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Comparative study on DNA sequences of ribosomal DNA and cytochrome c oxidase subunit 1 of mitochondrial DNA among five species of gnathostomes

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Abstract

The nucleotide sequences of partial 18S, complete internal transcribed spacer region 1 (ITS1), complete 5.8S, complete ITS2 and partial 28S of ribosomal DNA (rDNA) and cytochrome c oxidase subunit 1 of mitochondrial DNA (MCOI) from five species of gnathostomes (*G. spinigerum*, *G. doloresi*, *G. nipponicum*, *G. hispidum* and *G. binucleatum* with the former four species being distributed in Japan and Asia) that cause human gnathostomiasis were compared by direct polymerase chain reaction cycle-sequencing. The nucleotide sequences of each region of the18S (613 bp), 5.8S (158 bp) and 28S (598 bp) rDNA from the five species were almost identical. The ITS1 region was different in length for the five species. The nucleotide sequences of each region of ITS2 and partial MCO1 regions were different among the five species. Therefore, these two regions can be used as genetic markers for identification of worms.

Introduction

Gnathostomes are nematodes belonging to the genus *Gnathostoma* that require two intermediate hosts, a first intermediate host (*Cyclops*) and a second intermediate host (mainly freshwater fishes, frogs, snakes and birds), to complete their life cycles. Identification of gnathostome species is conducted by the shape and extent of cuticular spines on the body surface of adult worms (Miyazaki, 1960). At present, 13 species have been identified with some uncertainty, *G. spinigerum*, *G. hispidum*, *G. turgidum*, *G. americanum*, *G. doloresi*, *G didelphis*, *G. nipponicum*, *G. procyonis*, *G. brasiliense*, *G. miyazakii*, *G. malaysiae*, *G. vietnamicum* and *G. binucleatum*. Daengsvang (1980) considered *G. didelphis* and *G. brasilliens* as independent species, whereas Miyazaki (1991) considered them as synonyms of *G. turgidum.* This point of difference is not clarified yet.

Only one of the 13 identified species, *G. spinigerum*, is considered a causative agent of human gnathostomiasis and patients have been reported mainly from Thailand and Japan since the 1910s (Miyazaki, 1960; Daengsvang, 1981; Ando, 2003). However, three species, *G. hispidum*, *G. nipponicum* and *G. doloresi*, were added as causative agents of human gnathostomiasis in Japan in the 1980s. Gnathostomiasis occurring in urban areas since 1979 has been due to ingestion of loaches infected with larvae of *G. hispidum* imported from China (Akahane *et al*., 1982). *Gnathostoma nipponicum* infection by ingestion of domestic loaches (Ando *et al*., 1988) and *G. doloresi* infection by

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ingestion of brook trout (Nawa *et al.*, 1989) have also been reported.

On the other hand, in Latin America, the causative agent of the first human case of gnathostomiasis from Mexico (Pelaez & Perez-Reyes, 1970) and an outbreak in Ecuador (Ollague-Loaiza *et al.*, 1984) was a new species, *G. binucleatum*. (Koga *et al.*, 1999). Now five species are confirmed to cause gnathostomiasis and four of the five species are distributed in Japan and Asia.

Patients are diagnosed by the detection of worms, serum reactions, clinical manifestations and patient eating history. At present, five species of gnathostomes can be discriminated from each other by morphological features of the worms in histopathological sections (Akahane *et al.*, 1986, 1994; Ando *et al.*, 1990, 1991).

Recently, nucleotide sequence analysis of genes of worms has been reported for identification of parasites (Okamoto *et al.*, 1995; Hashimoto *et al.*, 1997; Ando *et al.*, 2001). Almeyda-Artigas *et al.* (2000) reported the nucleotide sequences of the ITS2 rDNA of *G. binucleatum* and *G. turgidum* distributed in the Americas and *G. spinigerum* distributed in Asia. In addition, the nucleotide sequences of the 18S and ITS2 regions of *G. procyonis*, and 18S, 5.8S and MCO1 regions of *G. binucleatum*, and 18S and 5.8S regions of *G. turgidum* were registered with GenBank. However this information is insufficient for identification of worms detected from humans and intermediate hosts because there is no information on three other important species, *G. hispidum*, *G. doloresi* and *G. nipponicum*. Therefore, in the present study, the genes of the 18S, ITS1, 5.8S, ITS2 and 28S regions of rDNA and MCO1 were analysed from four species, *G. spinigerum*, *G. hispidum*, *G. doloresi* and *G. nipponicum*, distributed in Asia and the ITS1, 5.8S, ITS2, 28S and MCO1 regions of *G. binucleatum* distributed in the Americas.

