Complete mitochondrial genomes of the ‘intermediate form’ of Fasciola and Fasciola gigantica, and their comparison with F. hepatica

Guo-Hua Liu, Robin B Gasser, Neil D Young, Hui-Qun Song, Lin Ai and Xing-Quan Zhu

Abstract

Background: Fascioliasis is an important and neglected disease of humans and other mammals, caused by trematodes of the genus Fasciola. Fasciola hepatica and F. gigantica are valid species that infect humans and animals, but the specific status of Fasciola sp. (‘intermediate form’) is unclear.

Methods: Single specimens inferred to represent Fasciola sp. (‘intermediate form’; Heilongjiang) and F. gigantica (Guangxi) from China were genetically identified and characterized using PCR-based sequencing of the first and second internal transcribed spacer regions of nuclear ribosomal DNA. The complete mitochondrial (mt) genomes of these representative specimens were then sequenced. The relationships of these specimens with selected members of the Trematoda were assessed by phylogenetic analysis of concatenated amino acid sequence datasets by Bayesian inference (B).

Results: The complete mt genomes of representatives of Fasciola sp. and F. gigantica were 14,453 bp and 14,478 bp in size, respectively. Both mt genomes contain 12 protein-coding genes, 22 transfer RNA genes and two ribosomal RNA genes, but lack an atp8 gene. All protein-coding genes are transcribed in the same direction, and the gene order in both mt genomes is the same as that published for F. hepatica. Phylogenetic analysis of the concatenated amino acid sequence data for all 12 protein-coding genes showed that the specimen of Fasciola sp. was more closely related to F. gigantica than to F. hepatica.

Conclusions: The mt genomes characterized here provide a rich source of markers, which can be used in combination with nuclear markers and imaging techniques, for future comparative studies of the biology of Fasciola sp. from China and other countries.

Keywords: Liver fluke, Fasciola spp, Mitochondrial genome, Phylogenetic analysis

Background

Food-borne trematodiases are an important group of neglected parasitic diseases. More than 750 million people are at risk of such trematodiases globally [1,2]. Fascioliasis is caused by liver flukes of the genus Fasciola, and has a significant adverse impact on both human and animal health worldwide [3]. Human fascioliasis is caused by the ingestion of freshwater plants or water contaminated with metacercariae of Fasciola [4]. It is estimated that millions of people are infected worldwide, and more than 180 million people are at risk of this disease worldwide [5]. To date, no vaccine is available to prevent fascioliasis. Fortunately, this disease can be treated effectively using triclabendazole [6], but there are indications of resistance developing against this compound [7].

The Fasciolidae is a family of flatworms and includes the genus Fasciola. Both F. hepatica and F. gigantica, which commonly infect livestock animals and humans (as definitive hosts), are recognized as valid species [8]. The accurate identification of species and genetic variants is relevant in relation to studying their biology, epidemiology...
and ecology, and also has applied implications for the diagnosis of infections. Usually, morphological features, such as body shape and perimeter as well as length/width ratio, are used to identify adult worms of Fasciola [9]. However, such phenotypic criteria are unreliable for specific identification and differentiation, because of considerable variation and/or overlap in measurements between F. hepatica and F. gigantica [10].

Due to these constraints, various molecular methods have been used for the specific identification of Fasciola species and their differentiation [5]. For instance, PCR-based techniques using genetic markers in nuclear ribosomal (r) and mitochondrial (mt) DNAs have been widely used [11-13]. The sequences of the first and second internal transcribed spacers (ITS-1 and ITS-2 = ITS) of nuclear rDNA have been particularly useful for the specific identification of F. hepatica and F. gigantica, based on a consistent level of sequence difference (1.2% in ITS-1 and 1.7% in ITS-2) between them and much less variation within each species [11,14]. Nonetheless, studies in various countries, including China [5], Iran [15], Japan [16], Korea [14], Spain [17] and Tunisia [18], have shown that some adult specimens of Fasciola sp., which are morphologically similar to F. gigantica [10], are characterized by multiple sequence types (or “alleles”) of ITS-1 and/or ITS-2, reflected in a mix between those of F. hepatica and F. gigantica [11,12]. Some authors [19-21] have suggested that such specimens (sometimes called ‘intermediate forms’) represent hybrids of F. hepatica and F. gigantica.

