Mitochondrial DNA sequence variation among geographical isolates of *Opisthorchis viverrini* in Thailand and Lao PDR, and phylogenetic relationships with other trematodes

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(*Received 5 July 2008; revised 25 August 2008; accepted 25 August 2008*)

SUMMARY

The present study compared the genetic variation among 14 different geographical isolates of *Opisthorchis viverrini sensu lato* from Thailand and Lao PDR using sequence data for 2 mitochondrial DNA genes, the subunit 1 of NADH dehydrogenase gene (*nad1*) and cytochrome c oxidase gene (*cox1*). Four different *nad1* haplotypes were detected among isolates, all of which were identical at the amino acid sequence level. Nucleotide sequence variation among 14 isolates ranged from 0 to 0·3% for *nad1*. Two different *cox1* haplotypes were detected among isolates. These two haplotypes differed at 2 nucleotide positions, one of which resulted in a change in the amino acid sequence. Nucleotide sequence variation among isolates for *cox1* ranged from 0 to 0·5%. Comparison of *cox1* sequences of *O. viverrini* to those of other trematodes revealed nucleotide differences of 13–31%. A phylogenetic analysis of the *cox1* sequence data revealed strong statistical support for a clade containing *O. viverrini* and 2 other species of opisthorchid trematodes; *O. felineus* and *Clonorchis sinsensis*.

Key words: *Opisthorchis viverrini*, trematodes, genetic variation, mitochondrial DNA, NADH dehydrogenase subunit 1 gene, cytochrome *c* oxidase subunit 1 gene, phylogenetic relationships.

INTRODUCTION

Fish-borne trematodiasis is becoming increasingly recognized as a serious public health problem in Southeast Asia. Three species within the family Opisthorchidae most commonly implicated as aetiological agents are *Opisthorchis viverrini, Opisthorchis felineus* and *Clonorchis sinensis*. These parasites infect at least 40 million people within a disease risk region estimated to include 700 million people (WHO, 1995; Keiser and Utzinger, 2005).

Of these three species of opisthorchid trematode, only *O. viverrini* is classified as a type 1 carcinogen because of its role as an initiator of chronic inflammation and the subsequent development of cholangiocarcinoma (CCA) (IARC, 1994; Sithithaworn *et al*. 1994; Honjo *et al*. 2005). This liver fluke is most commonly found in Southeast Asia (WHO, 1995). An estimated 9 million people are infected in Thailand with the region of highest endemicity being in the north and northeast (Sithithaworn and Haswell-Elkins, 2003). There is considerable variation, however, in parasite prevalence and disease presentation in different geographical areas (Sithithaworn and Haswell-Elkins, 2003; Sriamporn *et al*. 2004), the latter of which may be associated with genetic differences among parasites (Saijuntha *et al*. 2007).

Currently, there is inadequate information on the level of genetic variation within and among populations of *O. viverrini* throughout its geographical distribution. Previous studies (Ando *et al*. 2001; Saijuntha *et al*. 2006*a*, *b*; 2007; Sithithaworn *et al*. 2007) have reported genetic variation among isolates of *O. viverrini* from Thailand and the Lao PDR. These studies have been based on sequence analyses of the mitochondrial (mt) DNA cytochrome *c* oxidase subunit 1 gene (*cox1*) (Ando *et al*. 2001), RAPD analyses (Sithithaworn *et al*. 2007) and multilocus enzyme electrophoresis analyses (Saijuntha *et al*. 2006*a*, *b*, 2007). The findings of these studies have

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Parasitology (2008), **135**, 1479–1486.
doi:10.1017/S0031182008005015 Printed in the United Kingdom

suggested that there is population substructuring among *O. viverrini* populations from different geographical areas. Furthermore, Saijuntha *et al.* (2007) demonstrated that *O. viverrini* represents species complex of at least 2 species. Nonetheless, more DNA-based markers need to be established to determine the magnitude of genetic variation among populations of this liver fluke throughout different parts of its distributional range.