Materials and methods

Specimens of gnathostomes

Specimens of the following species were investigated: advanced third-stage larvae of *G. spinigerum* from swamp eels, *Fluta alba*, from Nakhon Nayok, Thailand; adults of *G. doloresi* from the stomach of wild boars, *Sus scrofa*, from Mie, Japan; adults of *G. nipponicum* from the oesophagus of weasels, *Mustela sibirica itasi*, from Mie, Japan; adults of *G. hispidum* reared in pig stomach from larvae in loaches imported from China (Akahane *et al.*, 1982); advanced third-stage larvae of *G. binucleatum* from fish, *Rhamdia cinerascens*, from Guayaquil, Ecuador.

Molecular techniques

Adults and larvae were homogenized on dry ice in a microcentrifuge tube using a hand made glass pestle. Genomic DNA from specimens was extracted by the phenol extraction technique. Mitochondrial DNA was also extracted by phenol extraction without separating the mitochondria. Partial 18S (613 bp), complete ITS1, complete 5.8S, complete ITS2 and partial 28S (598 bp) regions of rDNA and partial MCO1 (381 bp) region were amplified by polymerase chain reaction (PCR). PCR amplification was employed using 2 μl of gnathostome total DNA for each 50 μl reaction.

The PCR conditions for rDNA analysis were as follows: 94°C for 1 min, 52°C for 1 min and 72°C for 3 min for 30 cycles. The primers used were as follows: P6 (forward), 5′-AAAGCTGAAACTTAAAGGAAT-3′ (Katayama *et al.*, 1993); 18SR2 (reverse), 5′-ATAATGATCCTTCCGCAGGTTCA-3′ (newly designed); Lim1657 (f), 5′-CTGCCCTTTGTACACACCG-3′ (Almeyda-Artigas *et al.*, 2000); 58S2 (r), 5′-TCTTTATGCTCAATGTCTTCGC-3′ (newly designed); LC1 (f), 5′-CGAGTATCGATGAAGAACGCAGC-3′ (Navajas *et al.*, 1994); HC2 (r), 5′-ATATGCTTAAGTTCAGCGGG-3′ (Navajas *et al.*, 1994); 28SW (r), 5′-GCAACCCGACTCCAAGGAAC-3′ (newly designed); 28SY (f), 5′-CTAACCAGGATTCCCTCAGTAACGGCGAGT-3′ (Hillis *et al.*, 1996); 28SZ (r), 5′-AGACTCCTTGGTCCGTGTTTCAAGAC-3′ (Hillis *et al.*, 1996).

First, the ITS2 region was sequenced by using primers LC1 and HC2. These primers covered the partial 5.8S, complete ITS2 and partial 28S regions. Next, the 28S region was sequenced by using primers 28SY and 28SZ but the 5′ side of the sequence was not connected to the 3′ end of ITS2. A new primer 28SW was designed in the 28S region from the sequence results with primers 28SY and 28SZ. The sequence resulting from primers LC1 and 28SW overlapped the sequence obtained from primers 28SY and 28SZ. Therefore partial 5.8S, complete ITS2 and partial 28S regions were connected by these steps. Next, a new reverse primer 58S2 was designed for the ITS1 region in the 5.8S region from the sequence results with primers LC1 and HC2. Primers Lim1657 and 58S2 covered partial 18S, complete ITS1 and partial 5.8S. By these steps, partial 18S, complete ITS1, complete 5.8S, complete ITS2 and partial 28S were sequenced. However, partial 18S was very short so a new reverse primer 18SR2 was designed in the 18S region from sequence results with primers Lim1657 and 58S2 in order to obtain a longer 18S region. The primers P6 and 18SR2 covered a longer 18S region.

The PCR conditions for the MCO1 analysis were as follows: 95°C for 1 min, 40°C for 1 min and 72°C for 2 min for 30 cycles. Primers used were 5′-TTTTTTGGGCATCCTGAGGTTTA-3′ (FH5: forward) (Hashimoto *et al.*, 1997) and 5′-TAAAGAAAGAACATAATGAAAATGAGC-3′(MCO1B: reverse). The reverse primer was modified from FH3 by Hashimoto *et al.* (1997).

The PCR products were purified with QIA quick gel extraction kit (Qiagen, Germany) and used as templates for cycle sequencing. The sequences were analysed using an ABI sequencer (ABI 310). Some sequence data are quoted from GenBank.