In the present study, we undertook an independent, genetic comparison of Fasciola sp. (i.e. ‘intermediate form’) and F. gigantica with F. hepatica. To do this, we characterized the mt genomes of individual specimens of Fasciola sp. and F. gigantica whose identity was defined based on their ITS-1 and/or ITS-2 sequences, and assessed their relationships by comparison with F. hepatica and various other trematodes using complete, inferred mt amino acid sequence data sets.

**Methods**

**Ethics statement**

This study was approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Permit code: LVRIAEC2012-006). Adult specimens of Fasciola were collected from bovid, in accordance with the Animal Ethics Procedures and Guidelines of the People’s Republic of China.

**Parasites and isolation of total genomic DNA**

Adult specimens of Fasciola sp. were collected from the liver of a dairy cow (Bos taurus) in Heilongjiang province, China. Adult specimens of F. gigantica were collected from the liver of a buffalo (Bubalus bubalis) in Guangxi province, China. The worms were washed extensively in physiological saline, fixed in ethanol and then stored at −20°C until use. Single specimens were identified as Fasciola sp. or F. gigantica based on PCR-based sequencing of the ITS-1 and ITS-2 rDNA regions [11,12].

**Long-range PCR-based sequencing of mt DNA**

To obtain some mt gene sequence data for primer design, regions (400–500 bp) of the cox1 and nad4 genes were PCR-amplified and sequenced using relatively conserved primers JB3/JB4.5 and ALF/ALR [13,22], respectively. Using BigDy termino v.3.1 chemistry (Applied Biosystems, Weiterstadt, Germany), the amplicons were sequenced in both directions in a PRISM 3730 sequencer (ABI, USA). After sequencing regions of the cox1 and nad4 genes of both Fasciola sp. and F. gigantica, two internal pairs of conserved primers were designed (Table 1). These pairs were then used to long PCR-amplify the complete mt genome [23] in two overlapping fragments (cox1-nad4; ~9 kb and, nad4-cox1 = ~6 kb) from a portion of total genomic DNA (10–20 ng) from one individual of Fasciola sp. and another of F. gigantica. The cycling conditions used were 92°C for 2 min (initial denaturation), then 92°C for 10 s (denaturation), 58–63°C for 30 s (annealing), and 60°C for 5 min (extension) for 5 cycles, followed by 92°C for 2 min, 92°C for 10 s, 58–63°C for 30 s, and 66°C for 5 min for 20 cycles, and a final extension at 66°C for 10 min. Each amplicon, which represented a single band in a 0.8% (w/v) agarose gel, following electrophoresis and ethidium-bromide staining [23], was column-purified and then sequenced using a primer-walking strategy [24].

**Sequence analyses**

Sequences were manually assembled and aligned against each other, and then against the complete mt genome sequences of 11 other trematodes (see section on Phylogenetic analysis) using the program Clustal X 1.83 [25] and manual adjustment, in order to infer gene boundaries.

**Table 1 Sequences of primers used to amplify mt DNA regions from Fasciola spp.**

| Primer | Sequence (5’ to 3’) |
|--------|---------------------|
| F. gigantica |                    |
| FGCF1   | GTTTACTATTTGTTGTTGTTACTGT |
| FGNR1   | CAAACCTACAGAATATCCCTCCA |
| FGNF1   | GTATGAGTTGATCCCTTGAGGGAT |
| FGCR1   | CTTATCCAAAAGAGAAGCAGAACAGCA |
| FGKR1   | CGGTTTACGTTATATTGCTCTTCGCT |
| FGZNR1  | CCACTACGAACTATCCCTCCAAGACT |
| FGZNF1  | GGGTGTTATTGAGGCAGTTGAGGAT |
| FZCR1   | CAGAAAGCATAATACCCGTAACCCCA |
Open-reading frames (ORFs) were established using the program ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), employing the trematode mt code, and subsequently compared with those of *F. hepatica* [26]. Translation initiation and termination codons were identified based on comparisons with those of *F. hepatica* [26]. The secondary structures of 22 tRNA genes were predicted using tRNAscan-SE [27] with manual adjustment [28], and rRNA genes were predicted by comparison with those of *F. hepatica* [26].

