Updated molecular phylogenetic data for *Opisthorchis* spp. (Trematoda: Opisthorchioidea) from ducks in Vietnam

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**Abstract**

**Background:** An opisthorchiid liver fluke was recently reported from ducks (*Anas platyrhynchos*) in Binh Dinh Province of Central Vietnam, and referred to as "*Opisthorchis viverrini*-like". This species uses common cyprinoid fishes as second intermediate hosts as does *Opisthorchis viverrini*, with which it is sympatric in this province. In this study, we refer to the liver fluke from ducks as "*Opisthorchis* sp. BD2013", and provide new sequence data from the mitochondrial (mt) genome and the nuclear ribosomal transcription unit. A phylogenetic analysis was conducted to clarify the basal taxonomic position of this species from ducks within the genus *Opisthorchis* (Digenea: Opisthorchiidae).

**Methods:** Adults and eggs of liver flukes were collected from ducks, metacercariae from fishes (*Puntius brevis*, *Rasbora aurotaenia*, *Esomus metallicus*) and cercariae from snails (*Bithynia funiculata*) in different localities in Binh Dinh Province. From four developmental life stage samples (adults, eggs, metacercariae and cercariae), the complete cytochrome *b* (*cob*), nicotinamide dehydrogenase subunit 1 (*nad1*) and cytochrome *c* oxidase subunit 1 (*cox1*) genes, and near-complete 18S and partial 28S ribosomal DNA (rDNA) sequences were obtained by PCR-coupled sequencing. The alignments of nucleotide sequences of concatenated *cob* + *nad1* + *cox1*, and of concatenated 18S + 28S were separately subjected to phylogenetic analyses. Homologous sequences from other trematode species were included in each alignment.

**Results:** Phylogenetic trees were inferred from concatenated (*cob* + *nad1* + *cox1*) nucleotide sequences and combined 18S + 28S nucleotide sequences of five *Opisthorchis* sp. BD2013 samples and additional reference taxa. Both trees demonstrated the anticipated clustering of taxa within the superfamily Opisthorchioidea, the paraphyly of the genus *Opisthorchis* and the sister-species relationship of *Opisthorchis* sp. BD2013 with *O. viverrini*.

**Conclusions:** While it is likely that *Opisthorchis* sp. BD2013 is distinct from *O. viverrini*, it is clearly a sister taxon of *O. viverrini* within the limited number of *Opisthorchis* species for which appropriate sequence data are available. The new sequences provided here will assist the diagnosis and the taxonomic clarification of the opisthorchiid species.

**Keywords:** Mitochondrial gene, Ribosomal transcription unit, *Opisthorchis* sp. BD2013, Opisthorchiid, 18S rDNA, 28S rDNA, Phylogenetic analysis
Background
The family Opisthorchoiidae (Digenea: Opisthorchioidea) consists of 33 genera considered valid including the genera Opisthorchis and Clonorchis, in which O. viverrini, O. felineus and C. sinensis are known to infect humans [1]. Humans become infected by eating uncooked cyprinoid fish containing metacercariae. Opisthorchis viverrini has been reported in Central Vietnam, where Binh Dinh and Phu Yen Provinces are highly endemic for human opisthorchiasis [2–4].

In 2013, Dao et al. [5] found adults of an opisthorchiid species in ducks (Anas platyrhynchos) in areas of Binh Dinh Province where there are many human opisthorchiasis cases. This parasite was then given the working name “Opisthorchis viverrini-like”, because of its close similarity to O. viverrini [5, 6]. Subsequently, there has been a debate about the identity of this worm. Nawa et al. [7] argued that the duck liver fluke not be O. viverrini, but is most likely O. parageminus that was previously reported from ducks in Vietnam [8–10]. However, Dorny et al. [11] considered that their “Opisthorchis viverrini-like” species exhibited some morphological differences from O. parageminus. We now propose to use the working name “Opisthorchis sp. BD2013” instead of the earlier “Opisthorchis viverrini-like”.