Results

18S region

Primers P6 and 18SR2 amplified approximately 550 bp in the 18S region but this segment did not reach the 3′ end of the 18S region so a portion (63 bp) amplified by primers Lim1657 and 58S2 was connected with the 550 bp. Analysis of the up-stream region 613 bp from the 3′ end of the 18S region was conducted for seven species of gnathostomes after adding data for *G. binucleatum*,

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G. turgidum and *G. procyonis* registered with GenBank. This region was highly conserved and all nucleotides were identical except for only 13 sites in the alignment of seven species. Furthermore, when the four species distributed in Asia were compared, only three sites were not identical and two sites from the three species distributed in the Americas. Interestingly, there is a deletion of three continuous nucleotides near the 3′ end of 18S in the three species from the Americas.

ITS1 region

Primers Lim1657 and 58S2 covered the partial 18S, complete ITS1 and partial 5.8S regions. ITS1 regions of the five species analysed were different sizes; *G. spinigrum* – 633 bp, *G. doloresi* – 620 bp, *G. nipponicum* – 843 bp, *G. hispidum* – 581 bp and *G. binucleatum* – 667 bp. There is no information on this region for *G. procyonis* and *G. turgidum* in GenBank so analysis was conducted with

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G. spinigerum       CTTT-G-----T--TGAAAT-ATGTGATGGTGACA----CGACG-CG--AAG-C-AG-CG    41
G. doloresi         -...--------.G-.....-.--.C.............--G.-..C-.C.A.GG.-..    39
G. nipponicum       -...C.--AAA.------..G--.---..A.....-CGGT-G..CA.C-.--A---.-..    37
G. hispidum         --..--------.--C.....-.--.A...A.....-----.A.T.-.---C.A.-G.-..    36
G. binucleatum      --..-----AT.GA--.-..-...--.........-CGAT-G..--.-----A---.-..    34
G. procyonis        --..--------.--C.....-...-..C.A..-------..T.-.-----.-.-..-..    33
G. turgidum         -...T.AAAAA.GAC..C..G...--...A..-..GTGGT.GT.GT.CT.--ATG-.T..    53

G. spinigerum       AC-------GGC-TCT-CA--T-CTCGAG-G-CGATGA----GGCCTT--C-GT--TTG-    77
G. doloresi         ..-----AACAA.-...-..--.-....G.-.-...-..-TTTT.---.G-.T..--.C.T    81
G. nipponicum       .-----T--CT.G...CG.CG.GG.-.-.C.A...C..CGAGA.----GA.T-.CG.--C    81
G. hispidum         -------AT...-...-..--.-.T.G.-.-...-..-TCTT.---.--.-..--...C    72
G. binucleatum      .-GGGAT--CT.G...CG-C------.-.C.--.-C-----GA.----GT.T-.CG.--T    68
G. procyonis        .---------.---.--G.--.-T..----.--.-.---------...--.-..--...-    54
G. turgidum         ..GGCTTA-CT.G--.C-.C--GG.---.C.A...-.-GGAG-...--GC.TC.CA.-CC    98

G. spinigerum       T--AGAGTG-T-T-GTGTCGTCAT---TCA-TCGAGCGGCAAGTGA---TGTTG--ACGT   123
G. doloresi         .-----...-.-.--.-.-.-------...T.-C.......C....CGT.....--.-TA   119
G. nipponicum       .GC.--...-.----...-.---.-GG...T.-T.......G....---....--C.-TG   122
G. hispidum         .-----...-.-.--.-.-.---------...T.-C.......G....---.....--.-TG   107
G. binucleatum      .GC.--...-.-------...-.---.CAT...T.-T.......G....---....--G.-CG   111
G. procyonis        ----C....C.A.CT...-..T.----...T.T.-.-----.G....---.....AA..CC   101
G. turgidum         .CC.TT...-.A---.-.-.---.--G..GT.-TG.....C....---....--G.-TG   140

G. spinigerum       C-TTAA-A-T-GACT-G---CATCGC-TTGCTTA--GTGGA-CGAG--TCGT-ACGC-GA   167
G. doloresi         .-----C-T.C.-G.G.TTA.G..-.GA...------.----.-.--.--.G..-.--.   153
G. nipponicum       .T....-.------.G.TTG.....-.CA.....GT.....-.--.AA....G-...GT.   170
G. hispidum         .-----C-T.A.-A.G.TTG.....-.CA...------.----.-.--.--.G..-.--.   141
G. binucleatum      .C....-.------.G.TTG.....-.CA.....--.....T.-.--....G-...G-.   156
G. procyonis        .AAG..C.T.-.GT.-.---....C.-A.....--.....-.C..--....-...--T.   147
G. turgidum         .C....-.------.G.TTG.....-.GA.....GT-.-..-.---CA--..-----.GT.   180