**Sliding window analysis of nucleotide variation**

To detect variable nucleotide sites, pairwise alignments of the complete genomes, including tRNAs and all intergenic spacers, were performed using Clustal X 1.83. The complete mt genome sequences of *Fasciola* sp. and *F. gigantica* were aligned with that published previously for *F. hepatica* (NC_002546) [26], and sliding window analysis was conducted using DnaSP v.5 [29]. A sliding window of 300 bp (in 10 bp overlapping steps) was used to estimate nucleotide diversity *π* (n) across the alignment. Nucleotide diversity was plotted against mid-point positions of each window, and gene boundaries were identified.

**Phylogenetic analysis**

The amino acid sequences conceptually translated from individual genes of the mt genomes of each *Fasciola* sp. and *F. gigantica* were concatenated. For comparative purposes, amino acid sequences predicted from published mt genomes of selected members of the subclass Digenia, including *F. hepatica* (NC_002546) [26] [*Fasciolidae*]; *Clonorchis sinensis* (GeneBank accession no. FJ381664), *Opisthorchis felineus* (EU921260) [30] and *O. viverrini* (JF739555) [31] [*family Opisthorchiidae*]; *Paragonimus westermani* (NC_002354) [32] [*Paragonimidae*]; *Trichobilharzia regenti* (NC_009680) [33], *Orientobilharzia turkestanicum* (HQ283100) [33], *Schistosoma mansoni* (NC_002545) [34], *S. japonicum* (HM120846) [35], *S. mekongi* (NC_002529) [34], *S. spindale* (DQ157223) [36] and *S. haematobium* (DQ157222) [35] [*Schistosomatidae*], were also included in the analysis. A sequence representing *Gyrodactylus derjavinoides* (accession no. NC_010976) was included as an outgroup [37]. All amino acid sequences were aligned using the program MUSCLE [38] and subjected to phylogenetic analysis using Bayesian inference (BI), as described previously [39,40]. Phylogenograms were displayed using the program Tree View v.1.6.5 [41]. In addition, all publicly available sequences of NADH dehydrogenase subunit 1 gene (*nad1*) of *Fasciola* sp., *F. gigantica* and *F. hepatica* were aligned (over a consensus length of 359 bp) using MUSCLE, the alignment was modified manually, and then subjected to phylogenetic analysis by BI, applying the General Time Reversible (GTR) model. Nodal support values for the final phylogram were determined from the final 75% of trees obtained using a sample frequency of 100. The analysis was performed until the potential scale reduction factor approached 1 and the average standard deviation of split frequencies was less than 0.01. An *nad1* sequence of *Fascioloides magna* was used as an outgroup in phylogenetic analysis.

**Results**

**Identity of the two liver flukes, and features of the mt genomes**

The ITS-1 and ITS-2 sequences (GeneBank accession no. KF543341) of the specimen of *Fasciola* sp. from Heilongjiang province were the same as that of an ‘intermediate form’ of *Fasciola* from China (AJ628428, AJ557570 and AJ557571) reported previously [11,12], which is characterized by polymorphic positions at 10 positions in ITS-1 and ITS-2 (Additional file 1: Figure S1; Table 2). Based on these key polymorphic positions (cf. [11,12]), this specimen of *Fasciola* sp. from China was inferred to be a hybrid between *F. gigantica* and *F. hepatica*. The ITS-1 and ITS-2 sequences of the *F. gigantica* sample (accession no. KF543340) from Guangxi province were consistent with that of the same species from Niger (AM900371) and did not have any polymorphic positions (Table 2).

The complete mt genome sequences representing *Fasciola* sp. (GeneBank accession no. KF543343) and *F. gigantica* (accession no. KF543342) were 14,453 bp and 14,478 bp in size, respectively. Each mt genome contains 12 protein-coding genes (*cox1-3, nad1-6, nad4L, cytb and atp6), 22 transfer RNA genes and two ribosomal RNA genes (*rrnS* and *rrnL*), but lack an *atp8* gene (Figure 1). The mt genome arrangement of the two flukes is the same as that of *F. hepatica* [26], but as expected, distinct from *Schistosoma* spp. [36]. All genes are transcribed in the same direction and have a high A + T content (62.7%). The AT-rich regions of both mt genomes are located between tRNA-Glu and tRNA-Gly, and tRNA-Gly and *cox3*.