Mitochondrial DNA sequence data have been used widely to examine the population genetic structures of animals, including parasitic platyhelminths (e.g. McManus and Bowles, 1996; Le *et al.* 2000), because of the apparent maternal mode of inheritance and high mutation rates. Such sequences have proven useful for the analysis of within-species variation in parasitic flatworms, such as *Fasciola hepatica* (see Semyenova *et al.* 2006), *Paragonimus westermani* (Park *et al.* 2003), *Clonorchis sinensis* (see Lee and Huh, 2004; Park, 2007) and *Echinococus granulosus* (see Bowles *et al.* 1992, 1994; Maillard *et al.* 2007). For example, *cox1* and the NADH dehydrogenase subunit 1 gene (*nad1*) have been shown to be useful population genetic markers for *Schistosoma japonicum* (see Bowles *et al.* 1993; Sorensen *et al.* 1998). Bowles *et al.* (1993) examined the degree of genetic divergence in *cox1* between *S. japonicum* isolates from China and the Philippines using sequence data of *cox1*, whereas Sorensen *et al.* (1998) compared the level of genetic variation among populations of *S. japonicum* from 6 different geographical regions in mainland China. Morgan and Blair (1998) have also shown the utility of the *nad1* gene sequences for species identification and for comparing the rates of genetic divergence strains of echinostomes. Thus, *cox1* and *nad1* are of potential use to examine genetic variation among geographical isolates of *O. viverrini*.

In the present study, we investigated the level of genetic variation among isolates of *O. viverrini* from 14 different geographical areas in Thailand and the Lao PDR using *cox1* and *nad1* sequence data. In addition, we determined the phylogenetic relationships of *O. viverrini* to other trematodes based on analyses of sequence data of the *cox1* gene.

MATERIALS AND METHODS

Collection of adult O. viverrini

Metacercariae of *O. viverrini* were obtained from infected cyprinid fish from 14 different geographical localities within Thailand and the Lao PDR (Fig. 1 and Table 1). Fish were mixed and digested with 0·3% pepsin solution. Metacercariae were identified using a light microscope and 50–100 metacercariae were used to infect hamsters. After 4–6 months, adult worms were collected from the bile duct, identified using morphological characters, then washed extensively in physiological saline, placed in pools ($\geqslant$20 worms) into microcentrifuge tubes, frozen live and stored at −80 °C until used for the molecular analyses.

Molecular methods

Pools of adult worms ($\geqslant$20 worms) were crushed using a tissue grinder. Lysis buffer and proteinase K (200 μg/ml) were added to each homogenized sample. Genomic DNA (gDNA) was extracted using the phenol/chloroform method described by Sambrook *et al.* (1989). Part of the *nad1* gene was amplified by polymerase chain reaction (PCR) using the forward primer MNDI-A (5′-TAC GCA GGT GGT TTG GTT GG-3′) and the reverse primer MNDI-B (5′-CCC AAA GCT CAC ATC CTT GT-3′). These primers were designed based on a *nad1* sequence for *O. viverrini* (GenBank Accession number DQ119551). Part of the *cox1* gene was amplified by PCR using the forward primer MCOI-A (5′-TTT TTT GGG CAT CCT GAG GTT TA-3′) and the reverse primer MCOI-B (5′-TAA AGA AAG AAC ATA ATG AAA ATG AGC-3′) (Ando *et al.* 2001). PCR reactions were conducted in a total volume of 25 μl containing 25 mM $MgCl_2$, 10 × buffer, 2·5 mM dNTPs, 25 pmol of each primer, 0·15 U of Go *Taq* DNA polymerase (Promega) and 100–300 ng of gDNA. No gDNA (i.e. negative) controls were included in each run. PCR reactions were performed using a MyCycler Thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The cycling conditions used were: an initial denaturing cycle of 5 min at 94 °C, then 30 cycles of 94 °C for 1 min, 52 °C (for *nad1*) or 50 °C (for *cox1*) for 1 min and 72 °C for 2 min. Amplicons were compared electrophoretically on 1·5% agarose gels (Sambrook *et al.* 1989) stained with ethidium bromide and visualized using UV illumination. Specific bands were excised from agarose gels, purified using the UltraClean PCR kit (Mo-Bio Laboratories) and subjected to automated DNA sequencing using the same primers (in separate reactions) as employed for the primary PCR.