G. spinigerum       ---GA-GGAGATGTC-TAGCATCATCTCTTATCG---AG-GTGCGTACGCGT-GTGGCGC   217
G. doloresi         ----.-T-C.T.------C..--.--G.---.------C.C..--..--....-...T...   184
G. nipponicum       CGC..--.....-..A.--....-....-----..TAG..-.........A..-.......   217
G. hispidum         ---.-T-C.C.------..---.---.---..-.---T.C..--..--....-.......   172
G. binucleatum      ---..--.-.---..T.--....-...-----.-TA-.--.........A..-.......   194
G. procyonis        ---..TC....-.----..-...-.-..AA..-----.---.A.........-.......   187
G. turgidum         TGT..--..-.----A----.---.A.-------.--C.---.A..--..A..G..-....-   211

G. spinigerum       ATCGTCGG--GAAAT-G-GTAGCGA---TG--G-TGACG---A---TGATG-TT-G-AT-   257
G. doloresi         .........-T.-...---.--T.-.-----AT.A.....---.TGA..--.--.-.-..C   222
G. nipponicum       ...A..-.GC..-..--C---T.A.CGAC.AT.T.....ATGTTGA.....C-.C.G..-   268
G. hispidum         ......C.-T.-...---.--C.A.---..AT.A.....---.TGA.....-A.----..C   215
G. binucleatum      ---A..-.TC..G.---------A.TGGC.AT.A...T.ATA.TGT.....C-.C-G.--   237
G. procyonis        ...A....-T.-...G.C.CG.....---..AT.A.....A-C.T--.....C..-.C..-   237
G. turgidum         -.TA..-.GC..-.-------T----GAC.A---.---.A---TGA...----.--G..-   241

G. spinigerum       -GCTCGAATT--TT---G-TG--GC--A-A-ACGTTGAGGAACGTACG-GGGA-ATATCA   302
G. doloresi         G...-.-.---GC.---.CG.CC..AC.T.G...-.....T...T..---.-GG.-.---   266
G. nipponicum       G--.T.TG..GG..AGG.TG.TG..--.-.-...-.....T.....--..GA.....   319
G. hispidum         G.....-----GA.---.CG.CC..AG.T.G...-.....T...T..--.-GG.-...-   261
G. binucleatum      -------G-.-G..---.T---G...--.-.-...-.A....T.....--..GA.....   275
G. procyonis        -AT...--..-G..---.-..--.--.-.-...-.....T......AT.A.T.....G   282
G. turgidum         G--.T-CG--GG-.---.TG.CG.-------...-.....T...-.---------C.-.   273
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G. spinigerum       T-----GCTAC-AA-ATCGAGTTGATCACGCGGTGATGTCGT---C---GTT-AT--T--   344
G. doloresi         .-----.-.G.-------.C-----.---.---.C.-.----CG.---.C.G..G----   290
G. nipponicum       .GCCGCA..C.GC.C........C....--------C.AG..--G-A-T.-.--CG-.-C   362
G. hispidum         .-----.-.G.-----.-.C...--.--.----.G.-.-.-ACG.---.G.G..G-.--   292
G. binucleatum      .---G--.--AC.C.............--------..CT..--G-A-T.-.--CG-.-C   311
G. procyonis        .GCCGC.....-..T........C..................CCG.ATT...GC.GC.GC   341
G. turgidum         .G--G--.--GC-T..---.-.C.-..--------C----------T--.--CG----   294

G. spinigerum       -G----T--TG----C--TTATC-ATTGAG-CACGT-T-----T-ATCGCC---TT-GAC   378
G. doloresi         C.ATGA.-A.----T-G-----.-.....T.-G..-.GACAA--.-..-TTGG..-.--   328
G. nipponicum       --CTCA.--..TCGTTGC.....----...--.------------.---------.-.--   388
G. hispidum         T.-TCA.-C.--CGT.G--.-..G....-.T.-G..G.-TCAA.-.-.-----G..-.--   332
G. binucleatum      --GTCA.--.---GT.GC.....----.....-.....-.-----.-.--------..-.-.   342
G. procyonis        C.CTAA.GC..-C-T.GA.....G.-....G.....-.G-CAA.C.-....T-G..C...   394
G. turgidum         ----GA.--..ACG--G----.-------.---------------.---------.-.--   308

