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Molecular phylogenic location of the *Plagiorchis muris* (Digenea, Plagiorchiidae) based on sequences of partial 28S D1 rDNA and mitochondrial cytochrome C oxidase subunit I

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Abstract: To determine the molecular phylogenic location of *Plagiorchis muris*, 28S D1 ribosomal DNA (rDNA) and mitochondrial cytochrome C oxidase subunit I (mtCOI) were sequenced and compared with other trematodes in the family Plagiorchiidae. The 28S D1 tree of *P. muris* was found to be closely related to those of *P. elegans* and other *Plagiorchis* species. And, the mtCOI tree also showed that *P. muris* is in a separate clade with genus *Glypthelmins*. These results support a phylogenic relationship between members of the Plagiorchiidae, as suggested by morphologic features.

Key words:trematoda, ribosomal DNA, mitochondrial DNA, phylogeny, classification

Although *Plagiorchis muris* was first recovered from *Rattus norvegicus* (Seo et al., 1981) in Korea, eleven natural cases of human infection by the genus *Plagiorchis* have been reported (Chai, 1991). Molecular approaches to this worm are rare since it is difficult to acquire sufficient metacercariae. Since *P. muris* is unique species of which infection in human intestine has been reported among the family Plagiorchiidae, the phylogenic relationship with other worms in Plagiorchiidae is interesting. In the present study we determined molecular phylogenic location of *P. muris* with respect to other trematodes in the family Plagiorchiidae using the 28S D1 rDNA and mtCOI gene regions. It will be able to tell the consistency between morphologic and molecular phylogenic location of *P. muris* in the genus *Plagiorchis*.

Metacercariae of *P. muris* were obtained from dragonflies and adults were recovered from infected mouse intestines (Hong et al., 1999). Worms were kept in ethanol at -70°C until assayed. Frozen worms were lyophilized and lyzed with lysis buffer containing 1% SDS, proteinase K (500 g/ml), and RNase at 37°C for 2-3 hrs. DNA was extracted using the phenol/chloroform method and precipitated in ethanol. The 28S D1

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rDNA and mtCOI regions were amplified by PCR using the primer sets described by Qu et al. (1988), and by Garey and Wolstenholme (1989). PCR was conducted using a mixed solution (25l) of the above extracted DNA (2.5l) as a template (1-50 ng), and primers (1.0l, each 10 pmole) for each genes with ~1.5 units of *Taq* enzyme (TAKARA Shuzo Co., LTD, Japan) in a GeneAmp PCR System 9600 (Perkin Elmer, U.S.A.). PCR amplification was conducted over 40 cycles denaturing at 95°C for 20 seconds, annealing at 55°C (28S D1 rDNA) or 48°C (mtCOI) for 30 seconds, extending at 72°C for 30 seconds), followed by a final extension of 7 minutes.

Amplified PCR products were extracted and purified using a gel extraction kit and a PCR purification kit (QIAGEN Co., Germany) and were cloned into a pT7Blue Perfectly Blunt cloning vector using T4 DNA ligase and transformed into *E.coli* Nova Blue competent cells, according to the supplier's protocol (Novagen Co. USA). Positive recombinant clones were picked, and grown in 2 ml of LB broth (in the presence of 50 g/ml ampicillin) overnight at 37°C. The recombinant plasmid was screened using isopropyl—thiogalactoside (IPTG) and 5-bromo-4 chloro-3-indolyl—D-galactoside (X-gal). Positive plasmid DNA was purified using a QIAprep spin plasmid kit (QIAGEN Co., Germany). DNA sequencing was performed by the dideoxy chain termination method. Cycle sequencing reactions were performed using Thermo Sequenase dye terminator sequencing pre-mix kits (Amersham Life Science Co.). PCR products were run on an ABI 373A automated DNA sequencer (Applied Biosystems model 373A, Perkin Elmer) according to the manufacturer's instructions. The each gene was sequenced in both orientations using the universal sequencing primers T7 and U19. At least two clones were sequenced per gene; additional clones were sequenced as necessary to resolve ambiguous sites.

The analyses of 28S D1 rDNA and mtCOI region sequences were determined by comparison with those of a range of other related nematodes of the family Plagiorchiidae. NCBI (National Center for Biotechnology Information, NIH, Bethesda, USA) databases were used for sequence homology analysis using BLAST2. Multiple sequence alignments were performed using CLUSTA W program (European Bioinformatics Institute, http://www.ebi.ac.uk/clustalw/), and the fractional GC contents of nucleic acid sequences were determined using the EMBOSS GEECEE program provided by Dr. Richard Bruskiewich (Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SA, UK)(http://analysis.molbiol.ox.ac.uk/pise_html/geecee.html). Alignment gaps were treated as missing data. Other trematodes in the family Plagiorchiidae and their GenBank accession numbers used for comparative purposes in this study are listed in Tables 1 and 2. *Haematoloechus longiplexus* (Haematoloechidae, AY222280 for 28S rDNA) and *Paragonimus macrorchis* (Paragonimidae, AF159598 for mtCOI) were used as outgroups. Phylogenic analysis was performed using the Kimura 2-parameter model for distance correction; corrected for multiple substitutions (Kimura, 1980). Phylogenic trees were constructed using the Neighbor-joining algorithm (Swofford et al., 1996). In order to view these trees, we used PHYLIP version 1.6 for tree drawing using the parsimony, maximum likelihood, and distance methods.