**Annotation**

For the two liver flukes, the protein-coding genes were in the following order: *nad5 > cox1 > nad4 > cytb > nad1 > nad2 > cox3 > cox2 > atp6 > nad6 > nad5 > nad4L*, and the lengths of the all protein-coding genes are the same for *Fasciola* sp. and *F. gigantica* (Table 3). The inferred nucleotide and amino acid sequences of each of the 12 mt proteins of two liver flukes were compared. A total of 3,356 amino acids are encoded in the both mt genomes. All protein-coding genes have ATG, TTG or GTG as their initiation codon (Table 3). All protein-coding genes have TAG as their termination codon, except for *cox3* and *nad3*, which have TAA in *Fasciola* sp. (Table 3). No abbreviated stop codons, such as TA or T, were detected. Twenty-two tRNA genes were predicted from the mt genomes of the two liver flukes, and varied from 55 to

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69 bp in size. Of all tRNA genes, 20 can be folded into the conventional four-arm cloverleaf structures. The tRNA-tRNA-Ser(UCN) and tRNA-Ser(AGN) show unorthodox structures; their D-arms are unpaired and replaced by the loops of 8–11 bp.

The two ribosomal RNA genes (rrnL and rrnS) of Fasciola sp. and F. gigantica were inferred based on comparisons with sequences from those of F. hepatica. The rrnL of Fasciola sp. and F. gigantica is located between tRNA-Thr and tRNA-Cys, and rrnS is located between tRNA-Cys and cox2. The length of rrnL is 987 bp for both Fasciola sp. and F. gigantica. The size of the rrnS genes is 769 bp and 771 bp for Fasciola sp. and F. gigantica, respectively. The A + T contents of rrnL and rrnS are ~62% and ~61% for Fasciola sp. and F. gigantica, respectively.

Two AT-rich non-coding regions (NCR) in the mt genomes of Fasciola sp. and F. gigantica were inferred. In both mt genomes, the long NCR (841 bp) is located between the tRNA-Gly and cox3 (Figure 1), has an A + T content of ~53% and contains eight perfect, 86 bp tandem repeats (TR1 to TR8). The short NCR is 174–176 bp in length, is located between tRNA-Glu and tRNA-Gly (Figure 1) and has an A + T content of ~72%.

The complete mt genome sequences representing Fasciola sp. and F. gigantica are 9 bp shorter and 16 bp longer than F. hepatica (14,462 bp in length) [26], respectively. A comparison of the nucleotide sequences of each mt gene, and the amino acid sequences, conceptually translated from all mt protein-encoding genes of the three flukes, is given in Table 4. Across the entire mt genome, the sequence difference was 2.6% (380 nucleotide substitutions) between Fasciola sp. and F. gigantica, 11.8% (1712 nucleotide substitutions) between Fasciola sp. and F. hepatica, and 11.8% (1714 nucleotide substitutions).
between *F. gigantica* and *F. hepatica*. The difference across both nucleotide and amino acid sequences of the 12 protein-coding was 11.6% (1167 nucleotide substitutions) and 9.54% (320 amino acid substitutions) between the *Fasciola* sp. and *F. hepatica*; 11.6% (1167 nucleotide substitutions) and 9.83% (330 amino acid substitutions) between the *F. gigantica* and *F. hepatica*; and 2.8% (281 nucleotide substitutions) and 2.1% (71 amino acid substitutions) between the *Fasciola* sp. and *F. gigantica*, respectively.

