

# A molecular perspective on the genera *Paragonimus* Braun, *Euparagonimus* Chen and *Pagumogonimus* Chen

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## Abstract

The status of the genera *Euparagonimus* Chen, 1963 and *Pagumogonimus* Chen, 1963 relative to *Paragonimus* Braun, 1899 was investigated using DNA sequences from the mitochondrial cytochrome *c* oxidase subunit I (CO1) gene (partial) and the nuclear ribosomal DNA second internal transcribed spacer (ITS2). In the phylogenetic trees constructed, the genus *Pagumogonimus* is clearly not monophyletic and therefore not a natural taxon. Indeed, the type species of *Pagumogonimus*, *P. skrjabini* from China, is very closely related to *Paragonimus miyazakii* from Japan. The status of *Euparagonimus* is less obvious. *Euparagonimus cenocopiosus* lies distant from other lungflukes included in the analysis. It can be placed as sister to *Paragonimus* in some analyses and falls within the genus in others. A recently published morphological study placed *E. cenocopiosus* within the genus *Paragonimus* and probably this is where it should remain.

## Introduction

Lung flukes (family Paragonimidae Dollfus, 1939) occur as adults in the lungs of mammals in Asia, Africa and the Americas (reviewed in Blair *et al.*, 1999). Snail hosts occur in fresh and brackish waters. Mammals are usually infected by eating freshwater crustaceans containing metacercariae. Several species of lungflukes infect humans in various parts of the world. The taxonomy of the family presents some problems. At least fifty species

have been named, about half of these from China alone. Several generic groupings have been proposed for the family.

The type genus, *Paragonimus* Braun, 1899, was erected to contain *P. westermanni* (Kerbert, 1878), the human lung fluke from Asia. In the 1960s, Chen, working in China, proposed two additional genera. For *Paragonimus skrjabini* Chen, 1959 from Guangdong Province, he erected the genus *Pagumogonimus* Chen, 1963. Chen (1963, 1964a,b) distinguished *Pagumogonimus* from *Paragonimus* primarily by the fact that the metacercaria of the former possessed 72 flame cells (60 in *Paragonimus*). Later (Chen, 1977), he placed more emphasis on adult characters and in

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particular the elongate body shape and the location of the ventral sucker in the anterior one-third of the body. On the basis of these adult characters, he (1977) added to *Pagumogonimus* the species *P. yunnanensis*, *P. harinasutai*, *P. proliferus*, *P. bangkokensis*, *P. cheni* and *P. macrorchis*. Of these, at least two species, *P. macrorchis* and *P. cheni* have metacercariae with 60 flame cells. Chen was the editor of a monograph on paragonimids published in 1985. In the introductory section of this, he provided a key distinguishing between *Paragonimus* and *Pagumogonimus* solely on the basis of the adult characters mentioned above. The name *Pagumogonimus* continues to be used by Chinese workers but tends not to be used by taxonomists outside China (e.g. Miyazaki, 1974; Kurochkin, 1987) who place all species in the genus *Paragonimus*.

The genus *Euparagonimus* was erected by Chen (1963) for *E. cenocopiosus* Chen, 1962 from Guangdong Province. Metacercariae, juvenile and adult worms all possess an excretory bladder extending anteriorly only as far as the ventral sucker (Chen, 1962, 1965). This is a very distinctive feature. The excretory bladder in *Paragonimus* species extends to well in front of the ventral sucker. Apart from this, there are no major differences between *Euparagonimus* and *Paragonimus*. The genus was placed in a new subfamily, Euparagoniminae, by Chen in 1963. A second species, *E. hongziesiensis*, was described by Fu *et al.* (1990) from Jiangsu Province.

Most authors have retained *Euparagonimus* as a genus distinct from *Paragonimus*. There has been little critical discussion. Zhong *et al.* (1981) commented on some apparently unpublished work by Liu in saying 'Recently, Liu *et al.* (July 1980) have shown that the naming of *Euparagonimus cenocopiosus* Chen, 1962 as a new subfamily and new genus was erroneous. Liu *et al.* (July 1980) have found evidence to designate it *Paragonimus brevivesicarius*'. The specific name, *brevivesicarius*, clearly cannot be used even if the species is transferred to the genus *Paragonimus*: the name *cenocopiosus* has priority. Apart from this, the genus *Euparagonimus* has been retained as a

distinct entity in the literature. Metacercariae resembling those of *Euparagonimus* have been found in the Americas and Africa (Miyazaki *et al.*, 1980; Ollivier *et al.*, 1995).

Chen (1963) proposed two new subgenera within *Paragonimus*. These he called *Rodentigonimus* and *Megagonimus*. Later he proposed (Chen, 1985) to abandon the use of these names. They will not be considered further here.