Data analysis

The nucleotide sequences of the *nad1* and *cox1* for *O. viverrini* obtained during this study have been deposited in the GenBank databases (Accession numbers EU022337-EU022350 and EU022351-EU022364, respectively). Nucleotide sequences were translated into amino acid sequence using Translation Tool 1.3 (http://www.bioinformatics.vg/bioinformatics_tools/tranlatetool.shtml), employing the codon table of invertebrate mitochondrial code. BioEdit version 5.0.6 was used for both nucleotide and amino acid sequence alignments. In addition, included in the *nad1* alignment were sequence data

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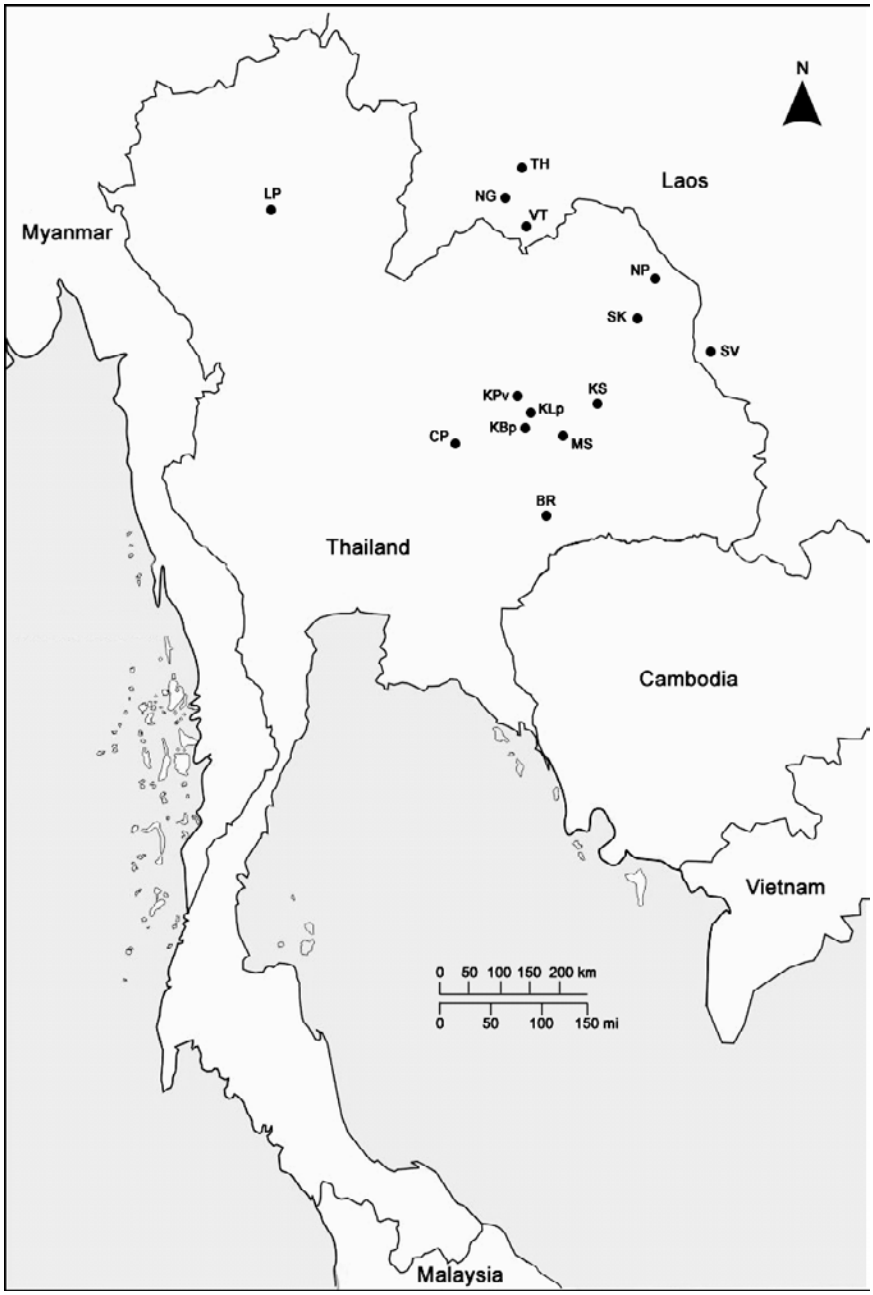

