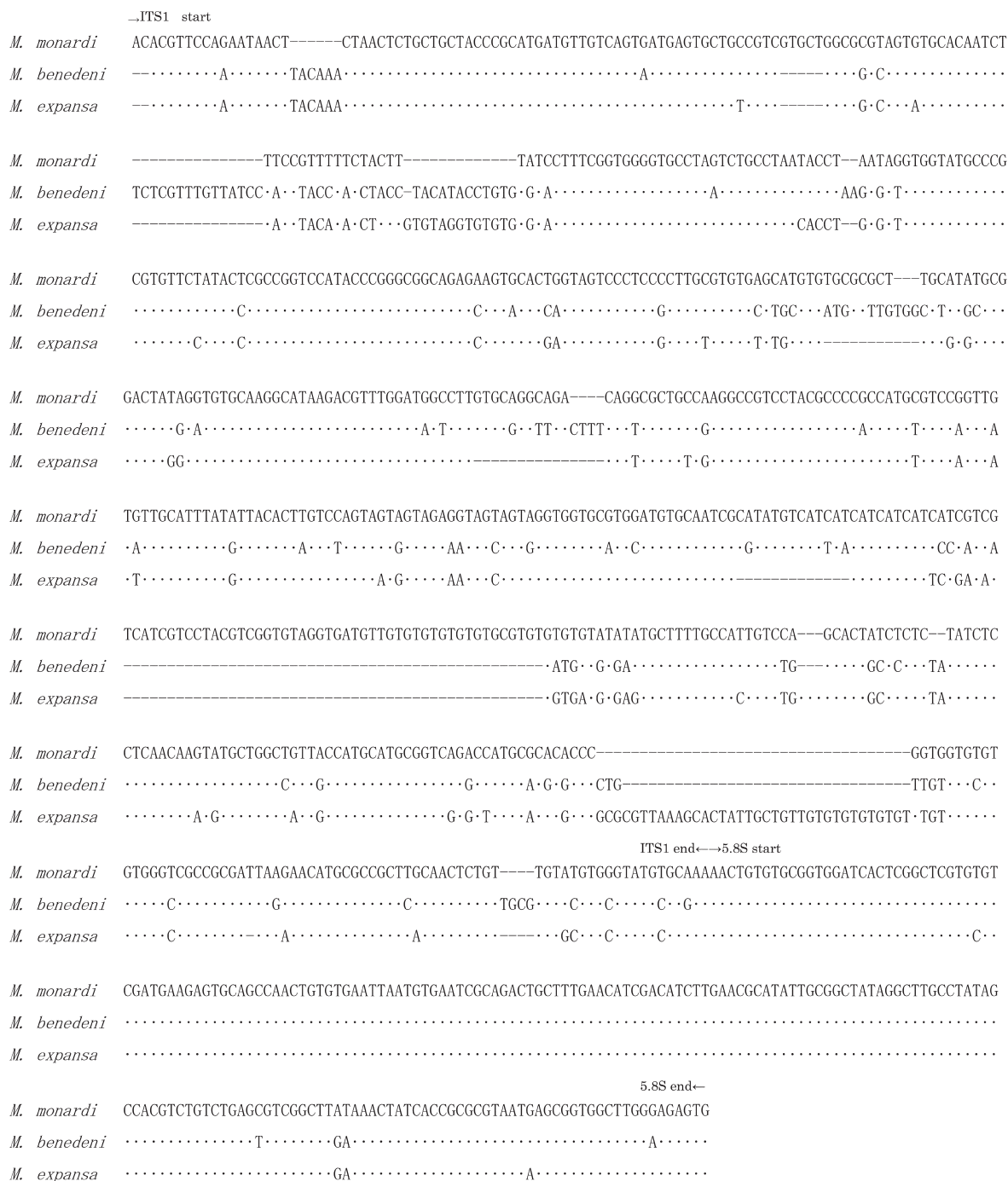


Fig. 1. Sequence alignments of the internal transcribed spacer (ITS) 1 region and 5.8S rRNA gene of 3 *Moniezia* species. Dots (·) show the identical nucleotide to *M. monardi*. “-” shows deletion of nucleotide.

constructed to select complete deletion for gaps/missing data treatment using MEGA5 [11]. For phylogenetic tree construction, the sequence of *Dipylidium caninum* (accession no. AM491338) was used as the outgroup. Bootstrap analyses were performed using 1,000 replicates.