In this study we investigate the relationships of *Pagumogonimus* and *Euparagonimus* to *Paragonimus* using DNA sequence data. We chose partial mitochondrial cytochrome c oxidase subunit I (COI) and complete nuclear rDNA internal transcribed spacer 2 (ITS2) gene.

## Materials and methods

Many sequences used in this study have been reported elsewhere (Blair *et al.*, 1997, 1998) and are given in table 1. Representatives of the nominal genus *Pagumogonimus* were *P. skrjabini* (Chen, 1959) from China and *P. macrorchis* (Chen, 1962) from Thailand. Sequences of these species were reported in Blair *et al.* (1998). The specimens of *P. mexicanus* sequenced were raised to adulthood in experimental hosts using metacercariae collected from freshwater crabs (*Hypolobocera* sp.) from El Cañero River, La Concordia, Quinindé, Esmeraldas Province, Ecuador and prepared by J.C. Vieira. Metacercariae of *E. cenocopiosus* were collected from the heart of the freshwater crab *Sinopotamon fujianense* (identification by Fujian Institute of Parasitic Diseases) from small streams in Qujiang Administrative Village, Shuyang Township, Nanjing County in southern Fujian Province, China and from East Chenyang Stream, Chendong Natural Village, Chenyang Administrative Village, Langu Township, Wuyishan City in the north of the same Province. The two localities are about 350 km apart. Adult worms were raised in experimentally infected dogs and frozen at  $-70^{\circ}\text{C}$  until required for PCR and sequencing. These adult worms were identified morphologically as *E. cenocopiosus* according to Chen & He (1965). Slide-mounted specimens

Table 1. Sources of material and published partial COI and ITS2 gene sequences for members of the nominal genera *Paragonimus* (*Pa.*), *Pagumogonimus* (*Pg.*) and *Euparagonimus*.

Species	Origin	ITS2	COI
<i>Pa. westermani</i>	Hyogo, Japan	U96907	U97205
	Leyte, Philippines	U96910	U97213
	Thailand	AF159604	U97212
	Malaysia	U96909	U97211
<i>Pa. siamensis</i>	Thailand	AF159605	AF159599
<i>Pa. heterotremus</i>	Thailand	AF159603	AF159597
<i>Pa. harinasutai</i>	Thailand	AF159609	AF159600
<i>Pa. miyazakii</i>	Miyazaki, Japan	U96912	U97215
<i>Pa. ohirai</i>	Tanegashima, Japan	U96911	U97214
<i>Pa. mexicanus</i>	Ecuador	AF159607	AF159596
<i>Pa. kellicotti</i>	USA	AF159606	–
<i>Pg. skrjabini</i>	Sichuan, China	U96913	U97216
<i>Pg. macrorchis</i>	Thailand	AF159608	AF159598
<i>E. cenocopiosus</i>	Nanjing, Fujian, China	AF159601	AF159595
<i>E. cenocopiosus</i>	Wuyishan, Fujian, China	AF159602	AF159594
<i>Fasciola hepatica</i>	USA	–	M93388

Table 2. Pairwise differences among CO1 nucleotide sequences (ignoring an insertion three nucleotides long in *Fasciola hepatica*). Values above the diagonal are transitions/transversions. Those below are amino acid differences. Abbreviations of generic names as in table 1.

Species	Origin	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 <i>Pa. westermani</i>	Japan	-	37/3	36/3	40/5	48/14	51/23	51/26	51/28	55/26	62/21	54/29	52/26	39/28	41/26	56/47
2 <i>Pa. westermani</i>	Malaysia	1	-	28/4	29/4	47/17	44/23	44/23	44/25	44/23	55/22	41/28	43/25	33/29	34/27	48/48
3 <i>Pa. westermani</i>	Philippines	1	1	-	23/2	38/17	37/24	37/25	40/27	38/25	36/22	37/28	40/27	26/27	26/25	36/48
4 <i>Pa. westermani</i>	Thailand	1	1	0	-	40/17	42/24	44/23	45/27	46/25	43/22	44/30	46/27	36/27	37/25	49/48
5 <i>Pa. siamensis</i>	Thailand	3	2	3	3	-	38/27	33/32	33/13	34/30	34/25	32/31	30/32	32/30	34/28	32/47
6 <i>Pa. heterotremus</i>	Thailand	2	3	3	3	5	-	22/25	33/18	35/23	29/14	34/14	30/15	33/23	33/21	33/46
7 <i>Pa. harrinasutai</i>	Thailand	2	3	3	3	5	1	-	34/18	39/10	32/23	29/23	31/28	33/24	35/22	31/51
8 <i>Pa. miyazaki</i>	Japan	2	3	3	3	5	1	0	-	38/14	38/15	30/5	33/14	36/26	37/24	30/51
9 <i>Pa. ohirai</i>	Japan	1	2	2	2	4	2	1	1	-	35/21	30/17	30/24	38/22	38/20	33/53
10 <i>Pa. mexicanus</i>	Ecuador	2	3	3	3	5	0	1	1	2	-	34/18	31/19	37/21	37/21	27/46
11 <i>Pg. skrjabini</i>	China	3	2	3	3	4	2	1	1	2	2	-	23/17	36/27	36/25	25/50
12 <i>Pg. macrorchis</i>	Thailand	2	3	3	3	5	1	0	0	1	1	1	-	31/28	33/26	31/47
13 <i>E. cenocopiosus</i>	Wuyishan, China	3	4	4	4	6	1	2	2	3	1	3	2	-	2/2	39/45
14 <i>E. cenocopiosus</i>	Nanjing, China	3	4	4	4	6	1	2	2	3	1	3	2	0	-	40/43
15 <i>F. hepatica</i>	USA	21	21	21	21	20	19	21	20	21	19	20	20	20	20	-