Fig. 1. Localities in Thailand and the Lao PDR where *Opisthorchis viverrini* were collected (see Table 1 for the details and abbreviations of each locality).

for 5 isolates of *O. viverrini* retrieved from GenBank: Isolate OVN3 (Accession number DQ119551) from Khon Kaen, Thailand; OVBD (Accession number DQ882172) from Binh Dinh, Vietnam; isolate OVDL3 (Accession number DQ882174) from Dak Lak, Vietnam; isolate OVPY3 (Accession number DQ882173) from Phu Yen, Vietnam; and isolate OVL (Accession number DQ882175) from Vientiane, Laos. Two species of *Taenia* (Accession numbers NC_004826 and AF338826) and *Ascaris suum* (Accession number NC_001327) were included as outgroups. Also included in the *cox1* alignment were publicly available sequence data for other trematodes, including *O. felineus* (Accession numbers DQ469316 and DQ469317), *Clonorchis sinensis* (Accession numbers AF181889 and AF188122), 2 species of *Metagonimus* (Accession numbers AF096231 and AF096230), 8 species of *Schistosoma* (Accession numbers NC_002544, NC_002529, AF295106, AF101196, NC_008074, DQ157223, AJ519521 and AY157201), 3 species of *Echinostoma* (Accession numbers AF025829, AF025824 and AF025823), 2 species of *Fasciola* (Accession numbers AB207180 and AF216697), *Fasciolopsis buski* (Accession number EF027094) and 7 species of *Paragonimus* (Accession numbers NC_002354, AY618842, AF538944, AY618838, AY618834, AY618806 and AF159599). Sequences of *cox1* for the nematode *A. suum* (Accession number NC_001327) and 5 species of taeniid cestode (Accession numbers

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Table 1. Details of the source of the 14 isolates of *Opisthorchis viverrini* used in this study

Code	Locality	Village/District	Province	Country
KLp	Prakeu Stream	Ban Lerngpleuy/Maung	Khon Kaen	Thailand
KBp	Kang Lawa Reservoir	Lawa/Ban Phai	Khon Kaen	Thailand
KPv	Ubonrattana Dam	Phuvaing	Khon Kaen	Thailand
CP	Godtom Reservoir	Nong Bau Dang	Chaiya Phum	Thailand
MS	Chi River	Din Dam/Maung	Mahasarakham	Thailand
KS	Lampao Dam	Maung	Kalasin	Thailand
LP	Kil Lom Dam	Maung	Lampang	Thailand
BR	Jawrakhae Mak Reservoir	Maung	Buri Ram	Thailand
SK	Nong Harn Reservoir	Nong Harn	Sakon Nakhon	Thailand
NP	Songkram River	Maung	Nakhon Phanom	Thailand
NG	Nam Ngum Dam	Nam Ngum	Vientiane	Lao PDR
VT	Nam Ngum Dam	Kampang Nakhon	Vientiane	Lao PDR
TH	Nam Ngum Dam	Tha Heur	Vientiane	Lao PDR
SV	Se Bang Heang River	Kaisorn	Savannakhet	Lao PDR

Table 2. Nucleotide and amino acid sequence differences in the *nad1* and *cox1* among haplotypes representing different isolates of *Opisthorchis viverrini*

	nad1													*cox1*		
	DNA[2]									Amino acid[3]				DNA[2]		Amino acid[3]
Genotypes[1]	41	91	112	119	434	470	543	608	638	30	37	181	Genotypes[1]	329	370	123
OVN1	T	T	G	G	T	C	C	C	A	M	G	L	OVC1	T	C	V
OVN2	T	T	G	G	T	T	C	C	A	M	G	L	OVC2	C	T	A
OVN3	T	T	G	G	C	T	C	C	A	M	G	L				
OVN4	T	T	G	G	C	C	C	C	A	M	G	L				
OVBD	T	C	T	G	C	T	T	C	A	T	V	F				
OVDL3	C	T	T	A	T	C	C	C	C	M	V	L				
OVL	T	T	T	G	C	C	T	C	A	M	V	F				
OVPY3	C	T	T	G	T	T	C	T	A	M	V	L				