G. spinigerum       GA-G----GACG-GCGATCAAC----G--ACTC-TC----TCGATCGA--ACA-CA-CCC   417
G. doloresi         .TC.CCTA....G.-...---.G--C.CA.----..-AA--...-...T----------   363
G. nipponicum       -----------------.---.GATC.--.-..-A.ACA-...----.---..-ACT...   415
G. hispidum         .TC.CCTA.G..A.-..----.G---.CG.--.-..--A---.--...T---.--.----   364
G. binucleatum      -----CT----------.-...GA-G.--.--.GG.-GA-.-..---.---.-GGCT...   371
G. procyonis        ..G.TCT-.G..-.........G-TT.C-.-..-..AAAG.-...-..TCT..-A.T...   445
G. turgidum         -----------------.----GATC---.-.-----CA-...---------..TGCTG..   329

G. spinigerum       GA--TCATGT-G-TCGACGA--AC-A-G-C---T--TCGC--CG-AT-A-C-AGT-GACG   456
G. doloresi         -.----.-----A.---.-.CG..G.--A-CAA.CA.--.GA..-.--.---...C.T..   398
G. nipponicum       ..TG----A.-.A---..-.TG-.-.T.-.-----A...-G-..A..G-T.--..-.-..   451
G. hispidum         ..----.-----A.---.-GCGG.G.--A-TAA.GA.--.GA..-----.---.-.-.T..   397
G. binucleatum      ..TG--.-A.C.-----..-.T--.-.T--.---.-A.------..-..G.A.--..---..   403
G. procyonis        ..TG..-G..C.A.......CG..G.C.A.GAA.-G....GG..-..----.T.-.---.-   494
G. turgidum         .--G-----.-------.-.TG---.T.-------A.---------.-G-T----.----.   346

G. spinigerum       --GTA--AGCGCCTCAT-TAT                                          472
G. doloresi         AT...TG....A.....-...                                          418
G. nipponicum       A--..CG..........-...                                          469
G. hispidum         AT...TG..........-...                                          417
G. binucleatum      A--..CG..........-.G.                                          421
G. procyonis        --...T-....T.....CA..                                          512
G. turgidum         -----C--.......G.-...                                          359
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Fig. 1. Nucleotide sequences of a region of the internal transcribed spacer region 2 of ribosomal DNA. *Gnathostoma turgidum* and *G. procyonis* sequences were quoted from GenBank. A dot (·) indicates that the sequence at that point is the same as in *G. spinigerum*. Hyphens (-) indicate alignment spaces.

the five species mentioned above. The anterior half was less conserved and the posterior half was relatively conserved.

5.8S region

The nucleotide sequence of the 5.8S region was obtained by connection of the partial 3′ segment amplified by primers used for the ITS1 region and partial 5′ segment amplified by primers used for the ITS2 region. The nucleotide sequence of this region of *G. spinigerum*, *G. binucleatum* and *G. turgidum* registered with GenBank racked 29 bp so the analysis was conducted with only our sequence results of five species shown in the ITS1 region. This region consisted of 158 bp and was highly conserved. Only two nucleotides were different in the alignment of the five species.

ITS2 region

Primers LC1 and HC2 covered the partial 5.8S, complete ITS2 and partial 28S regions. ITS2 regions of the five species were different sizes; *G. spinigrum* – 472 bp, *G. doloresi* – 418 bp, *G. nipponicum* – 469 bp, *G. hispidum*– 417 bp and *G. binucleatum* – 421 bp. Analysis was conducted on the seven species after adding data for *G. turgidum* (359 bp) and *G. procyonis* (512 bp) registered with GenBank. As shown in fig. 1, this region was less conserved. The highest and lowest G + C content were 52.15 for *G. doloresi* and 49.64 for *G. binucleatum*. Intraspecific variation was not observed in the 18S, ITS1, 5.8S, ITS2 and 28S regions of each species from the same geographical location in our samples but our results (472 bp) were different from that (476 bp) registered with GenBank at five sites including deletions at four sites in the ITS2 region of *G. spinigerum*.

28S region

Primers 28SY and 28SZ covered a partial 28S region but this segment did not contain the 5′ end so a portion amplified with primers LC1 and 28SW was used to connect the 5′ end. As there is no information of this region from *G. procyonis* and *G. turgidum* in GenBank,

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analysis was conducted with the 598 bp from the 5′ end of the 28S region for the five species. This region was highly conserved and sites with the nucleotides not identical were only 31 sites in the alignment of the five species.