### Table 3 The organization of the mt genomes of *Fasciola* sp., *Fasciola gigantica* and *F. hepatica*

| Genes | Positions and nt sequence lengths (bp) | Ini/Ter codons |
|-------|----------------------------------------|----------------|
|       | *Fasciola* sp. | *Fasciola gigantica* | *F. hepatica* | *Fasciola* sp. | *Fasciola gigantica* | *F. hepatica* |
| **cox3** | 1-642 | 1-642 | 1-642 | ATG/TAA | ATG/TAG | ATG/TAG |
| tRNA-His | 650-713 (64) | 650-713 (64) | 650-713 (64) | ATG/TAG | ATG/TAG | ATG/TAG |
| **cytb** | 715-1827 | 715-1827 (62) | 715-1827 | ATG/TAG | ATG/TAG | ATG/TAG |
| **nad4L** | 1836-2108 | 1836-2108 | 1836-2108 | GTG/TAG | GTG/TAG | GTG/TAG |
| **nad4** | 2069-3337 | 2069-3337 | 2069-3340 | GTG/TAG | GTG/TAG | GTG/TAG |
| tRNA-Gln | 3339-3404 (66) | 3339-3404 (66) | 3342-3404 (63) | GTG/TAG | GTG/TAG | GTG/TAG |
| tRNA-Phe | 3420-3484 (65) | 3417-3481 (65) | 3417-3482 (66) | GTG/TAG | GTG/TAG | GTG/TAG |
| tRNA-Met | 3491-3556 (66) | 3488-3553 (66) | 3494-3561 (68) | GTG/TAG | GTG/TAG | GTG/TAG |
| **atp6** | 3557-4075 | 3554-4072 | 3562-4080 | ATG/TAG | ATG/TAG | ATG/TAG |
| **nad2** | 4088-4954 | 4085-4951 | 4093-4959 | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Val | 4959-5021 (63) | 4957-5020 (64) | 4965-5027 (63) | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Ala | 5035-5099 (65) | 5035-5099 (65) | 5042-5104 (63) | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Asp | 5103-5167 (65) | 5103-5167 (65) | 5107-5172 (66) | ATG/TAG | ATG/TAG | ATG/TAG |
| **nad1** | 5171-6073 | 5171-6073 | 5176-6078 | GTG/TAG | GTG/TAG | GTG/TAG |
| tRNA-Asn | 6079-6146 (68) | 6084-6153 (67) | 6089-6158 (67) | GTG/TAG | GTG/TAG | GTG/TAG |
| tRNA-Pro | 6152-6220 (69) | 6163-6230 (68) | 6168-6234 (67) | GTG/TAG | GTG/TAG | GTG/TAG |
| tRNA-Ile | 6221-6282 (62) | 6231-6292 (62) | 6235-6296 (62) | GTG/TAG | GTG/TAG | GTG/TAG |
| tRNA-Lys | 6287-6352 (66) | 6297-6363 (67) | 6301-6367 (67) | GTG/TAG | GTG/TAG | GTG/TAG |
| **nad3** | 6353-6709 | 6364-6720 | 6368-6724 | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Ser [JCN] | 6714-6768 (55) | 6725-6780 (56) | 6731-6788 (58) | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Trp | 6771-6833 (63) | 6790-6852 (63) | 6796-6858 (63) | ATG/TAG | ATG/TAG | ATG/TAG |
| **cox1** | 6837-8378 | 6865-8397 | 6871-8403 | GTG/TAG | GTG/TAG | GTG/TAG |
| tRNA-Thr | 8391-8458 (68) | 8419-8486 (68) | 8420-8488 (69) | GTG/TAG | GTG/TAG | GTG/TAG |
| tRNA-Cys | 8460-9445 | 8488-9473 | 8489-9475 | GTG/TAG | GTG/TAG | GTG/TAG |
| **rml** | 9446-9510 (65) | 9474-9538 (65) | 9476-9538 (63) | GTG/TAG | GTG/TAG | GTG/TAG |
| **rrnL** | 9511-10279 | 9539-10309 | 9539-10304 | GTG/TAG | GTG/TAG | GTG/TAG |
| **rrnS** | 10280-10882 | 10310-10912 | 10305-10907 | ATG/TAG | ATG/TAG | ATG/TAG |
| **nad6** | 10929-11381 | 10959-11411 | 10950-11402 | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Tyr | 11389-11445 (57) | 11419-11475 (57) | 11411-11467 (67) | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Leu [LUN] | 11456-11520 (65) | 11486-11550 (65) | 11478-11543 (66) | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Ser [AGN] | 11521-11579 (59) | 11551-11607 (57) | 11542-11603 (62) | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Leu [LUR] | 11588-11651 (64) | 11616-11678 (63) | 11609-11673 (64) | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Arg | 11653-11718 (66) | 11680-11745 (66) | 11673-11738 (66) | ATG/TAG | ATG/TAG | ATG/TAG |
| **nad5** | 11720-13282 | 11747-13309 | 11737-13305 | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Glu | 13305-13372 (68) | 13332-13399 (68) | 13327-13395 (69) | ATG/TAG | ATG/TAG | ATG/TAG |
| **Short non-coding region** | 13373-13548 (176) | 13400-13573 (174) | 13396-13582 (187) | ATG/TAG | ATG/TAG | ATG/TAG |
| tRNA-Gly | 13549-13612 (64) | 13574-13637 (64) | 13583-13645 (63) | ATG/TAG | ATG/TAG | ATG/TAG |
| **Long non-coding region** | 13613-14453 (841) | 13638-14478 (841) | 13646-14462 (817) | ATG/TAG | ATG/TAG | ATG/TAG |
Nucleotide variability in the mt genome among *Fasciola* sp., *F. gigantica* and *F. hepatica*