[1] Genotypes OVN1 = isolated from CP, KPv, NG, TH, VT and SV; OVN2 = KS, BR, NP and LP; OVN3 = KLp, SK and DQ119551; OVN4 = KBp and MS; OVBD = DQ882172; OVDL3 = DQ882174; OVL = DQ882175; OVPY3 = DQ882173; OVC1 = KBp, KPv, CP, SK, NP, BR, LP, NG, TH and SV; OVC2 = KLp, KS, MS and VT.
[2] Position of DNA sequence differences.
[3] Position of amino acid sequence difference.

AF297617, AB018440, NC_004826, AB107245 and AB271234) were included as outgroups for the phylogenetic analyses. Phylogenetic trees were constructed using the PHYLIP program version 3.6.6. A distance matrix was calculated using the Kimura two-parameter model and the tree constructed using the neighbor-joining method (Saitou and Nei, 1987). The relative support for the clades in the NJ analyses was determined using 1000 bootstrap replicates.

RESULTS

The partial *nad1* nucleotide sequences derived from all 14 geographical isolates were compared over an alignment length of 668 bp. There were only 2 alignment positions (nos. 434 and 470 Table 2) at which nucleotide variation was detected among the isolates included in the present study. However, comparison with previously published sequences revealed sequence variation at 9 alignment positions (Table 2). Four different haplotypes (designated as OVN1-OVN4) were detected among the 14 isolates of the present study. Haplotype OVN1 was detected in 6 isolates of *O. viverrini*, 2 from Thailand (CP and KPv) and 4 from Laos (NG, TH, VT and SV). Haplotype OVN2 was detected in 4 isolates from Thailand (KS, BR, NP and LP). Haplotype OVN3 was detected in 2 isolates from Thailand (KLp and SK), whereas haplotype OVN4 was found in another 2 Thai isolates (KBp and MS) (Table 2). One of the publicly available sequences (Accession number DQ119551) was identical in sequence to that of haplotype OVN3. The 4 other *O. viverrini* sequences available publicly represented a unique haplotype compared with the sequences of the isolates examined in the present study (Table 2). The *nad1* nucleotide sequences of 8 different haplotypes were conceptually translated to peptide sequences (221

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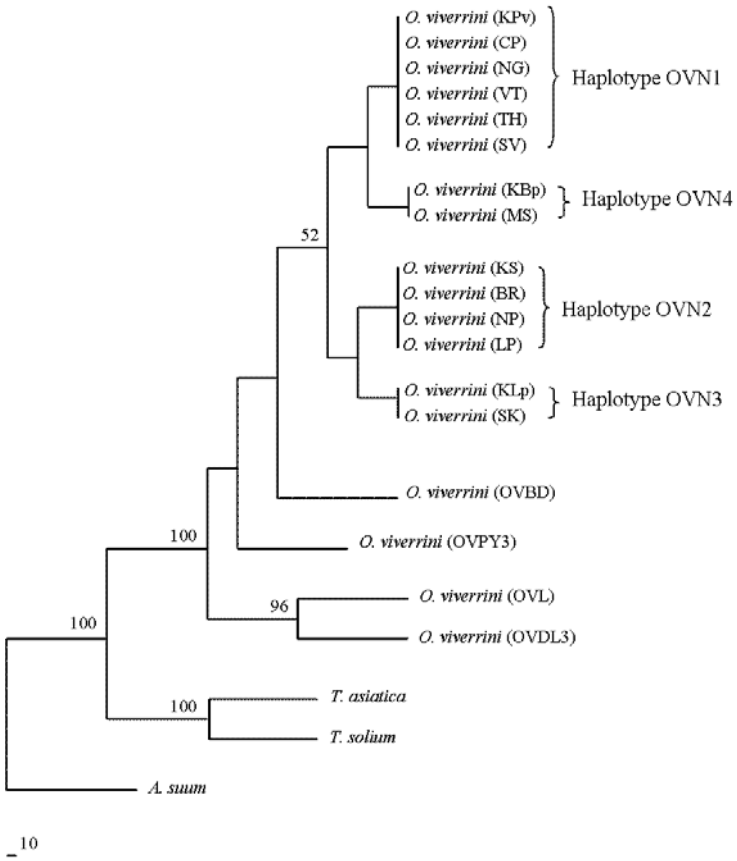