Molecular phylogenetic/systematic studies are excellent aids for taxonomy [12–15]. Such studies require homologous sequences from as many taxa as possible within the group of interest. In the genus Opisthorchis, a number of genetic markers from complete mitochondrial sequences and the nuclear ribosomal transcription units including, ITS1, ITS2, 18S rDNA and partial 28S rDNA have been generated for O. viverrini, O. felineus and Clonorchis sinensis. These genetic markers have greatly contributed to molecular diagnostic, epidemiological, phylogenetic and evolutionary studies of the species in Opisthorchiidae and trematodes [3, 13, 16–19]. However, Opisthorchis is a very large genus [7], and molecular data are available for only a few species. Moreover, given difficulties with the morphological taxonomy within the genus, it is not always certain that names assigned to samples are accurate. The only molecular data claimed to be from O. parageminus consist of two sequences recently deposited in GenBank (accession numbers KX258656, KX258657) by Nguyen and Nguyen (otherwise unpublished data). Although their worms came from ducks in Vietnam, no information is available on the morphological basis for the identification. Both of these sequences (mitochondrial partial mt cox1 and nuclear ribosomal ITS2) are very similar to earlier sequences available for Opisthorchis sp. BD2013 published by [5]. Here, we provide additional mitochondrial sequences, i.e. complete cytochrome b (cob), nicotinamide dehydrogenase subunit 1 (nad1) and cytochrome c oxidase subunit 1 (cox1) genes, and near-complete 18S rDNA and partial 28S rDNA sequences in an effort to better resolve the affinities of Opisthorchis sp. BD2013 within the family Opisthorchiidae and the superfamily Opisthorchioidea.

Methods
Opisthorchis sp. BD2013 samples collected from the field
Adult specimens and eggs of Opisthorchis sp. BD2013 were collected from naturally infected domestic ducks (Anas platyrhynchos) originating from 4 localities (Phu Cat, Phu My, An Nhơn and Tuy Phuoc Districts) in Binh Dinh Province of Central Vietnam [6, 20] (Table 1). Each adult worm, unstained or stained with acetic carmine, was morphologically identified by light microscopy [5]. Up to three adult worms from each locality were individually fixed in 70% ethanol, and one or two worms from each locality were separately subjected to genomic DNA extraction and molecular analysis.

Fishes (harbouring metacercariae) and snails (shedding cercariae) were collected from My Tho Lake in the lowlands of Binh Dinh Province [20]. Infected fishes were identified as Puntius brevis, Esomus metallicus, Rasbora aurataenina, and the snail as Bithynia funiculata [20] (Table 1). For molecular analysis, metacercariae and cercariae were individually fixed in RNAlater™ buffer (Qiagen, Texas, USA) at 4 °C. Individual parasites from each intermediate host and each locality were used for extraction of DNA and molecular study.

Eggs were individually collected from the gallbladder of naturally infected ducks by washing and centrifuging the bile ten times in normal saline (0.9% NaCl), then three times in phosphate buffered saline (PBS) before storage at -20 °C until use (Table 1).

Genomic DNA extraction and primers
Total genomic DNA was extracted from individual adults, metacercariae, cercariae or pooled eggs (approximately 2000–3000 eggs) using the GeneJET™ Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., MA, USA), according to the manufacturer’s instructions. A slight modification applied for eggs was to increase the incubation period by 3–4 h after enzymatic lysis. Genomic DNA was eluted in 50 μl of the elution buffer provided in the kit and stored at -20 °C. The DNA concentration was estimated using a GBC UV/visible 911A spectrophotometer (GBC Scientific Equipment Pty. Ltd., Braeside, Australia) and diluted to a working concentration of 50 ng/μl (about 10 ng/μl for DNA from eggs). From this genomic DNA, 2–3 μl was used as template in a PCR of 50 μl volume.

Primers used both for amplification and sequencing of the mitochondrial and nuclear ribosomal genes are listed in Table 2. The primer pair OACOBF/OACO1R
amplified approximately 7.8 kb of mtDNA. Based on the sequence obtained from this amplicon, three primer pairs specific for the individual target protein-coding genes were designed. Primer pairs OACOBF/OACOBR, OAND1F/OAND1R, OACO1F/OACO1R amplified complete cob, nad1 and cox1 genes, respectively. The primer pairs U18SF/U18SR were used for obtaining major fragments of ribosomal 18S and U28SF/U28SR for 28S, respectively [12]. Additional internal primers were designed and used as needed (Table 2).