Sliding window analysis across the mt genomes of *Fasciola* sp., *F. gigantica* and *F. hepatica* provided an estimation of nucleotide diversity $\pi$ for individual mt genes (Figure 2). By computing the number of variable positions per unit length of gene, the sliding window indicated that the highest and lowest levels of sequence variability were within the genes *nad6* and *cytb*, respectively. Conserved regions were identified within *nad1* and *cox1* genes. In this study, the *cytb* and *nad1* genes are the most conserved protein-coding genes, and *nad6*, *nad5* and *nad4* are the least conserved.

**Phylogenetic analysis**

Phylogenetic analysis of the concatenated amino acid sequence data for all 12 mt proteins (Figure 3) showed that the Fasciolidae clustered to the exclusion of representatives of the families Paragonimidae (*P. westermani*) and Opisthorchiidae (*O. viverrini*, *O. felineus* and *C. sinensis*); the Schistosomatidae clustered separately with strong nodal support (posterior probability (pp) = 1.0). Within the Fasciolidae, *Fasciola* sp. and *F. gigantica* clustered together with strong support (pp = 1.0), to the exclusion of *F. hepatica*, with the former two taxa being more closely related than either was to *F. hepatica*. In addition, phylogenetic analysis using the *nad1* data supports clustering of

![Figure 2](image-url)
the *Fasciola* sp. with aspermic *F. gigantica* x *F. hepatica* hybrids characterised previously [42] (Additional file 2: Figure S2).

**Discussion**

The present comparative, genetic investigation of representative specimens of *Fasciola* sp. (i.e. the ‘intermediate form’), *F. gigantica* and *F. hepatica* using whole mt genomic and protein sequence data sets showed that *Fasciola* sp. and *F. gigantica* were more closely related than either was to *F. hepatica*. This finding was also supported by an analysis of nad1 sequence data (cf. Additional file 2: Figure S2). Although this evidence might suggest that *Fasciola* sp. is a population variant of *F. gigantica*, previous studies [19-21] have proposed that *Fasciola* sp. is a hybrid of *F. gigantica* and *F. hepatica*. The combined use of mtDNA (if indeed maternally inherited in fasciolids) and nuclear DNA markers [43] should assist in exploring the "hybridization/speciation" hypotheses [44]. Clearly, there is consistent evidence from various studies [11,12,14] of mixed ITS-1 and ITS-2 sequence types, representing both *F. gigantica* and *F. hepatica* among the multiple rDNA copies, within individual specimens of *Fasciola* sp. (i.e., the ‘intermediate form’). Although the number or proportion(s) of different sequence types within individual adults of *Fasciola* sp. has not yet been estimated using a mutation scanning- or cloning-based sequencing [45], the polymorphic positions in the sequences determined by direct sequencing [11,14] indicate a clear pattern of introgression between the *F. gigantica* and *F. hepatica*. Although mt genomic (11.8%) and inferred protein (9.83%) sequence differences between these two species is substantial, the explanation that *Fasciola* sp. represents a hybrid between these two recognized species seems plausible, given that the karyotypes of both diploid *F. hepatica* and *F. gigantica* are the same (2n = 20) [46,47] and that the magnitude of sequence variation (1.7%) in ITS-2 (a species marker) between *F. gigantica* and *F. hepatica* is comparable with the highest level (1.3-1.6%) in this rDNA region between some schistosome species for which hybrids (i.e. *S. haematobium* x *S. bovis; S. haematobium* x *S. guineenis; *S. haematobium* x *S. intercalatum*) have been reported [48-50]. While hybridization seems possible, another explanation might be ITS rDNA "lineage sorting and retention of ancestral polymorphism" [51,52], but this is perhaps less likely, given a clear pattern of mixing of ITS sequences seen in *Fasciola* sp. (cf. Additional file 1: Figure S1).

