Jpn. J. Trop. Med. Hyg., Vol. 31, No. 1, 2003, pp. 1 - 3

Phylogenetic status of a lung fluke in the Philippines based on mitochondrial genome

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Accepted 26, February, 2002

Abstract

Based on a near-complete mitochondrial DNA sequence, the phylogenetic status of a lung fluke collected in the Philippines was evaluated. The lung fluke from Leyte Island, Philippines, resembles *Paragonimus westermani* morphologically and is sometimes regarded as a subspecies, *P. westermani filipinus.* In this study, all mitochondrial genes of the Leyte form were sequenced: 12 subunits of mitochondrial enzymes, 2 ribosomal RNA genes and 21 transfer RNAs. The gene order is the same as that of the previously-published *P. westermani* from Korea. All genes are transcribed in the same direction. The sequence from Leyte was 88.8% identical to the previously-published sequence (accession No. AF219379). This is further evidence that the Philippine form should be regarded as specifically distinct from the form in East Asia (China, Japan and Korea). This conclusion is strengthened by the observation that the molluscan host of the Philippine form is of a different family from that of the East Asian form.

Key words: mt DNA, complete sequence, *Paragonimus westermani*, *P. filipinus,* Philippines

INTRODUCTION

The lung fluke, *Paragonimus westermani* (Kerbert, 1878) is a well known zoonotic agent found in eastern and southern Asia and has been the focus of many taxonomic and biological studies (e.g. Blair *et al.,* 1999). Several previous studies have suggested that some regional populations of *P. westermani* should be regarded as distinct species. For example, Miyazaki (1981) proposed on morphological grounds that the Philippine form should be recognized as a distinct subspecies, *P. westermani filipinus.* Allozyme analysis and partial mitochondrial DNA sequences demonstrated that Philippine isolates are genetically distant from strains found in Taiwan, Japan and North East China (Agatsuma *et al,* 1988; Blair *et al,* 1997). Previous analyses of partial sequences (e.g. Iwagami *et al,* 2000) used only short sequence tracts. Comparisons of complete genome sequences can provide more reliable results and also permit comparisons of features such as gene order and the structure of non-coding regions. In this study, we present and discuss the complete sequence of the coding region of the mitochondrial genome for the Philippine lung fluke. In the light of this, we make recommendations concerning its phylogenetic status.

MATERIALS AND METHODS

Adult specimens of a Philippine species of *Paragonimus* used in this study are those previously reported (Agatsuma *et al.,* 1988). DNA extraction of the specimens is as described previously (Iwagami *et al.,* 2000). Based on the 14,967bp of mitochondrial DNA sequence from *P. westermani* (Korean triploid form - database accession No. AF 219379), several PCR primer sets were designed and applied for the present study. Amplified PCR products were purified by gel-electrophoresis and ethanol-precipitation, ligated into pGEM-T plasmid vector (Promega) and cloned inserts sequenced using a dye terminator cycle-sequencing kit (Applied Biosystems) and an automated sequencer (ABI 310, Applied Biosystems). Multiple sequence alignments were performed using the programs CLUSTAL V and GENETYXMAC.

RESULTS AND DISCUSSION

In total, 14,199bp were amplified and sequenced. As

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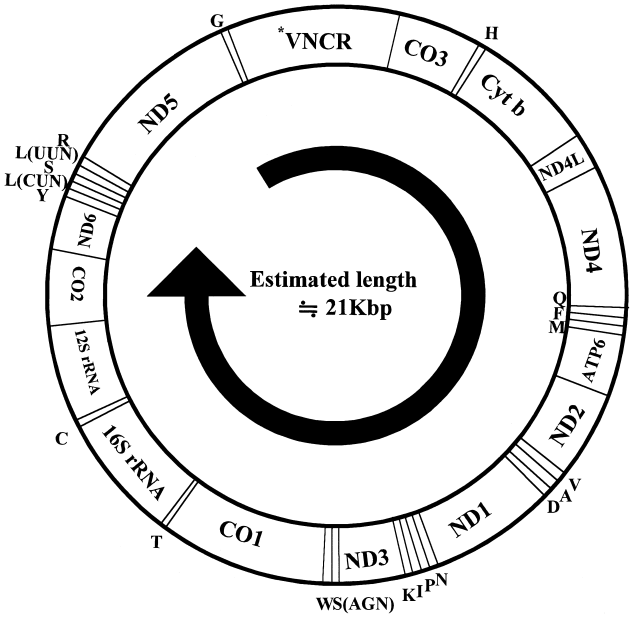

Fig. 1. Circular map of the mitochondrial genome of a lung fluke of *Paragonimus* from Leyte, the Philippines. There are 12 protein-encoding, 2 ribosomal (12S and 16S subunits) and 21 transfer RNA genes. Transfer RNA genes are described with amino acid one letter code. An arrowhead indicates putative direction for transcription. *VNCR: variable non-coding region.

shown in Fig.1, the putative mitochondrial genes included 12 subunits of mitochondrial enzymes (ATP6, CO1～3, Cytb, ND1～6, ND4L), two ribosomal RNA genes (12S rRNA and 16S rRNA) and 21 transfer RNAs (tRNA). As is the case in other trematodes, serine and leucine are each specified by two different tRNAs. No tRNA corresponding to glutamic acid (Glu: E) was found. The gene order and direction of transcription was determined by comparison with the previously reported sequences (Le *et al.,* 2000).