### Amplification of mitochondrial and ribosomal genes

**The 7.8 kb mt genomic region**

Long PCR reactions were prepared using 25 μl of Fusion High-Fidelity PCR Master Mix (2×) (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 2 μl of each primer (10 pmol/μl), 2 μl DNA template of the adult sample (50 ng/μl), 2 μl DMSO (dimethyl sulfoxide) and 17 μl H2O up to a final volume of 50 μl. All PCRs were performed in an MJ PTC-100 thermal cycler with initiation at 98 °C for 30 s, followed by 35 cycles consisting of denaturation for 10 s at 98 °C, annealing at 56 °C for 30 s, extension at 72 °C for 6 min.

**Individual mt and ribosomal DNA genes**

PCR reactions of 50 μl were prepared using 25 μl of DreamTaq PCR Master Mix (2×) (Thermo Fisher Scientific Inc., Waltham, MA, USA), 2 μl of each primer (10 pmol/μl), 2 μl DNA template (50 ng/μl for adults; 50 ng/μl for metacercariae; 10–20 ng/μl for cercariae and eggs), 2 μl DMSO (dimethyl sulfoxide) and 17 μl H2O. All PCRs were performed in an MJ PTC-100 thermal cycler with initiation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation for 30 s at 94 °C, annealing at 56 °C for 30 s, extension at 72 °C for 3 min.

| Life-cycle stage | Site collected (district) | Host | Scientific name | Sample abbreviation for use in this study |
|------------------|---------------------------|------|-----------------|------------------------------------------|
| Adult worm       | Phu Cat                   | Duck | Anas platyrhynchos | Opisthorchis sp. BD2013-PC6aduBD         |
| Adult worm       | Phu My                    | Duck | Anas platyrhynchos | Opisthorchis sp. BD2013-PM10aduBD        |
| Adult worm       | An Nhon                   | Duck | Anas platyrhynchos | Opisthorchis sp. BD2013-PC6aduBD         |
| Adult worm       | Tuy Phuoc                 | Duck | Anas platyrhynchos | Opisthorchis sp. BD2013-PM10aduBD        |
| Metacercariae    | Phu My                    | Fish | Puntius brevis    | Opisthorchis sp. BD2013-PCmetaBD         |
| Metacercariae    | Phu My                    | Fish | Rasbora aurataenia | Opisthorchis sp. BD2013-PCmetaBD        |
| Metacercariae    | Phu My                    | Fish | Esomus metallicus | Opisthorchis sp. BD2013-PCmetaBD         |
| Cercariae        | Phu My                    | Snail| Bithynia funiculata | Opisthorchis sp. BD2013-PCcercaBD        |
| Eggs             | Phu My                    | Duck | Anas platyrhynchos | Opisthorchis sp. BD2013-PCeggBD          |

### Table 2 Primers for amplification and sequencing of the mitochondrial protein-coding and nuclear ribosomal genes used in this study

| Primer name   | Sequence (5′–3′) | Target gene | Amplicon by PCR | Length of sequence (bp) | Reference |
|---------------|-----------------|-------------|-----------------|-------------------------|-----------|
| OACOBF        | AGCCGGAGAGTCTTTGTTG | cob         | 1.4 kb          | 1110                    | This study |
| OACOBR        | TGAATCCCAACACCGCTTA |             |                 |                         |           |
| OACOBR2a      | TACGGTTAAGGACGGTTG |             |                 |                         |           |
| OAND1F        | CGTGTGGTGGGCGCAAGATAG | nad1       | 1.2 kb          | 903                     | This study |
| OAND1R        | CACACACAGCTTCCTCAAGT |             |                 |                         |           |
| OACO1F        | GAGGTTTACGTGGTGTGAG | cox1        | 1.8 kb          | 1551                    | This study |
| OACO1R        | CAACCTCTAAGCCACACACG |             |                 |                         |           |
| OACO1R2a      | GGATCCAAAAAGCCTCACG |             |                 |                         |           |
| U18SF         | GCGAATGGCTCATATAATCG | 18S         | 1.8 kb          | ~ 1790                  | [12]      |
| U18SR         | GGAAACATCGAGGCGCTACTG |             |                 |                         |           |
| NS2F          | GCAAGTCTGGTGCACGCAGCC |             |                 |                         |           |
| U28SF         | CTCAACAGGATTCCCTTAAGCAC | 28S         | 1.3 kb          | ~ 1100                  | [12]      |
| U28SR         | GTCTTCGCCCCCTATACCAC |             |                 |                         |           |