In addition, polyploidy or diplody in aspermic *Fasciola* [20] needs to be considered, and warrants future investigation. Perhaps the aspermic *Fasciola* specimens described in the literature [53] were infertile hybrids of *F. gigantica* and *F. hepatica* (in situations where both species occur in sympatry). Questions that might be addressed directly in relation to *Fasciola* sp. are: Are eggs from *Fasciola* sp. fertilized and viable? If miracidia develop and emerge from these eggs, are they infective to snails? If they do infect snails, do the ensuing adult worms (in the definitive host) contain sperm and are these worms fertile, and what is their ploidy? These questions should be addressed, and could be complemented by detailed light and transmission electron microscopic investigations of a relatively large number of adult specimens of *Fasciola* sp., *F. gigantica* and *F. hepatica* (preferably from different countries), which have been unequivocally and individually identified based on their ITS-1 and ITS-2 sequences. Such a study...
should pay particular attention to the morphology of the reproductive organs, sperm and oocytes, and the karyotypes of worms, and establish whether or not *Fasciola* sp. from China is polyploid and/or aspermic [20].

Moreover, although challenging, laborious and time-consuming, it would be highly informative to conduct hybridization studies *in vivo*, whereby individual miracidia from eggs from adults of each *Fasciola* sp., *F. gigantica* and *F. hepatica* would be used to infect (separately) their lymnaeid snail hosts, to raise clonal populations of cercariae and metacercariae of these three taxa, so that mixed infections (in different combinations and with monospecific controls) could be established in, for example, sheep or goats, to attempt to cross-hybridize the three taxa in a pairwise manner. Using such an experimental design, eggs and adult worms could then be examined in detail at both the electron microscopic, karyotypic and molecular levels. Importantly, in these experiments, ITS-1 and/or ITS-2 could be used to establish the genotypes of subsamples of individuals, and mt markers derived from mt genomes determined here and of *F. hepatica* could be used to determine haplotypes and mtDNA inheritance if the cross-hybridization studies were successful. Therefore, the present markers could be employed, in combination, to establish the biological relationship of the three taxa through *in vivo* experiments, but also in the field in sympatric and allopatric populations, if they occur. Combined with the use of markers in nuclear and mt genomes, advanced genomic sequencing, optical mapping and micro-imaging techniques might assist studies of *Fasciola* sp. in China and other countries.

**Conclusion**

The findings of this study provide robust genetic evidence that *Fasciola* sp. is more closely related to *F. gigantica* than to *F. hepatica*. The mtDNA datasets reported in the present study should provide useful novel markers for further studies of the taxonomy and systematics of *Fasciola* from different hosts and geographical regions.

**Additional files**

**Additional file 1:** Figure S1. Polymorphic positions in the internal transcribed spacer regions (ITS-1 and ITS-2) of nuclear ribosomal DNA of *Fasciola* sp.

**Additional file 2:** Figure S2. Phylogenetic tree of *Fasciola* spp. inferred from the mitochondrial nad1 sequence data by Bayesian inference (B). *Fascioloides magna* was used as an outgroup. Nodal support values were determined from the final 75% of trees using a sampling frequency of 100.

**Competing interests**

The authors declare that they have no competing interests.
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