Fig. 2. Phylogenetic tree depicting relationships between *Opisthorchis viverrini* isolates inferred from a neighbor-joining method of analysis of the *nad1* nucleotide sequence data using two species of *Taenia* and *Ascaris suum* as outgroups. Bootstrap values (>50%) are indicated above branches.

amino acids in length). An alignment of these amino acid sequences revealed differences at 4 positions (Table 2). The 8 nucleotide sequences translated to 5 different amino acid sequences.

A phylogenetic analysis of nucleotide sequence data revealed that haplotype OVN1 was genetically more similar to OVN4, whereas haplotype OVN2 was more similar to OVN3. These 4 groups were more closely related to each other than 3 isolates from Vietnam and 1 isolate from Laos (Fig. 2).

The *cox1* sequences from all 14 isolates of *O. viverrini* were compared over an alignment length of 394 bp. Only 2 sequence variants were detected (designated as haplotypes OVC1 and OVC2) and these differed at 2 alignment positions (Table 2). The mutational change at alignment position 370 resulted in a change in amino acid sequence (Table 2). Haplotype OVC1 was detected in 7 isolates from Thailand (designated KBp, KPv, CP, SK, NP, BR and LP) and 3 isolates from Laos (NG, TH and SV), whereas haplotype OVC2 was detected in 3 isolates from Thailand (designated KLp, KS and MS) and 1 isolate from Laos (VT).

A phylogenetic analysis of the 2 *cox1* haplotypes of *O. viverrini* and other species of trematode (Fig. 3) revealed strong statistical support (i.e. bootstrap value of 91%) for a clade comprising sequences from *O. viverrini*, *O. felineus* and *C. sinensis* (members of the family Opisthorchidae). There was also some support (i.e. bootstrap value of 61%) for a clade that included 2 species of the genus *Metagonimus* with the opisthorchid nematodes. There were 3 other clades with varying levels of statistical support. The second clade contained all 7 species of *Paragonimus* (bootstrap value of 96%) that differed in sequence by 9–19%. The third clade, with moderate bootstrap support (71%) comprised 3 genera *Echinostoma*, *Fasciola* and *Fasciolopsis*, with sequence divergence among species ranging from 6 to 25%. The fourth clade with limited bootstrap support (65%) comprised the 8 species of *Schistosoma*, which differed in sequence by 7–23%.

DISCUSSION

Although genetic variation within *O. viverrini* sequences of the mtDNA gene *cox1* has been reported previously (Ando *et al.* 2001), this is a first study that has used sequence data of the *nad1* gene to investigate genetic variation within this parasite species. In the present study we detected limited genetic variation among 14 geographical isolates of *O. viverrini* for the parts of the *cox1* (394 bp) and *nad1* (668 bp) genes. For both *cox1* and *nad1*, only 2 nucleotide

