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Molecular evidence for the synonymy of three species of *Paragonimus*, *P. ohirai* Miyazaki, 1939, *P. iloktsuenensis* Chen, 1940 and *P. sadoensis* Miyazaki *et al.*, 1968

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Abstract

The *Paragonimus ohirai* group, named after *P. ohirai* Miyazaki, 1939, consists of three nominal species. *Paragonimus iloktsuenensis* Chen, 1940 and *P. sadoensis* Miyazaki *et al.*, 1968, the other members of the group, were proposed primarily because of perceived differences in metacercarial morphology and/or host preferences. It has long been recognized that adults of the three were virtually indistinguishable. With the application of genetic techniques, it has become clear that the three forms can exchange genes freely, and that differences in metacercarial morphology constitute a polymorphism probably due to a single gene inherited in Mendelian fashion. Here, additional genetic data (DNA sequences from the second internal transcribed spacer of the nuclear ribosomal gene cluster and from the mitochondrial cytochrome *c* oxidase subunit I gene) are presented in support of the synonymy.

Introduction

Miyazaki (1939) found metacercariae of a *Paragonimus* species in brackish water crabs of the genus *Sesarma* in Japan. The metacercarial cyst possessed a two-layered wall. Adult worms were raised experimentally in several mammal species, and the name *P. ohirai* was proposed. In the following year, Chen (1940a,b) described *P. iloktsuenensis* from rats in southern China. Adults of the two species were very similar, but there were marked differences in the metacercarial cysts with those of the latter species possessing only a single wall (see also Lou *et al.*, 1992). Metacercariae corresponding to those of Chen's species were reported from Japan by Miyazaki in 1944 and subsequently from Taiwan by Miyazaki & Chiu (1962) and Korea by Yokogawa *et al.* (1971). Yoshimura *et al.* (1969) observed different protein banding patterns between Japanese *P. ohirai* and *P. iloktsuenensis* from Taiwan. This might represent intraspecific geographic variation.

Miyazaki *et al.* (1968) described *P. sadoensis* as a new species from Sado Island, off the coast of Honshu, Japan. Prior to experimental completion of the life cycle, adult specimens of these worms had been regarded as belonging to *P. ohirai* (see Miyazaki *et al.*, 1968). Adult morphology and all life-cycle stages were very similar to those of *P. ohirai*. Specific status was inferred from differences in host specificity (snail host a freshwater pomatiopsid whereas *P. ohirai* utilized brackish water assimineids: crustacean host the freshwater species *Geothelphusa* (=*Potamon*) *dehaani* whereas *P. ohirai* utilized brackish water crabs) and structure of the cercaria, with adult and metacercarial morphology being of secondary importance. Reports of differences from *P. ohirai* in the excretory system of the cercaria were later demonstrated to be wrong (Ito *et al.*, 1969).

Adults of the three nominal species have long been recognized as very similar and their separate identity frequently called into question (see comments in

Agatsuma & Habe, 1986). Yoshimura (1969a,b) and Yoshimura *et al.* (1969, 1970c) investigated patterns of soluble whole-body proteins separated by disc electrophoresis. Whereas clearly different patterns were observed among *P. westermani*, *P. ohirai* and *P. miyazakii*, the patterns observed for *P. ohirai* and *P. sadoensis* were very similar. On this evidence, Yoshimura suggested that the Sado Island species represented a race of *P. ohirai*. Yoshimura *et al.* (1970a,b) experimentally completed the life-cycles of these two nominal species, finding that each could utilize the normal intermediate hosts of the other and that worms of both species from experimental infections produced identical electrophoretic patterns regardless of the intermediate host through which they had been passaged. Yokogawa *et al.* (1968), comparing *P. ohirai* and *P. sadoensis* using immunoelectrophoresis, found minor differences between them and stated that differentiation of the two species seemed difficult based only on this method.