**Abbreviations:** F forward, R reverse

*Internal primer used for sequencing*
Sequencing and sequence analyses
PCR products were obtained from at least two individual samples for each template (i.e. adults, metacercariae, cercariae and eggs) originating from different geographical localities. The PCR products (10 μl of each) were examined on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light (Wealtec, Meadowvale Way Sparks, USA).

All the purified or gel-extracted amplicons were subjected to direct sequencing by automated sequencers using amplifying/flanking and internal primers (Table 2) by primer-walking in both directions (Macrogen Inc., Seoul, South Korea). Sequences (two from each sample) were aligned to obtain the final sequence for characterization. All sequences of Opisthorchis sp. BD2013 were identical, regardless of the life-cycle stage or locality.

The concatenated nucleotide and amino acid sequences of three protein-coding genes, i.e., cob + nad1 + cox1, were used to infer the pairwise genetic distances between 10 opisthorchiids (Table 3). These isolates included Opisthorchis sp. BD2013 and the reference sequences from Laos (JF739555), Vietnam (MF287777–MF287779) and Thailand (MF287780–MF287782). The genetic distances were inferred by pair-wise analysis using the MEGA6.0 software, and the number of base substitutions per site was calculated by the most simplified method (uncorrected p-distance) [21].

Phylogenetic analysis
Preparation of DNA sequences
Phylogenetic analysis using three mitochondrial protein-coding (cob, nad1, cox1) and two nuclear ribosomal (18S and 28S rDNA) genes was conducted to examine the taxonomic placement of Opisthorchis sp. BD2013 from ducks within the superfamily Opisthorchioidea. Sequences of trematode species/isolates of the Opisthorchidae, Heterophyidae, Fasciolidae and Schistosomatidae (as the outgroup) were used. Summary data of species/isolates, mainly from the available complete mitochondrial genomes are presented in Table 3. Accession numbers for the target and reference 18S and 28S rDNA sequences are listed in Table 4. For Opisthorchis sp. BD2013, we decided to use only two sequences of adults, and one each from metacercariae, cercariae and eggs for phylogenetic analyses.

Concatenated nucleotide sequences of mt protein-coding genes (cob, nad1, cox1) from adults, metacercariae, cercariae, and eggs of Opisthorchis sp. BD2013, and from additional taxa (available in GenBank; see Table 3) were imported into GENEDOC 2.7 (available at http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html) and aligned for phylogenetic analysis. Additionally, the sequences of opisthorchiids were translated (using the echinoderm/flatworm mitochondrial genetic code: translation Table 9 in GenBank), and the deduced amino acid sequences were aligned for pairwise genetic distance analysis.

DNA sequences of 18S rRNA and 28S rRNA genes (listed in Table 4) were aligned separately using GENEDOC 2.7. The sequences were trimmed at both ends to the shortest length of the representative sequences. For 18S rDNA, in this study, the final alignment was 2005 nucleotides (nt) long of which 87 nt positions were trimmed at 5’ end and 114 nt at 3’ end, leaving 1804 characters for analyses. For 28S rDNA, the final alignment was 1449 nt long of which 122 nt positions were trimmed at 5’ end and 123 nt at 3’ end, leaving 1202 characters for analyses. The two sequences were then concatenated as indicated in Table 4, preferably from the same strains/isolates. The concatenated 18S + 28S rDNA sequences representing species/isolates were imported into GENEDOC 2.7 and phylogenetic analysis and tree construction were done by MEGA6.0 [21].