MCO1 region

Primers MCO1A and MCO1B amplified 381 bp of the CO1 gene of the mitochondorial DNA. This segment corresponds to the central portion of the complete CO1 gene inferred from other data of parasites registered with GenBank. The CO1 region of *G. binucleatum* is registered with GenBank but the amplified segment is not identical to our segment so analysis was conducted with only our sequence data for the five species. As shown in fig. 2, the sites with nucleotides not identical were 86 sites in the alignment. When two species were compared, the sites with nucleotides not identical were 52 sites (max.) between *G. spinigerum* and *G. nipponicum* and *G. nipponicum* and *G. hispidum*, and 36 sites (min.) between *G. spinigerum* and *G. binucleatum*. Intraspecific variation was found at six sites in *G. doloresi* from the same geographical location. Amino acid sequence alignment of this region was different at 5 of 127 sites among the five species.

Discussion

At present, 13 species of gnathostomes have been identified in the world but only seven have been shown to be important, that is, *G. spinigerum*, *G. doloresi*, *G. hispidum* and *G. nipponicum* distributed in Asia and *G. turgidum*, *G. procyonis* and *G. binucleatum* distributed in the Americas, on human diagnosis and epidemiological survey. Almeyda-Artigas *et al.* (2000) attempted to analyse the nucleotide sequences of ITS2 rDNA for *G. turgidum*, *G. procyonis*, *G. binucleatum* and *G. spinigrum* and reported that the ITS2 region is a good marker for identification of worms. However, as there is insufficient information for identification of worms we analysed the nucleotide sequences of *G. spinigerum*, *G. hispidum*, *G. doloresi*, *G. nipponicum* and *G. binucleatum*. Accumulation of these data clarified nucleotide sequences of the rDNA

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G. spinigerum       ATTTTAATTTTGCCTGCTTTTGGAATTGTTAGTCAGAGTAGTTTGTATTTGACAGGTAAA  60
G. doloresi         .....G.................T.....G........A..............T.....G
G. nipponicum       .....G........A........G..C........A..A..A...........T.....G
G. hispidum         ........................T..............A..............T.....G
G. binucleatum      .....G.................G.................G........A..T.....G

G. spinigerum       AAAGAGATTTTTGGTTCCTTAGGTATGGTTTATGCTATTTTAAGGATTGGTTTGATTGGT 120
G. doloresi         ..G...G.A.....A..T..G........................T.....GC.......
G. nipponicum       ..G..A...........T..G..G..A..A..............A........A......
G. hispidum         ..G...........G..T.....A.....A...........G...........A......
G. binucleatum      ..G..A........C..T..G..G..A..G...........G...........A......

G. spinigerum       TGTGTGGTTTGGGCTCATCATATATATACGGTGGGGATGGATTTGGATTCTCGTGCTTAT 180
G. doloresi         ..C.........A..G........G.........T..T..A..............G..C...
G. nipponicum       ........................G.........A..T..A.....A...............
G. hispidum         ........................G.....A..A............................
G. binucleatum      ........................G...........T..A.....A.....G.........

G. spinigerum       TTTACAGCTGCTACTATGGTGATTGCTGTACCTACGGGGGTGAAGGTTTTTAGATGGTTG 240
G. doloresi         .....G...........A...........T.....T........A................
G. nipponicum       .....T..............A........T..C..T..A..T..A...........A...
G. hispidum         ....................A........G..G..T.....T.....A.....T......
G. binucleatum      .....G........................G.....T.....T...........G..A...

G. spinigerum       GCTACTTTGTATGGTTTTCGTATGATGTTTTCTCCT-TTGTTGTTGTGGGTAT-T-GGGT 297
G. doloresi         ..............G....A....G.T.........-.....A..A.....-.A.-...G
G. nipponicum       ........A..............A............C..-..A..A.....T.-.A...-
G. hispidum         ..G...........G.....G...G.T.........-.........A..A..G.-.-....
G. binucleatum      ............................A.........-...C.A........T.-.-....

G. spinigerum       TTTATTTTTTTGTTTACTGTTGGG-G-GG-TTGACTGGCGTAATGTTGTCTAATTCTAGT 354
G. doloresi         ..................-AG.-...-.A..T........T..T..A...........G...
G. nipponicum       ...........A......-AG.-...-.A..T..A.....T..T..A...............
G. hispidum         ..................-A....T.-..-..A..A..A..T..............A...
G. binucleatum      ...........A......A..-..T.-..-........G..G..................