Agatsuma and co-workers took up the study of Japanese populations using allozymes and breeding experiments. Agatsuma & Habe (1986) analysed 15 enzymes (18 loci) and obtained genetic distance values (Nei's *D*) among allopatric populations of the three nominal species comparable with those expected between conspecific populations and far lower than between geographical strains of *P. westermani* (see review in Blair, 1993). In Sendai, where the two forms were sympatric, *P. ohirai* and *P. iloktsuenensis* seemed to be exchanging genes freely. This was rather surprising given the considerable difference in metacercarial morphology between these species. Agatsuma & Habe (1985) set up experimental crosses among the three species and analysed two enzyme systems in the progeny. In each case, offspring of crosses between any two of the nominal species inherited alleles from both parents, confirming the absence of reproductive barriers. Similarly, experimental crosses between *P. ohirai* and *P. iloktsuenensis* (Habe *et al.*, 1985) and between *P. sadoensis* and *P. iloktsuenensis* (Habe *et al.*, 1992) demonstrated that the strikingly different metacercarial morphologies represented a polymorphism probably due to a pair of alleles at a single locus, with the *P. iloktsuenensis* type recessive.

Hirai *et al.* (1985) examined the karyotypes of a number of species of *Paragonimus*, including the three nominal species considered here. A C-band polymorphism occurred on chromosome 4 in all three, supporting the view that they are conspecific.

It is important to resolve the taxonomic puzzle presented by these species. *Paragonimus ohirai* is relatively easy to maintain in the laboratory. It is primarily a parasite of rats, and adult worms can be raised quickly in this host. Species pathogenic in humans, and in particular *P. westermani* and *P. skrjabini*, are difficult and/or expensive to maintain in the laboratory. *Paragonimus ohirai* has therefore become a valuable laboratory model for studies on paragonimiasis and needs to be characterized as fully as possible. Here, we present DNA sequences from the second internal transcribed spacer of the nuclear ribosomal gene cluster and from the mitochondrial cytochrome *c* oxidase subunit I gene in support of the synonymy of the three members of the *P. ohirai* group.

Materials and methods

All localities are in Japan. Strains of nominal *P. ohirai* used came from Kinosaki (northern coast of Hyogo Prefecture, Honshu), Tanegashima (in the Pacific Ocean, south of Kyushu) and Yakushima (in the Pacific, close to Tanegashima). Metacercariae with morphology typical of *P. ohirai* were obtained from the crab *Sesarma dehaani*. The strain of nominal *P. iloktsuenensis* (metacercariae with typical morphology from the crab species *Sesarma dehaanii*) was from Amami (an island south of Tanegashima) and of *P. sadoensis* (crab host *Geothelphusa dehaani*) from Sado Island (in the Sea of Japan, off northern Honshu). Extensive searches for other species of *Paragonimus* on Sado Island have been unsuccessful (Kawashima *et al.*, 1967). Specimens of *P. westermani* and *P. miyazaki*, used for comparative purposes, came from Hyogo (Hyogo Prefecture) and Okuyanai (Kochi Prefecture) respectively. Adult worms raised experimentally in rats, cats or dogs, were used as sources of DNA. Data for the ITS2 region was obtained from all the above strains. Partial COI sequences were obtained from all except the strain of *P. ohirai* from Yakushima.

DNA extraction and purification of mtDNA were as described previously (Agatsuma *et al.*, 1994). A single worm was used from each locality. Gene regions were amplified using the polymerase chain reaction (PCR). For the COI region, the primers used were as in Bowles *et al.* (1993). For the ITS2, primers used were BD2 and 3S (Bowles *et al.*, 1995). An additional primer, A28 (5′ GGGATCCTGGTTAGTTTCTTTTCCTCCGC 3′), was sometimes used instead of BD2.

All sequences were determined directly from the PCR products. Cycle sequencing reactions were run on an ABI 373A automated sequencer. PCR primers were used as sequencing primers.

For the COI region, published sequence for *Fasciola hepatica* was used for comparison (Garey & Wolstenholme, 1989). Codon usage was derived from the same source, except that the codon ATA was translated to I rather than M (Bowles *et al.*, 1992) and AAA translated to N rather than K (Ohama *et al.*, 1990). A tree showing relationships among the species studied was constructed using a distance matrix approach in TREECON (Van De Peer & De Wachter, 1993).