Single amplicons of approximately 1,700 bp were pro-

duced in PCR using 13 DNAs of the three *Moniezia* species. The amplicons of 7 *M. monardi* and 3 *M. benedeni* could be sequenced from the 5'-end of ITS1 region to the 3'-end of 5.8S using the three forward primers; however, they could not be analyzed using the two reverse primers. No difference in the ITS1-5.8S sequences obtained from 7 *M. monardi* was

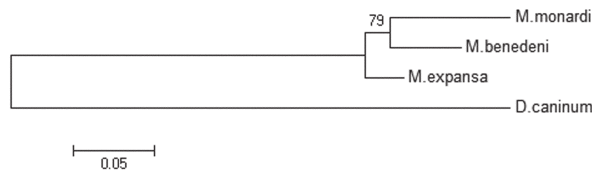


Fig. 2. A neighbor-joining phylogenetic tree based on the internal transcribed spacer (ITS) 1 sequences of *Moniezia expansa*, *M. benedeni* and *M. monardi*.

observed. Similarly, no diversity was observed among the sequences of 3 *M. benedeni*. The amplicons of 3 *M. expansa* could be sequenced through ITS1-5.8S-ITS2 using the five primers, and no difference in these sequences was detected. The ITS1-5.8S sequences determined for the three *Moniezia* species are shown in Fig. 1. There were many nucleotide indels and substitutions in the ITS1 sequences, and their length were 680, 675 and 651 bp for *M. monardi*, *M. benedeni* and *M. expansa*, respectively. These results suggest that the ITS1 sequence could serve as a potential marker for differentiating the 3 *Moniezia* species. However, intraspecific diversity of the ITS1 sequences should be confirmed by sequencing clones of the ITS1 region in a future study, because the occurrence of intragenomic variation has been known in the ITS region. Further diversity analysis is also needed using additional samples of the 3 *Moniezia* species isolated from distinct geographical areas, because in this study, the number of cestodes (N=13) and locality from which they were isolated (Iwate and Akita Prefectures) were limited. The 5.8S sequences were 200 bp long in the three species and had no indels. The sequences obtained between the three species showed 98% similarity. These sequences were deposited in the DNA data bank of Japan (DDBJ) with accession numbers AB367791–AB367793.

In the phylogenetic tree based on the ITS1 sequences, *M. monardi* and *M. benedeni* showed a genetically closer relationship to each other than to *M. expansa* (Fig. 2). A similar topography was also observed in the NJ tree based on the mixed sequences of ITS1 and 5.8S (data not shown). In classical taxonomy of the genus *Moniezia*, Skrjabin and Schulz [9] divided this genus into 3 subgenera as follows: *Moniezia*, *Blanchariezia* and *Baeriezia* on the basis of the presence and morphological characteristics of the interproglottidal glands. Spassky [10] categorized *M. expansa* and *M. monardi* into the same subgenus (*Moniezia*), in which the interproglottidal glands were grouped around depressions that opened to the surface of the segment (so-called rosette-type) and thus differentiated them from the other subgenus (*Blanchariezia*), which included *M. benedeni* with linear-type interproglottidal glands and not gland that are grouped as rosettes. These findings indicate that the genetic relation-

ships among *Moniezia* spp. were not entirely relevant to the morphological similarity of their diagnostic interproglottidal glands. However, the phylogenetic relationship needs to be confirmed by using sequence data of other *Moniezia* species belonging to the 2 subgenera.

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