of *E. cenocopiosus* are held at the Institute for Parasitic Diseases, Shanghai.

Total genomic DNA was prepared from frozen adult worms using standard extraction techniques. A single adult worm was used in each case. The two gene regions were amplified by the polymerase chain reaction (PCR) from 20~50 ng of worm DNA. For the CO1 region, the primers used were JB3 and JB4.5 as described by Bowles *et al.* (1993). For the ITS2, primers used were 3S (Bowles *et al.*, 1995) and A28 (Blair *et al.*, 1997). All sequence data from adult worms were determined directly from the PCR products. Cycle sequencing reactions were run on a Licor automated sequencer twice in both directions to confirm the sequence. In the case of *P. mexicanus*, sequencing reaction products were separated on an ABI 373 automated sequencer. PCR primers were used as sequencing primers (after fluorescent-labelling if appropriate).

Sequence alignments were performed manually using the sequence editor ESEE3 (Cabot & Beckenbach, 1989). Codon usage (CO1 gene) was derived from Garey & Wolstenholme (1989), except that the codon ATA was translated to isoleucine rather than methionine (Bowles *et al.*, 1992) and AAA was translated to asparagine rather than lysine (Ohama *et al.*, 1990). Phylogenetic trees were constructed in PAUP 3.1 using heuristic searches. Bootstrapping was done (1000 resamplings). For the CO1 data set, a sequence from *Fasciola hepatica* was used as an outgroup. For the ITS2 data set, no suitable outgroup was available. The trees inferred from ITS2 data were therefore rooted at the midpoint. Numbers of transitions, transversions and amino acid differences in pairwise comparisons among CO1 sequences and numbers of nucleotide differences in pairwise comparison among ITS2 sequences were calculated in MEGA (Kumar *et al.*, 1993). Insertions and deletions were omitted from calculations.

### Results and discussion

The CO1 alignment was 393 bases long including an insertion of one codon (3nt) in the outgroup, *F. hepatica*, relative to all the *Paragonimus* species/strains. Table 2 gives additional information about the CO1 sequences. The single most-parsimonious tree constructed using all sites in the alignment had a length of 431, a consistency index of 0.543 (0.494 when uninformative characters were omitted). The same tree topology was found by bootstrapping, and the bootstrap values are shown on the tree (fig. 1, right hand side). The third codon position is expected to be the most variable in a protein coding gene. Removal of all third codon positions from the analysis might increase the consistency index. When this was done, 30 trees of length 58 and consistency index of 0.69 were found. The strict consensus tree collapsed all branches indicated by an asterisk in fig. 1. This indicates that much of the phylogenetic information is contained in sites at third codon positions. PAUP found 3 equally most parsimonious trees (length 27 and consistency index 0.963) using inferred amino acid sequences. The strict consensus tree derived from these collapsed many internal branches among the lungflukes and therefore provided little information about relationships among



Sequences from the sole representative of *Euparagonimus*, *E. cenocopiosus*, were rather distant from all other lung flukes in the trees in fig. 1. However, in all trees, the *Euparagonimus* sequences are nested among the lungflukes in a way that would render *Paragonimus* paraphyletic if *Euparagonimus* were to be recognized as a distinct taxon. Although the metacercariae and adults of *Euparagonimus* differ from *Paragonimus* species in the length of the excretory bladder, their anatomy does not otherwise set them apart from *Paragonimus*. Indeed, in a recent morphometric analysis of lungflukes (Zhan *et al.*, 1997), *E. cenocopiosus* was nested among species assigned to the genera *Paragonimus* and *Pagumogonimus*. It seems that this species should not be placed in a distinct genus.

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