G. spinigerum       TTGGATATTATTCTT-CATGATACTTAT                                 381
G. doloresi         ............-..A............
G. nipponicum       ..A............-..C.........
G. hispidum         ............-..A............
G. binucleatum      ..............-............
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Fig. 2. Nucleotide sequences of a 381 bp fragment of the mitochondrial cytochrome c oxidase subunit 1 gene from five species of gnathostomes.

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and MCOI regions of at least five species which cause human infection.

The sequence data reported in this paper are available in the GenBank database. rDNA sequences are found under the following accession numbers: *G. spinigerum*, AB181155; *G. doloresi*, AB181156; *G. nipponicum*, AB181157; *G. hispidum*, AB181158; *G. binucleatum*, AB181159. Partial MCO1 sequences are found under the following accession numbers: *G. spinigerum*, AB180099; *G. doloresi*, AB180100; *G. nipponicum*, AB180101; *G. hispidum*, AB180102; *G. binucleatum*, AB180103.

Nucleotide sequences for each of the regions 18S, 5.8S and 28S in the five species were almost identical. Therefore, these regions are not available for identification of worms. Nucleotide sequences of the ITS1 region differed in the five species, but this region was a little difficult to sequence so is also inadequate for identification. The nucleotide sequences of the ITS2 region differed in the five species. Furthermore this region was easy to sequence so it is a good marker for identification of worms.

Nucleotide sequence of the partial MCO1 (381 bp) was different in the five species and this region is also a good marker for identification of worms. Mitochondrial DNA is known to have a faster evolutionary rate than nuclear DNA and mitochondrial genes such as CO1 or CO2 have been used for identification and to study the phylogenetic relationships between numerous related organisms (Okamoto *et al.*, 1995; Hashimoto *et al.*, 1997). However, the ITS2 region showed marked differences in the present results and those of Almeyda-Artigas *et al*. (2000). This may be the consequence of a very fast evolutionary rate for this ITS2 in gnathostomes.

At present, sequence data of the ITS1 region of five species, ITS2 region of seven species including data in GenBank and MCO1 region of five species are accumulated. However, the difference of size in ITS2 regions among seven species were so great that no phylogenetic analysis was performed. For ITS1 and MCO1 regions, the number of species on which sequence analysis had been performed was too small to perform phylogenetic analysis.

Gnathostoma nipponicum has been considered to be distributed only in Japan but Sohn *et al.* (1993) detected larvae of this species from the muscle of loach imported from China to Korea. Further, Han *et al.* (2003) detected larvae from the snake, *Rhabdophis tigrina*, in Korea. These results indicate that *G. nipponicum* may be distributed in countries other than Japan. We expect the discovery of new species of gnathostomes and recognition of new distributions of known species will occur in the future. The sequence results reported in this paper will be very useful for this future work.

References

Akahane, H., Iwata, K. & Miyazaki, I. (1982) Studies on *Gnathostoma hispidum* Fedchenko, 1872 parasitic in loaches imported from China. *Japanese Journal of Parasitology* **31**, 507–516 (in Japanese with English summary).

Akahane, H., Sano, M. & Mako, T. (1986) Morphological difference in cross sections of the advanced third-stage larvae of *Gnathostoma spinigerum*, *G. hispidum* and *G. doloresi*. *Japanese Journal of Parasitology* **35**, 465–467.

Akahane, H., Lamote-Argumedo, R., Martinez-Cruz, J.M., Osorio-Sarabia, D. & Garcia-Priento, L. (1994) A morphological observation of the advanced third-stage larvae of Mexican *Gnathostoma*. *Japanese Journal of Parasitology* **43**, 18–22.

Almeyda-Artigas, R.J., Bargues, M.D. & Mas-Coma, S. (2000) ITS-2 rDNA sequencing of *Gnathostoma* species (Nematoda) and elucidation of the species causing human gnathostomiasis in the Americas. *Journal of Parasitology* **86**, 537–544.

Ando, K. (2003) Gnathostomiasis (2) *Gnathostoma nipponicum*. pp. 507–518 *in* Otsuru, M., Kameya, S. & Hayashi, S. (*Eds*) *Progress of medical parasitology in Japan 8*. Tokyo, Japan, Meguro Parasitological Museum.

Ando, K., Tanaka, H., Taniguchi, Y., Shimizu, M. & Kondo, K. (1988) Two human cases of gnathostomiasis and discovery of a second intermediate host of *Gnathostoma nipponicum* in Japan. *Journal of Parasitology* **74**, 623–627.

Ando, K., Tokura, H. & Chinzei, Y. (1990) Morphological features in cross section of early and advanced third-stage larvae of *Gnathostoma nipponicum*. *Japanese Journal of Parasitology* **39**, 482–487.