Phylogenetic reconstruction
The alignments of the concatenated nucleotide (cob, cox1, nad1) and 18S +28S sequences, respectively, were trimmed to the length of the shortest sequence and imported into the MEGA 6.06 software [21]. Maximum likelihood (ML) analyses were performed in each case. For DNA sequences, we used the general time-reversible model of evolution with gamma distributed rate heterogeneity and a proportion of invariant sites (GTR + Γ + Ω). This model was given the best Bayesian information criterion score by MEGA. For amino acid sequences, the Jones-Taylor-Thornton (JTT) model with uniform rates and Nearest-Neighbor-Interchange (NNI) method was used. The confidence in each node was assessed using 1000 bootstrap resamplings [21].

Results
Mitochondrial cob, nad1, cox1 and genetic distances among opisthorchid species/sequences
For Opisthorchis sp. BD2013, lengths of the complete cob, nad1 and cox1 genes were 1110, 903 and 1551 nucleotides, respectively. Among opisthorchid species, cob genes ranged in length from 1110 to 1116 nt, and cox1 genes were 1551 to 1563 nt in length. The primer pairs U18SF/U18SR were used for obtaining major fragments of ribosomal 18S and U28SF/U28SR for 28S rDNA.

Nucleotide and amino acid pairwise comparisons of the concatenated mt genes among ten opisthorchid isolates/species are presented in Tables 5 and 6. The concatenated cob + nad1 + cox1 nucleotide sequences of Opisthorchis sp. BD2013 differed at 14.4–14.5% of nucleotide sites and 10.3–10.6% of amino acid positions from the reference sequences of O. viverrini (Vietnam,
Table 3 Summary data for complete mitochondrial genomes of species providing cytochrome b (cob), nicotinamide dehydrogenase subunit 1 (nad1) and cytochrome c oxidase subunit 1 (cox1) used in the phylogenetic analysis including Opisthorchis sp. BD2013 in ducks in Vietnam

| Family/Species          | Isolates/Strains | Country         | GenBank ID         | Reference |
|-------------------------|------------------|-----------------|--------------------|-----------|
| **Opisthorchiidae**     |                  |                 |                    |           |
| Opisthorchis sp. BD2013 | PC6aduBD         | Vietnam         | MF287762-MF287764  | This study|
| Opisthorchis sp. BD2013 | PM10aduBD        | Vietnam         | MF287765-MF287767  | This study|
| Opisthorchis sp. BD2013 | PCmetaBD         | Vietnam         | MF287768-MF287770  | This study|
| Opisthorchis sp. BD2013 | PCCercaBD        | Vietnam         | MF287771-MF287773  | This study|
| Opisthorchis sp. BD2013 | PeggBD           | Vietnam         | MF287774-MF287776  | This study|
| Opisthorchis viverrini' | na               | Laos            | JF739555           | [19]      |
|                         | Binh Dinh 1      | Vietnam         | MF287777-MF287779  | This study|
|                         | Khon Koen        | Thailand        | MF287780-MF287782  | This study|
| Opisthorchis felineus   | Ust-Tula (Novosibirsk) | Russia     | EU921260           | [16]      |
| Clonorchis sinensis     | Nam Dinh         | Vietnam         | MF287783-MF287785  | This study|
|                         | Guangdong        | China           | JF729303           | [19]      |
|                         | na               | South Korea     | JF729304           | [19]      |
|                         | Amur - Khabarovsk | Russia          | FJ381664           | [16]      |
| Metorchis orientalis    | Heilongjiang     | China           | KT239342           | [22]      |
| **Heterophyidae**       |                  |                 |                    |           |
| Haplorchis taichui      | na               | Laos            | KF214770           | [24]      |
|                         | Quang Tri 3      | Vietnam         | MF287786-MF287788  | This study|
| **Metagonimus yokogawai** | na               | South Korea     | KC330755           |           |
| **Fasciolidae**         |                  |                 |                    |           |
| Fasciola hepatica       | Geelong          | Australia       | AF216697           | [25]      |
| Fasciola gigantica      | Guangxi          | China           | KF543342           | [26]      |
|                         | Thua Thien-Hue   | Vietnam         | MF287789-MF287791  | This study|
| Fasciola sp. (intermediate form) | GHL-Heilongjiang | China           | KF543343           | [26]      |
| Fasciolopsis buski      | Jiangxi          | China           | KX169163           | [27]      |
|                         | Ha Tay           | Vietnam         | MF287792-MF287794  | This study|
| Fascioloides magna      | Kokolínko        | Czech Republic  | KU060148           | [28]      |
| **Schistosomatidae**    |                  |                 |                    |           |
| Schistosoma haematobiuma | N10 Village      | Mali            | DQ157222           | [29]      |