Results

The new data we present are ITS2 sequences and COI sequences. The COI alignment (fig. 1) is 393 bases long. Alignment was straightforward. The outgroup, *Fasciola hepatica*, has an insertion of one codon (3 nt) relative to all the *Paragonimus* species. This insertion was not included in any of the calculations. Among members of the *P. ohirai* group, a maximum of two nucleotide, but no amino acid, differences were noted (table 1). Members of the *P. ohirai* group differed from *P. miyazakii* at 50–52 nucleotide sites, but only at one amino acid site. Corresponding figures for differences from *P. westermani* are 79–81 nucleotide sites and one amino acid site (table 1). *Fasciola hepatica* differed from *P. ohirai* at only a slightly greater number of nucleotide sites, but at many amino acid sites. Figure 2 presents these differences graphically. The tree was

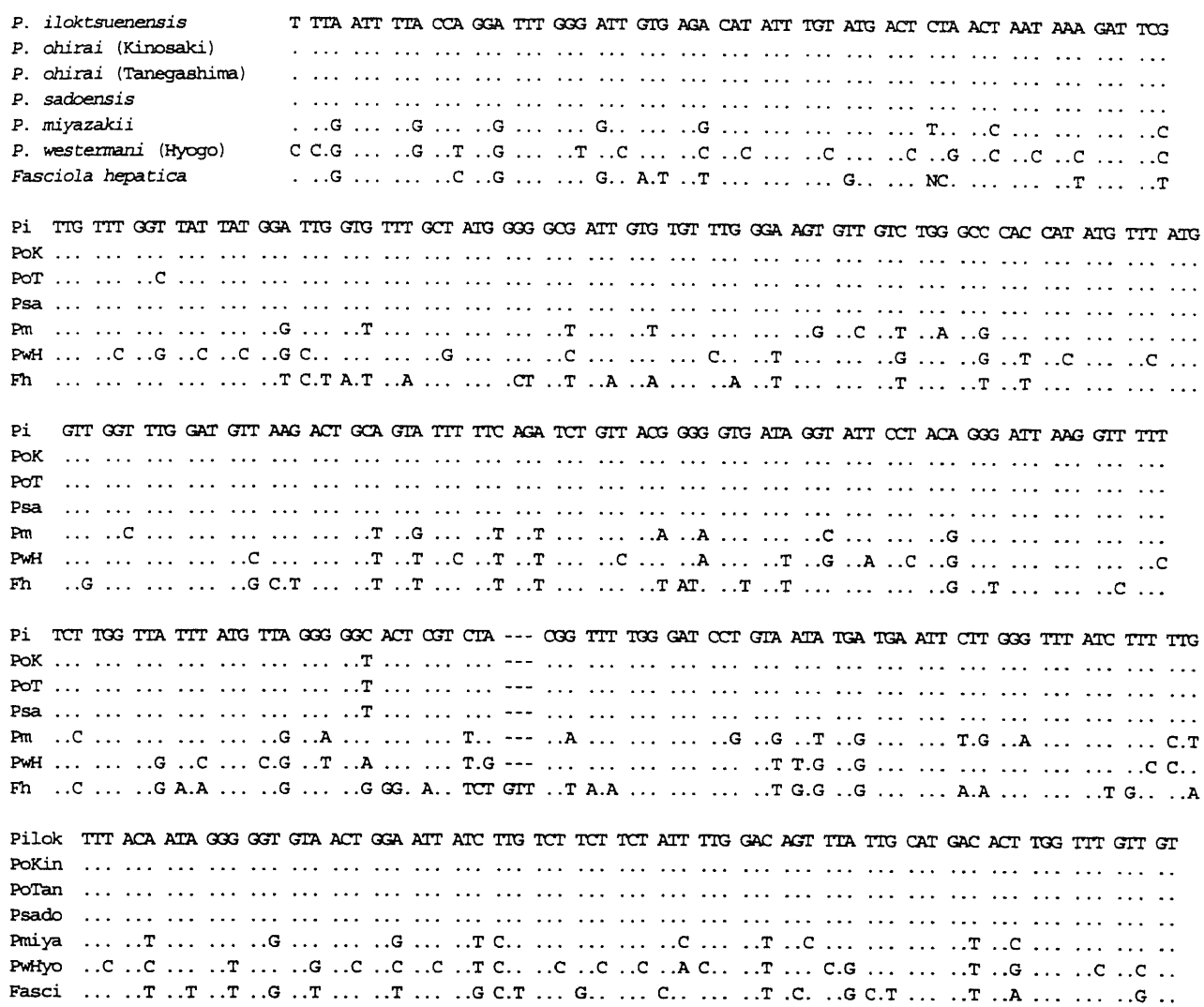

Fig. 1. Alignment of mitochondrial cytochrome *c* oxidase subunit I sequences (GenBank numbers U97205, U97214, U97215, AF008188-AF008190) showing codons. A dot "." indicates identity with base on top line. Alignment gaps are indicated by "-".

inferred using the COI nucleotide alignment and shows that the *P. ohirai* group forms a tight cluster.

The alignment of ITS2 sequences (fig. 3) is 363 bases long. Again, alignment was straightforward. *Paragonimus miyazakii* exhibited a deletion, 2 nt long, not found in the other species. The ITS2 sequences are identical among all isolates in the *P. ohirai* group but differ at 29 and 35 sites from *P. miyazakii* and *P. westermani* respectively

Table 1. Nucleotide (above diagonal) and amino acid (below diagonal) differences in COI region among *Paragonimus* species.