Sequence comparisons between Philippine (2n) and Korean (3n) forms are shown in Table1. The overall difference in DNA sequences of protein-coding genes was 12.40%, in tRNA genes 6.10% and in ribosomal RNAs, 8.03%.

The complete mitochondrial genome of *P. westermani* is estimated to be about 21Kbp (Agatsuma *et al.,* 1994). Of the 14,199 bp we sequenced, 13,196 bp coded for genes and 1,003 bp consisted of non-coding regions. The portion remaining to be sequenced is therefore about 6,800 bp and must constitute the long variable non-coding region (VNCR). Complete sequencing of the VNCR has not been possible to date, probably because of the presence of many repeats. This region corresponds to origin of replication of mitochondrial genomes in many other organisms, attracting interest as to why the length of the site in *Paragonimus* seems to be much longer than in others.

Table 1. Sequence analysis of the mitochondrial genome of a lung fluke from the Philippines, compared to a triploid type of *Paragonimus westermani* from Korea*

Protein-coding region	Number of bases	Number of substitution	Rate of difference (%)
ATP6	513	62	12.09
CO1	1,536	151	9.83
CO2	600	62	10.33
CO3	645	70	10.85
Cytb	1,119	126	11.26
ND1	891	114	12.79
ND2	867	131	15.11
ND3	357	47	13.17
ND4	1,263	180	14.25
ND4L	258	18	6.98
ND5	1,584	225	14.20
ND6	453	65	14.35
Total	10,086	1,251	12.40
ribosomalRNA			
16S rRNA	988	76	7.69
12S rRNA	744	63	8.47
Total	1,732	139	8.03

tRNA	Number of bases	Number of substitution	Rate of difference (%)
Ala	73	0	0
Arg	72	5	4.17
Asn	71	1	0
Asp	67	6	7.46
Cys	64	6	9.23
Gln	62	1	1.61
Gly	71	4	5.71
His	62	11	12.70
Ile	62	1	1.61
Leu (CUN)	62	0	1.61
Leu (UUN)	65	3	4.62
Lys	65	3	7.58
Met	66	0	0
Phe	70	6	8.57
Pro	66	10	13.43
Ser (AGN)	60	5	8.33
Ser (UCN)	65	2	3.08
Thr	66	3	4.55
Trp	64	4	6.25
Tyr	62	3	4.84
Val	63	10	15.38
Glu	Not found	Not found	Not found
Total	1,378	84	6.10

*Accession No. AF219379

As mentioned above, Miyazaki (1981) regarded the Philippine lung fluke as a subspecies of *Paragonimus westermani*, namely *P. westermani filipinus.* The justification was that one lobe of one or both testes is detached from the remainder and that the molluscan host belongs to a different family from that of *P. westermani.* Blair (2000) reviewed phylogenetic relationships among species and strains of the genus *Paragonimus* and showed *P. westermani filipinus* to be distinct from populations from Japan, Taiwan and Malaysia. His study used only 393 bp of the mitochondrial CO1 gene. Partial CO1 sequences previously deposited in the public databases showed 88.7% identity between *Paragonimus* isolates from the Philippines and Korea, suggesting that *Paragonimus* in the Philippines can be regarded as a species distinct from *P. westermani* from East Asia (China, Japan and Korea). However this suggestion was thought to be weak because such short sequence tracts were used. Our results enabled full comparison of all genes of the mitochondrial genome for the first time and found an overall identity of 88.8% between Philippines and Korean worms (Table 1).This result resembles that of the partial sequence analysis discussed above. Despite the lack of clear molecular criteria to determine species-boundaries within a genus such as *Paragonimus*, we consider that our results, and the differences in molluscan host specificity, are good evidence that the Philippine form is specifically distinct. The name *P. filipinus* Miyazaki, 1981 should be applied to it.

ACKNOWLEDGEMENTS

We would like to thank Associate professor D. Blair (James Cook University) and Associate professor Joanna D. Hare (Kochi Medical School) for reviewing the manuscript. We also would like to thank Dr. S. Habe (Fukuoka University) for collecting materials in this study. This study was supported in part by research grants (Nos. 60041052 and 61043047) under the International Scientific Research Program from the Ministry of Education, Science, Sports and Culture of Japan.

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