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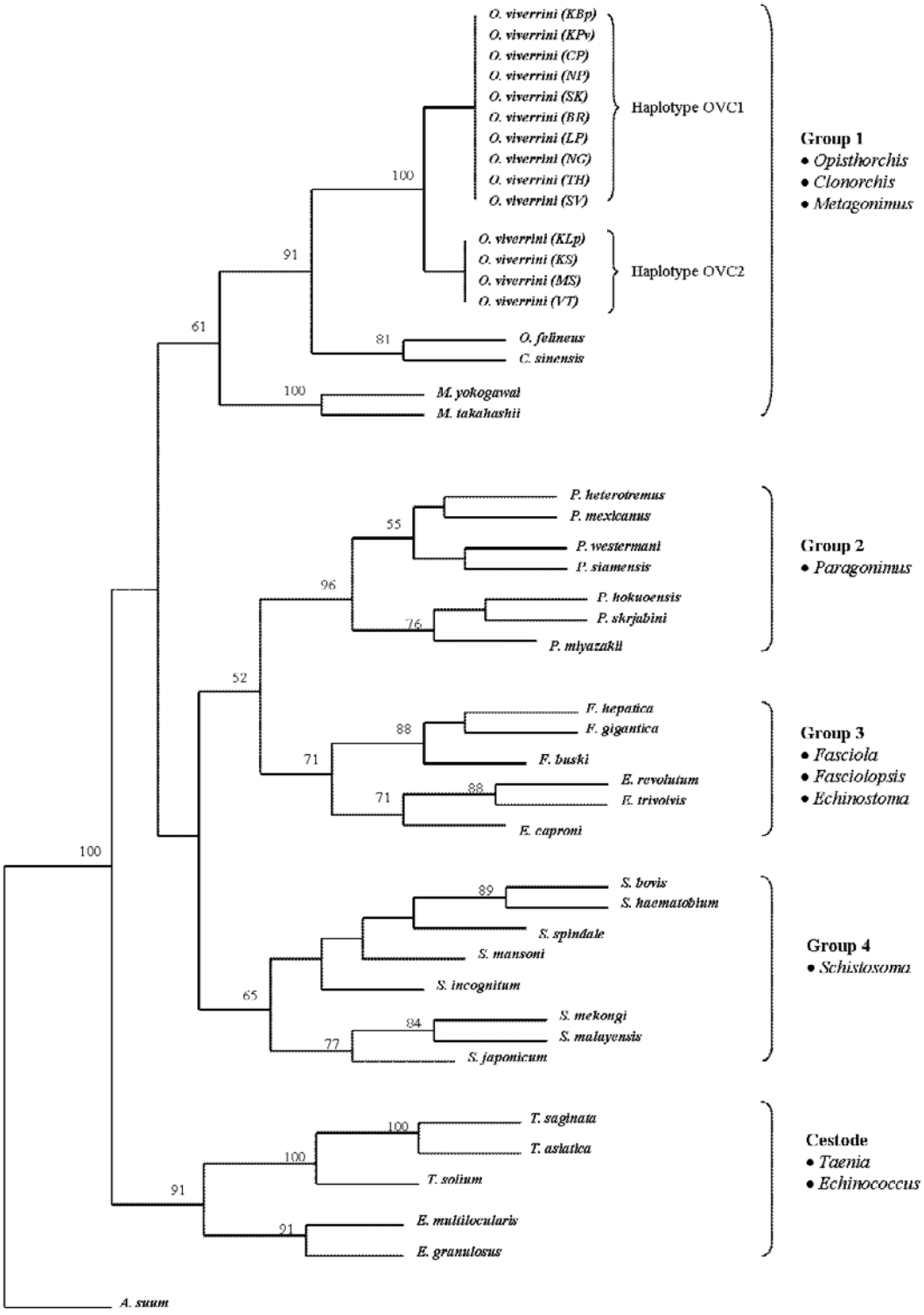

Fig. 3. Phylogenetic relationships of *Opisthorchis viverrini* with the other parasitic trematodes based on a neighbor-joining analysis of *cox1* nucleotide sequence data. *Ascaris suum* (Nematoda) was used as the outgroup in the analyses. Bootstrap values (>50%) are indicated above branches.

positions (0·5% and 0·3% respectively) were variable among *O. viverrini* isolates. However, in both genes, there were changes in amino acid sequences among isolates. A comparison of the *nad1* sequences with those for 4 *O. viverrini* isolates deposited in the GenBank database revealed additional variable positions in the sequence alignment. Thus, at the nucleotide level there are at least 8 *nad1* haplotypes

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for *O. viverrini*, representing 4 different amino acid sequence types.