*aSequence used as the outgroup
*bSequences of the opisthorchids used for pairwise genetic distance calculation (Tables 5 and 6)

Thailand and Laos isolates) [19]; 17.9–18.2% for nucleotides and 13.3–13.7% for amino acids from C. sinensis (Russia, China, South Korea and Vietnam isolates); 18.1% (nucleotides) and 13.7% (amino acids) from O. felineus (a Russian isolate) [16] and 15.4% (nucleotides) and 11.6% (amino acids) from Metorchis orientalis (China isolate) [23].

Within each opisthorchiid taxon, pairwise genetic distances were small, only 0.4–0.7% for nucleotides and 0.5–0.6% for amino acids within O. viverrini; 0.3–0.6% (nucleotides) and 0.2–0.8% (amino acids) within C. sinensis. Opisthorchis sp. BD2013 in ducks differs from O. viverrini by more than 10%, a figure comparable to those separating species within the genus Opisthorchis and the family Opisthorchiidae (Tables 5 and 6).

**Phylogenetic analysis**

Phylogenetic reconstruction based on the complete cob + nad1 + cox1 amino acid sequences

A phylogenetic tree was constructed from 25 nucleotide sequences inferred from complete cob + nad1 + cox1 of 13 trematode species belonging to 4 families with...
Schistosoma haematobium of the Schistosomatidae as the outgroup (Table 3, Fig. 1). The superfamily Opisthorchioidea in this study comprises the Heterophyidae and Opisthorchiidae (no appropriate sequences from the third family, Cryptogonimidae, were available), with the strong nodal support of 99%, clearly separate from the family Fasciolidae. The Opisthorchis sp. BD2013 clade was placed as a sister of O. viverrini from...
Table 5  Pairwise genetic distances (%) between Opisthorchis sp. BD2013 sample from ducks in Vietnam and the sequences for O. viverrini, Clonorchis sinensis, O. felineus and Metorchis orientalis of the concatenated mitochondrial genes cob, nad1 and cox1

| Species                          | GenBank ID  | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 |
|----------------------------------|-------------|----|----|----|----|----|----|----|----|----|----|
| 1 Opisthorchis sp. BD2013 (PM10aduBD/Vietnam) | MF287767 | –  |    |    |    |    |    |    |    |    |    |
| 2 O. viverrini (Binh Dinh 1/ Vietnam) | MF287779 | 14.4 | –  |    |    |    |    |    |    |    |    |
| 3 O. viverrini (Khon Kaen/ Thailand) | MF287782 | 14.5 | 0.4 | –  |    |    |    |    |    |    |    |
| 4 O. viverrini (Laos) | JF739555 | 14.4 | 0.5 | 0.7 | –  |    |    |    |    |    |    |
| 5 C. sinensis (Amur-Khabarovsk/Russia) | FJ381664 | 17.9 | 18.1 | 18.1 | 17.9 | –  |    |    |    |    |    |
| 6 C. sinensis (Guangdong/ China) | JF279303 | 18.0 | 18.1 | 18.1 | 17.9 | 0.4 | –  |    |    |    |    |
| 7 C. sinensis (South Korea) | JF729904 | 18.2 | 18.2 | 18.3 | 18.0 | 0.5 | 0.3 | –  |    |    |    |
| 8 C. sinensis (Nam Dinh/ Vietnam) | MF287784 | 18.0 | 18.1 | 18.2 | 18.0 | 0.5 | 0.5 | 0.6 | –  |    |    |
| 9 O. felineus (Ust-Tula/ Russia) | EU921260 | 18.1 | 18.8 | 18.9 | 18.7 | 15.4 | 15.6 | 15.8 | 15.5 | –  |    |
| 10 Metorchis orientalis (Heilongjiang/China) | KT239342 | 15.5 | 13.7 | 13.7 | 13.5 | 17.0 | 17.2 | 17.2 | 17.0 | 16.8 | –  |