The sizes of amplified genomic DNA fragments of 28S D1 rDNA and mtCOI were 0.3 kb and 0.45 kb, respectively. The sequences length was 309 bp for the 28S D1 rDNA gene, and 397 bp for the mtCOI gene excluding primer sequences (results not shown). The GC contents were 52% (28S D1 rDNA) and 48% (mtCOI) exlcuding primers. The 28S D1 rDNA and mtCOI coding regions were highly conserved by multiple sequence alignment without additional nucleotides, Overall nucleotide similarity between *P. muris* and other *Plagiorchiidae* species ranged 61.1%~63.7% for 28S D1 rDNA and 51.4%~63.9% for mtCOI (results not shown). Several insertions, a 79 bp insertion in *P. muris* 28S D1 rDNA, a 16 bp insertion in *P. muris* mtCOI, with gaps, 1 bp gap in *P. muris* 28S D1, in the same or in different positions were detected within and between species (results not shown). The most-parsimonious tree was obtained when gaps were treated as missing data. The 28S D1 tree of *P. muris* was closely related with that of *P. elegans* and

Table 1. Plagiorchiidae species used in this study, GenBank accession numbers for corresponding sequences, sequence lengths of the 28S D1 rDNA gene

Species	GenBank No.	Sequence length (bp)	Aligned sequence length (bp)[b)]	Reference
Astiotrema monticellii	AF184253	1,261	231	Littlewood and Bray, 2001
Glypthelmins californiensis	AY278052	1,275	222	
G. facioi	AY278046	1,275	222	
G. hyloreus	AY278050	1,274	222	
G. pennsylvaniensis	AF433676	1,250	230	Tkach et al., 2001
G. quieta	AY222278	1,256	227	Olson et al., 2003
G. tuxtlasensis	AY278048	1,274	223	
Haplometra cylindracea	AF151933	1,254	230	Tkach et al., 2000a, 2001
Lecithopyge rastellus	AF151932	1,254	230	Tkach et al., 2000a, 2001
Leptophallus nigrovenosus	AF151914	1,256	229	Tkach et al., 2000a
Macrodera longicollis	AF151913	1,257	229	Tkach et al., 2000a
Metaleptophallus gracillimus	AF151912	1,256	229	Tkach et al., 2000a
Neoglyphe locellus	AF300330	1,255	230	
N. sobolevi	AF300329	1,255	230	Tkach et al., 2001
Paralepoderma cloacicola	AF151910	1,255	229	Tkach et al., 2000a
Plagiorchis eleganse	AF151911	1,263	230	Tkach et al., 2000b
P. koreanus	AF151930	1,254	230	Tkach et al., 2000ab, 2001
P. muelleri	AF184250	1,254	230	Tkach et al., 2000b
P. muris	AF096222[a)]	309	309	Present paper
P. vespertilionis	AF151931	1,254	230	Tkach et al., 2000ab, 2001
Skrjabinoeces similis	AY222279	1,255	232	Olson et al., 2003

[a)]Sequences generated as part of the current study.

[b)]Aligned sequence length indicates 28S D1 domain rDNA sequence from 28S rDNA region.

Table 2. Plagiorchiidae species used in this study, GenBank accession numbers for corresponding sequences, sequence lengths for the mtCOI gene

Species	GenBank No.	Sequence length (bp)	Aligned sequence length (bp)	Reference
Glypthelmins californiensis	AY278058	381	381	–
G. facioi	AY278053	383	383	–
G. hyloreus	AY278059	381	381	–
G. quieta	AY278056	381	381	–
G. tuxtlasensis	AY278054	381	381	–
Plagiorchis muris	AF096236[a)]	443	397	Present paper

[a)] Sequences generated as part of the current study.

other *Plagiorchis* species (Fig. 1). The mtCOI tree of *P. muris* was in the separate clade with genus *Glypthelmins* (Fig. 2).