Ando, K., Hatsushika, R., Akahane, H., Matsuoka, H., Taylor, D., Miura, K. & Chinzei, Y. (1991) *Gnathostoma nipponicum* infection in the past human cases in Japan. *Japanese Journal of Parasitology* **40**, 184–186.

Ando, K., Ishikura, K., Nakakugi, T., Shimono, Y., Tamai, T., Sugawa, M., Limviroj, W. & Chinzei, Y. (2001) Five cases of *Diphyllobothrium nihonkaiense* infection with discovery of plerocercoids from an infective source, *Oncorhynchus masou ishikawae*. *Journal of Parasitology* **87**, 96–100.

Daengsvang, S. (1980) A monograph on the genus *Ganathostoma* and gnathostomiasis in Thailand. Tokyo, SEAMIC.

Daengsvang, S. (1981) Gnathostomiasis in Southeast Asia. *Southeast Asian Journal of Tropical Medicine and Public Health* **12**, 319–332.

Hashimoto, K., Watanabe, T., Liu, C.X., Init, I., Blair, D., Ohnishi, S. & Agatsuma, T. (1997) Mitochondrial DNA and nuclear DNA indicate that the Japanese *Fasciola* species is *F. gigantica*. *Parasitology Research* **83**, 220–225.

Han, E.T., Lee, J.H., Choi, S.Y., Park, J.H., Shin, E.H. & Chai, J.Y. (2003) Surface ultrastructure of the advanced third-stage larvae of *Gnathostoma nipponicum*. *Journal of Parasitology* **89**, 1245–1248.

Hillis, D.M., Mable, B.K. & Moritz, C. (1996) Nucleic acids IV: sequencing and cloning. pp. 321–383 *in* Hills, D.M., Moritz, C. & Mable, B.K. (*Eds*) *Molecular systematics*. Sunderland, Massachusetts, Sinauer.

Katayama, T., Yamamoto, M., Wada, H. & Satoh, N. (1993) Phylogenetic position of acoel turbellarians inferred from partial 18S rDNA sequences. *Zoological Science* **10**, 529–536.

Koga, M., Akahane, H., Ogata, K., Lamothe-Argumedo, R., Osorio-Satabia, D., Garcia-Prieto, L. & Martizez-Cruz, J.M. (1999) Adult *Gnathostoma* cf. *binucleatum* obtained from dogs experimentally infected with larvae as an etiological agent in Mexican gnathostomiasis; external

morphology. *Journal of the Helminthological Society of Washington* **66**, 41–46.

Miyazaki, I. (1960) On the genus *Gnathostoma* and human gnathostomiasis, with special reference to Japan. *Experimental Parasitology* **9**, 338–370.

Miyazaki, I. (1991). *An illustrated book of helminthic zoonoses*. pp. 369–409 Tokyo, International Medical Foundation of Japan.

Navajas, M., Gutierrez, J., Bonato, O., Bolland, H.R. & Mapangou-Divassa, S. (1994) Intraspecific diversity of the cassava green mite *Mononychellus progresivus* (Acari: Tetranychidae) using comparisons of mitochondrial and nuclear ribosomal DNA sequences and cross-breeding. *Experimental and Applied Acarology* **18**, 351–360.

Nawa, Y., Imai, J., Ogata, K. & Otsuka, K. (1989) The first record of a confirmed case of *Gnathostoma doloresi* infection. *Journal of Parasitology* **75**, 166–169.

Okamoto, M., Bessho, Y., Kamiya, M., Kurosawa, T. & Horii, T. (1995) Phylogenetic relationships within *Taenia taeniaeformis* variants and other taeniid cestodes inferred from the nucleotide sequence of the cytochrome c oxidase subunit I gene. *Parasitology Research* **81**, 451–458.

Ollague-Loaiza, W., Ollague-Torres, J., Guevara, A., Veliz, D.E. & Penaherrera, S. (1984) Human gnathostomiasis in Ecuador (nodular migratory eosinophilic panniculitis): first finding of the parasite in South America. *International Journal of Dermatology* **23**, 647–651.

Pelaez, D. & Perez-Reyes, R. (1970) Gnathostomiasis humana en America. *Revista Latino-Americana de Microbiologia* **12**, 83–91 (in Spanish with English abstract).

Sohn, W.M., Kho, W.G. & Lee, S.H. (1993) Larval *Gnathostoma nipponicum* found in the imported Chinese loaches. *Korean Journal of Parasitology* **3**, 347–352.

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