	P.i.	*P.o.* (K)	*P.o.* (T)	*P.s.*	*P.m.*	*P.w.*	*Fasc.*
P.i.	–	1	2	1	50	81	87
P.o. (K)	0	–	1	0	51	81	87
P.o. (T)	0	0	–	1	52	81	88
P.s.	0	0	0	–	51	81	87
P.m.	1	1	1	1	–	79	83
P.w.	1	1	1	1	2	–	105
Fasciola	21	21	21	21	20	21	–

P.i., P. iloktsuenensis; *P.o.* (K), *P. ohirai* from Kinosaki; *P.o.* (T), *P. ohirai* from Tanegashima; *P.s., P. sadoensis*; *P.m., P. miyazakii*; *P.w., P. westermani*.

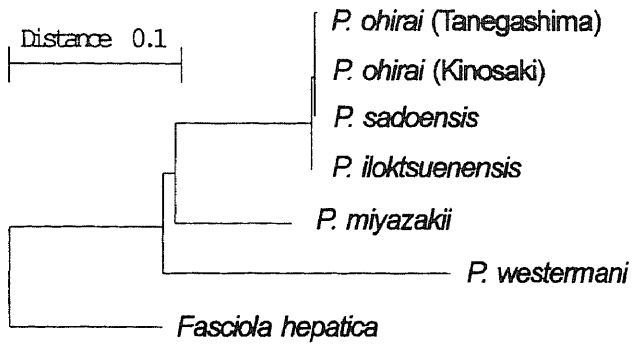

Fig. 2. Tree depicting relationships among *Paragonimus* species inferred from COI data. A distance matrix was calculated using the Kimura 2-parameter model and the tree constructed using the neighbour-joining approach in TREECON.

(table 2). Alignment with the ITS2 of *Fasciola hepatica* is impossible.

Discussion

Intraspecific variation in ITS2 sequences is virtually unknown among trematodes. Where different nominal species exhibit identical ITS2 sequences, gene exchange between them can often be demonstrated. For example, within the *Schistosoma haematobium* group, *S. intercalatum*, *S. bovis* and *S. curassoni* have identical ITS2 sequences (Després *et al.*, 1992). At least the last two of these species are capable of producing viable hybrids (Rollinson *et al.*, 1990), and gene exchange between them, along with concerted evolution, might explain the identity of sequences observed. In the genus *Echinostoma*, *E. caproni* and *E. liei* have identical ITS2 sequences (Morgan & Blair, 1995). These species are usually regarded as synonymous and can produce viable hybrids.

That members of the *P. ohirai* group have identical ITS2 sequences is therefore an indication that they are

Table 2. Nucleotide differences in ITS2 among *Paragonimus* species.