Discussion

In this study, we used two concatenated datasets to infer the molecular phylogenetic position of Opisthorchis sp. BD2013 (formerly named “Opisthorchis viverrini-like” or as O. parageminus by several authors). We did not have samples of O. lobatus [17] and the so-called O. parageminus [8, 9] for analysis in the present study, therefore, we were not able to establish the relationship between Opisthorchis sp. BD2013 and these species.

The genus Opisthorchis is very large [7], but relevant sequence data are limited to only a few species. It was necessary to determine whether Opisthorchis sp. BD2013 from ducks is distinct from O. viverrini, a zoonotic liver fluke known to infect and to cause cholangiocarcinoma in humans [23]. The data presented in this study strongly imply that the two are distinct species. The sister-species relationship demonstrated between Opisthorchis sp. BD2013, and O. viverrini might simply be because O. felineus is the only other member of the genus for which data are available. Opisthorchis felineus renders Opisthorchis paraphyletic in our trees, indicating that much systematic work remains to be done in the

Table 6  Pairwise genetic distances (%) between Opisthorchis sp. BD2013 sample from ducks in Vietnam and O. viverrini, Clonorchis sinensis, O. felineus and Metorchis orientalis of the concatenated mitochondrial amino acid sequence of cob, nad1 and cox1

| Nucleotide sequences                          | Accession No. | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 |
|-----------------------------------------------|---------------|----|----|----|----|----|----|----|----|----|----|
| 1 Opisthorchis sp. BD2013 (PM10aduBD/Vietnam) | MF287767 | –  |    |    |    |    |    |    |    |    |    |
| 2 O. viverrini (Binh Dinh 1/ Vietnam) | MF287779 | 10.6 | –  |    |    |    |    |    |    |    |    |
| 3 O. viverrini (Khon Kaen/ Thailand) | MF287782 | 10.6 | 0.5 | –  |    |    |    |    |    |    |    |
| 4 O. viverrini (Laos) | JF739555 | 10.3 | 0.6 | 0.6 | –  |    |    |    |    |    |    |
| 5 Clonorchis sinensis (Amur-Khabarovsk/Russia) | FJ381664 | 13.3 | 12.4 | 12.4 | 12.4 | –  |    |    |    |    |    |
| 6 C. sinensis (Guangdong/ China) | JF279303 | 13.5 | 12.8 | 12.8 | 12.8 | 0.3 | –  |    |    |    |    |
| 7 C. sinensis (South Korea) | JF279304 | 13.7 | 12.7 | 12.7 | 12.7 | 0.3 | 0.2 | –  |    |    |    |
| 8 C. sinensis (Nam Dinh/ Vietnam) | MF287784 | 13.6 | 12.6 | 12.6 | 12.6 | 0.4 | 0.8 | 0.8 | –  |    |    |
| 9 O. felineus (Ust-Tula/ Russia) | EU921260 | 13.7 | 13.8 | 13.9 | 13.9 | 9.3 | 9.7 | 9.7 | 9.5 | –  |    |
| 10 Metorchis orientalis (Heilongjiang/China) | KT239342 | 11.6 | 8.8 | 8.8 | 8.7 | 9.8 | 10.2 | 10.2 | 10.1 | 11.0 | –  |
Fig. 1 Phylogenetic tree for *Opisthorchis* sp. BD2013 (indicated by diamond symbol) and other opisthorchiids and representative trematodes from 4 families, the Opisthorchiidae, Heterophyidae, Fasciolidae and Schistosomatidae (the latter used as an outgroup), based on concatenated nucleotide sequences of complete cytochrome b (cob), nicotinamide dehydrogenase subunit 1 (nad1) and cytochrome c oxidase subunit 1 (cox1) genes. Phylogenetic reconstruction was performed using maximum likelihood analysis with the general time-reversible model with a gamma distributed rate heterogeneity and a proportion of invariant sites (GTR + Γ + I) in the MEGA6.06 software package. Support for each node was evaluated using 1000 bootstrap resamplings [21]. The scale-bar indicates the number of substitutions per site. Accession numbers (where available) are given at the end of each sequence name. Isolates/geographical localities are given in parentheses (if available). Country abbreviation codes (2-letter) are given prior to the accession numbers: AU, Australia; CN, China; CZ, Czech Republic; KR, Korea; LA, Lao PDR; RU, Russia; TH, Thailand; VN, Vietnam.