We found sequence variability in both the 28S rDNA and mtCOI region of the family Plagiorchiidae, but the 28S D1 rDNA region was more conserved than the mtCOI region. With respect to the 28S D1 rDNA region, it is also worth noting that *P. muris* had longer sequences (about 68bp at 5′ end) than other species of the *Plagiorchis* genus. The phylogenic tree of

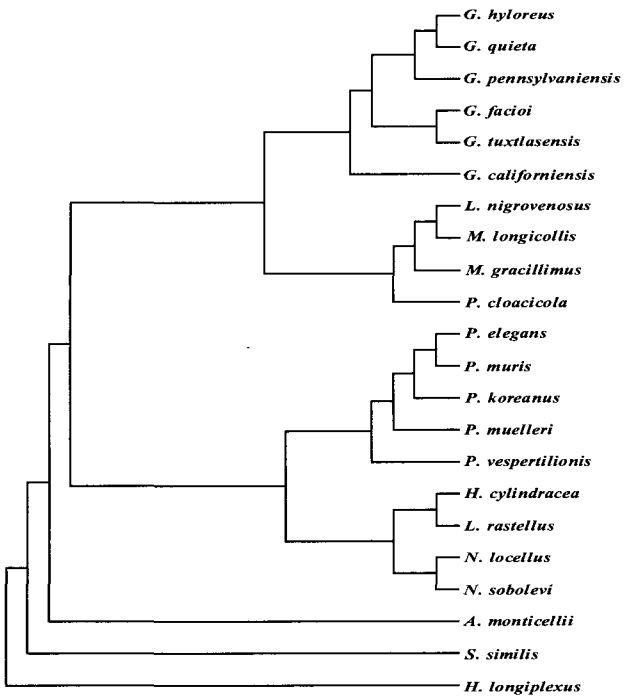

Fig. 1. Phylogenic relationships between the 28S D1 rDNA gene of *Plagiorchis muris* and other Plagiorchiidae. This parsimonious tree was analyzed by neighbor-joining method using PHYLIP program. *G, Glypthelmins; A, Astiotrema; H, Haplometra; L. rastellus, Lecithopyge rastellus; L. nigrovenosus, Leptophallus nigrovenosus; M. longicollis, Macrodera longicollis; N, Neoglyphe; P. cloacicola, Paralepoderma cloacicola; P, Plagiorchis; S, Skrjabinoeces; H. longiplexus, Haematoloechus longiplexus.*

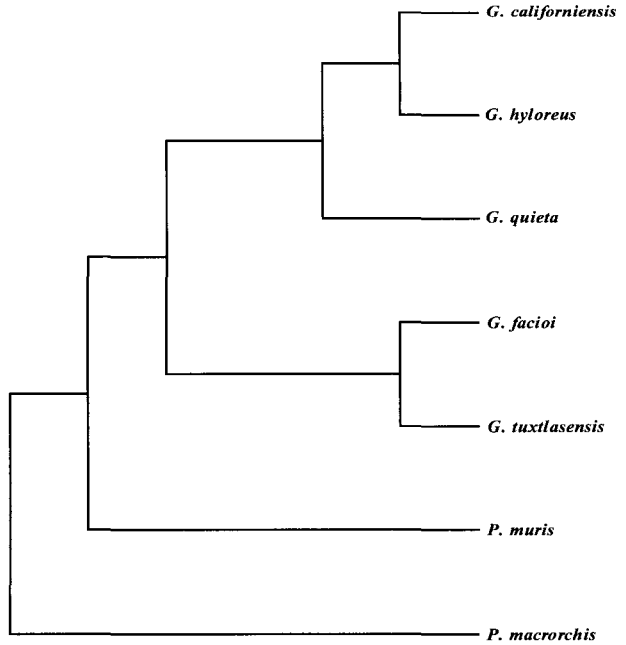

Fig. 2. Phylogenic relationships of the mtCOI gene of *Plagiorchis muris* with other Plagiorchiidae. This parsimonious tree was analyzed by the neighbor-joining method using PHYLIP program. *G, Glypthelmins; P. muris, Plagiorchis muris; P. macrorchis, Paragonimus macrorchis.*

the family Plagiorchiidae is consistent with a previous molecular analysis of *Haematoloechus* species and *Plagiorchis* species using internally transcribed spacer 1 (ITS1), and with large subunit sequence data (Snyder and Tkach, 2001). Tkach et al. (2000a, 2000b, 2001) reported that the suborder Plagiorchiata is composed of two-supported clades, which can be considered superfamilies, namely, Plagiorchioidea including the Plagiorchiidae, and Microphalloidea based on partial lsr DNA sequences. In Plagiorchiidae, a close phylogenic relationship was observed between two *Plagiorchis* species (*P. koreanus, P. vespertilionis*), *Lecithopyge rastellus*, and *Haplometra cylindracea*. Since there is a little data on mtCOI Plagiorchiidae worms, it is not possible to determine whether *P. muris* is in the same clade as *Glypthelmins* spp. However, we infer that *P. muris* is probably in a separate clade (Fig. 2).

The present partial 28S rDNA sequence-base phylogenic analysis of the family Plagiorchiidae including *P. muris* places the genera *Plagiorchis* into a well-defined separate clade within the family Plagiorchiidae. The positions of the majority of the taxa of Plagiorchiidae are consistent with the traditional systematic views, the above molecular data also supports the traditional morphology-based conclusion that *P. muris* belongs to *Plagiorchis* spp.

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