	P.o. (K)	*P.o.* (T)	*P.o.* (Y)	*P.s.*	*P.m.*	*P.w.*
P.i.	0	0	0	0	29*	35
P.o. (K)		0	0	0	29*	35
P.o. (T)			0	0	29*	35
P.o. (Y)				0	29*	35
P.s.					29*	35
P.w.						26*

*Includes a deletion 2 nt long.
P.i., P. iloktsuenensis; P.o. (K), *P. ohirai* from Kinosaki; *P.o.* (T), *P. ohirai* from Tanegashima; *P.o.* (Y), *P. ohirai* from Yakushima; *P.s., P. sadoensis; P.m., P. miyazakii; P.w., P. westermani.*

conspecific or at least capable of exchanging genes. The group stands out as very distinct from other members of the genus in ITS2 sequence (table 2). A similar situation occurs with the COI sequences. The very small amount of nucleotide variation among members of the *P. ohirai* group, all of it synonymous (no amino acid changes), is dwarfed by the magnitude of differences between this group and the other taxa. Differences among amino acid sequences within the *Paragonimus* species used here are few (maximum two) compared with the 20–21 differences observed between *Paragonimus* spp. and *Fasciola hepatica*. This is despite the fact that *F. hepatica* differs from *Paragonimus* species at only a few more nucleotide sites than species of *Paragonimus* differ among themselves. The sequences may be approaching saturation at synonymous sites within the genus *Paragonimus*.

Members of the *Paragonimus ohirai* group can utilize snails of two different families (Davis *et al.*, 1994), and a number of species of crabs. The original distinction between *P. sadoensis* and *P. ohirai* was based largely on the occurrence of the former in freshwater (as opposed to brackish water) molluscs and crustaceans. However, experimental infections show that Japanese populations of *P. ohirai* and *P. sadoensis* can infect each other's snail and crustacean host species (Yoshimura *et al.*, 1970a,b).

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P. ohirai                ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCGCGACGCCCAAAA
P. miyazakii             ....................................................................
P. westermani (Hyogo)    ....................................................................

Po    AGTCGCGGCTTGGGTTTTGCCAGCTGGCGTGATTTCCCCAATCTGACCATGTGTTGGTGGGGTGCCAGATCTATGGCGTTTCCCTAACCTATCCGGG
Pm    ..........................................C...G..TC...GCT.............G.............A.......
PwHyo ..............................C...........GT.T....CCT.............G.............A..CT....

Po    CGTACCCATGTTGTGGCTGAAGGCCTTGGTGGGGATGTGGCAACGGAATCGTGGCTCAGTGGATTATTTATGTGCGCGTTCCGTTGTCCTATCATCA
Pm    ......................A......A.....................................AT.G....--.............C.A.........G
PwHyo ..C.....C....C.......A......AC.....................................A..G.................C......G..T...

Po    TCTATGGTTGATGCTGCGCATGGTGTGCGTCCGACGCCAACCTACGTATGGGCCGTGTGGCTCATTCTCCTGACCTCGGATCAGACGTGAGTACC
Pm    ............T.....G.......T.C....T..TG.....T.....T...A.....................................N.......
PwHyo ...G.....T...T.....G....C...T.T...T..TG...........T...A.....T.....................................
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Fig. 3. Alignment of ITS2 sequences (GenBank numbers U96907, U96911, U96912). All members of the *P. ohirai* group were identical. A dot "." indicates identity with base on top line. Alignment gaps are indicated by "-".

Paragonimus iloktsuenensis populations from China (Chen 1940a,b), Japan (see Yoshida & Kawashima 1961; Sato *et al.*, 1969) and Korea (see Yokogawa *et al.*, 1971; Seo *et al.*, 1977) utilize assimineid snails and crustaceans associated with lower reaches of rivers and brackish water. However, the Taiwan population of this nominal species occurs in pomatiopsid snails and freshwater crustaceans which are taxonomically and ecologically close to the hosts of *P. sadoensis* in Japan (Miyazaki & Chiu, 1962).

We conclude that *P. iloktsuenensis* and *P. sadoensis* should be regarded as junior synonyms of *P. ohirai*.

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