Surprisingly, isolates separated by small geographical distances, did not necessarily have the same *nad1* haplotype. For example, for the 3 isolates from Khon Kaen province, KPv had the haplotype OVN1, KLp the haplotype OVN3 and KBp the haplotype OVN4. This finding does not agree with the results of a multilocus enzyme electrophoretic study of isolates from the same geographical areas using 32 enzyme loci (Saijuntha *et al*. 2007). These authors found that *O. viverrini* isolates separated by small geographical distances and within the same defined wetland formed a distinct cluster. This difference may be a reflection of the different number of genes examined. The addition of sequence data for more, independent genes may indeed provide more reliable results and hence biological interpretations regarding the level of genetic variation detected among isolates *O. viverrini* examined in this study and their phylogenetic relationships to other trematodes.

A previous study (Ando *et al*. 2001) using *cox1* sequences compared 5 *O. viverrini* isolates from Ubon Ratana, Lerngpleuy, Ban Phai, Mahasarakham, and Chatturat. Each isolate had a different *cox1* sequence. The *cox1* sequences for these isolates could not be incorporated in the present study because the data were not available in any public database. In the present study of 14 isolates, 2 different haplotypes were detected, and these differed at 2 nucleotide positions. A possible explanation for the differences between studies may relate to the reproductive turn-over rate in *O. viverrini* populations, which occurs seasonally, as shown in the different rate of infection in cyprinid fish in each month (Sithithaworn *et al*. 1997). Alternatively, preferential selection could result in lower numbers of haplotypes of *O. viverrini* being detected between the two studies.

The results of our study show that sequence data of *cox1* can be used to establish evolutionary relationships of parasitic trematodes at the genus and species levels. Campos *et al*. (1998) used the sequence data of the 18S nuclear ribosomal DNA gene to infer the phylogenetic relationships of taxa within the phylum Platyhelminthes. They found a clade comprising *Fasciola*, *Fasciolopsis* and *Echinostoma*, and that *Opisthorchis* was not closely related to the other parasitic trematodes included in our study. The phylogenetic tree derived using sequence data of the *cox1* showed similar relationships. Furthermore, each cluster of different genera of parasitic trematodes was separated into clades that were correlated with the type of second intermediate host they utilize and their mode of transmission to the definitive host. Of the three clades of food-borne trematodes, the first (the genera *Opisthorchis*, *Clonorchis* and *Metagonimus*; order Opisthorchiida) use cyprinid fish as their second intermediate host, the second (*Paragonimus* spp.; Order Plagiorchiida) parasitize crabs, and the third (the genera *Fasciola*, *Fascolopsis* and *Echinostoma*; order Echinostomida) lack a second intermediate host (i.e. *Fasciola*, and *Fascolopsis*) and the definitive host consumes the metacercariae attached to water plants, or they use snails, mussels and frogs as second intermediate hosts (i.e. *Echinostoma* spp.). The fourth clade represents the waterborne trematodes (*Schistosoma* spp.; order Strigeatida) which lack a second intermediate host in their life cycle. The definitive host is infected by the ceracariae that directly penetrate the skin. This mode of transmission may be due to co-adaptation between clades of parasites and specific species of intermediate hosts. For example, it has been suggested by Saijuntha *et al*. (2007) that *O. viverrini* may co-evolve with their first intermediate hosts, snails of the genus *Bithynia*. They found congruence between the phenogram of the genetic variation in *O. viverrini* from different geographical areas and the phenogram of their gastropod intermediate hosts based on analyses using multiple enzyme loci.

In conclusion, the number of different *nad1* haplotypes of *O. viverrini* was greater than that previously reported for this parasite based on *cox1* sequences. Hence, *nad1* may prove useful to examine in greater detail the magnitude of population variation within *O. viverrini*, whereas the *cox1* gene is more useful for examining of the phylogenetic relationships of food-borne trematodes.

This research was supported by a Wellcome Trust Collaborative Research Initiative Grant, the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (grant no. PHD/0221/2544 to W.S.) and the Faculty of Medicine, Khon Kaen University Overseas Visiting Professor Program. Dr Petney's travel was supported by Deutsche Forschungsgemeinschaft grant (PE 1611/1-1).

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