Fig. 2 Phylogenetic tree for *Opisthorchis* sp. BD2013 (indicated by diamond symbol) and other opisthorchiids and representative trematodes from 4 families, the Opisthorchiidae, Heterophyidae, Fasciolidae and Schistosomatidae (the latter used as the outgroup), based on combined nucleotide sequences of the nuclear small ribosomal subunit (18S rDNA) and large ribosomal subunit (28S rDNA). Phylogenetic reconstruction was performed using maximum likelihood analysis with the general time-reversible model and a gamma distributed rate heterogeneity and proportion of invariant sites (GTR + Γ + I) in the MEGA6.06 software package. Support for each node was evaluated using 1000 bootstrap resamplings [21]. The node for the superfamily (infraorder) Opisthorchioidea is indicated by an arrow. The scale-bar indicates the number of substitutions per site. Accession numbers are given at the end of each sequence name. Isolates or geographical localities and country isolated are given in parentheses (if available).
Opisthorchiidae. A further unresolved question is the relationship between *Opisthorchis* sp. BD2013 and *O. parageninus*. Both were found in ducks in Vietnam, but some morphological differences seem to exist [11]. At this stage, we prefer to leave the question open, pending future morphological and molecular work.

Our previous phylogenetic analysis using short sequences of ITS2 and cox1 revealed close affinities between *O. viverrini*, *O. lobatus* and *Opisthorchis* sp. BD2013 [5]. In the current study, we are unable to resolve the status of *O. lobatus* compared to *Opisthorchis* sp. BD2013 and other opisthorchiids.

**Conclusions**

Based on mitochondrial *cob* + *nad1* + *cox1* and ribosomal 18S + 28S rRNA sequence analyses, *Opisthorchis* sp. BD2013 was distinct from *O. viverrini*, although the two species are closely related. The genus *Opisthorchis* itself appears as paraphyletic. Data from additional *Opisthorchis* species are vital to create a phylogeny with higher resolution within *Opisthorchis* and the Opisthorchiidae.

**Abbreviations**

cob: cytochrome b; cox1: cytochrome c oxidase subunit 1; MEGA: Molecular Evolutionary Genetics Analysis; ML: maximum likelihood; mt: mitochondrial; nad1: nicotinamide dehydrogenase subunit 1; rTU: ribosomal transcription unit

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**Availability of data and materials**

The data sets supporting the alignment and phylogenetic analysis are included in the article. Nucleotide sequences obtained in the present study have been deposited into the GenBank database with the following accession numbers: MF077358–MF077362 (18S rDNA; *Opisthorchis* sp. BD2013); MF110001–MF110005 (28S rDNA; *Opisthorchis* sp. BD2013); MF287762–MF287776 (cob, nad1, cox1; *Opisthorchis* sp. BD2013).

**Authors’ contributions**

THTD, PD and THL conceived the study, analyses of final data and wrote the manuscript. TGN, KLB and SG conducted field collections, laboratory, and preliminary sequence analyses. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Appropriate permission was obtained from the commune authorities and local households before the collection of parasite specimens from their stocks.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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