Complete mitochondrial genome of the giant liver fluke *Fascioloides magna* (Digenea: Fasciolidae) and its comparison with selected trematodes

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**Abstract**

**Background:** Representatives of the trematode family Fasciolidae are responsible for major socio-economic losses worldwide. *Fascioloides magna* is an important pathogenic liver fluke of wild and domestic ungulates. To date, only a limited number of studies concerning the molecular biology of *F. magna* exist. Therefore, the objective of the present study was to determine the complete mitochondrial (mt) genome sequence of *F. magna*, and assess the phylogenetic relationships of this fluke with other trematodes based on the mtDNA dataset.

**Findings:** The complete *F. magna* mt genome sequence is 14,047 bp. The gene content and arrangement of the *F. magna* mt genome is similar to those of *Fasciola* spp., except that *trnE* is located between *trnG* and the only non-coding region in *F. magna* mt genome. Phylogenetic relationships of *F. magna* with selected trematodes using Bayesian inference (BI) was reconstructed based on the concatenated amino acid sequences for 12 protein-coding genes, which confirmed that the genus *Fascioloides* is closely related to the genus *Fasciola*; the intergeneric differences of amino acid composition between the genera *Fascioloides* and *Fasciola* ranged 17.97–18.24 %.

**Conclusions:** The determination of *F. magna* mt genome sequence provides a valuable resource for further investigations of the phylogeny of the family Fasciolidae and other trematodes, and represents a useful platform for designing appropriate molecular markers.

**Keywords:** *Fascioloides magna*, *Fasciola*, Mitochondrial genome, Phylogenetic analysis

**Background**

*Fascioloides magna* (Bassi, 1875), the type- and only species of the genus *Fascioloides* Ward, 1917, was first described as *Distomum magnum* in 1875 [1]. Latter in 1917, Ward erected the genus *Fascioloides* for *Fasciola magna* (Bassi, 1875) [2]. *Fascioloides magna*, known as the large American liver fluke, giant liver fluke or deer fluke, is an important digenetic trematode of the family Fasciolidae [3, 4]. This species, which is of North America origin [5, 6] and invasive in European countries [7], has high potential to colonize new geographic territories (a variety of wild and domestic ungulates [3, 8–10]), and can establish expanding populations from a natural epidemic focus through translocated hosts [5, 6, 11]. Migration of *F. magna* immature flukes within the host body often leads to profound damage to the liver and other organ tissues [8, 12], causing economic losses worldwide [13].

The consequences of infection of various intermediate and definitive hosts by *F. magna* has been intensively studied [8, 12], but the relevant molecular research of this fluke has not received enough attention [4, 9]. To date, a sequence of nuclear ribosomal DNA (rDNA) of *F. magna* was obtained in 2008 [14], partial sequences of mitochondrial (mt) genes, such as cytochrome *c* oxidase...
subunit I (cox1) and nicotinamide dehydrogenase subunit I (nad1) were characterized [3]. According to these data, *F. magna* was divided into two mt haplotype groups [5, 14, 15], the first haplotype representing isolates from western North America and Italy, and the second haplotype representing isolates from eastern North America and some European countries such as Czech Republic, Poland and Croatia [3, 5]. Recently, the *F. magna* transcriptome was reported, which provides a useful platform for further fundamental studies of this fluke [16], but complete mt genome of *F. magna* is still unavailable.

Molecular tools, using genetic markers in mitochondrial DNA (mtDNA) sequences, have been proven reliable in identification and differentiation of trematode species [17–20]. In the present study we determined the mitochondrial genome sequence of *F. magna* (Czech isolate) using PCR-coupled sequencing technique combined with bioinformatic analysis, and for the first time assessed its phylogenetic relationship with selected trematodes based on the nucleotide- and inferred amino acid sequences of the protein-coding genes.

**Methods**

**Sampling and DNA extraction**

Three adult *F. magna* worms were isolated from livers of naturally infected red deer (*Cervus elaphus*), hunted at Kokořínsko area, Czech Republic. Worms were washed in 0.1 M phosphate-buffered saline (PBS), pH 7.2, fixed in 70 % (v/v) ethanol and preserved at -20 °C, until further use. Total genomic DNA was extracted from individual *F. magna* specimens using sodium dodecyl sulfate (SDS)/proteinase K treatment [21] and column-purification (Wizard® SV Genomic DNA Purification System, Promega, Madison, USA), according to the manufacturer’s protocol.

**Acquisition of ITS rDNA and sample identification**

The internal transcribed spacer (ITS) rDNA region of each of the three *F. magna* specimens, spanning partial 18S rDNA, the complete ITS-1, 5.8S rDNA, ITS-2, and partial 28S rDNA, was amplified using primers BD1 (forward; 5’-GTC GTA ACA AGG TTT CCG TA-3’ and BD2 (reverse; 5’-ATG CTT AAA TTC AGC GGG T-3’) [22] and sequenced using the same primers. These *F. magna* samples had ITS-1 and ITS-2 sequences identical to the corresponding sequences available on GenBank (EF051080).

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

The primers were designed based on relatively conserved regions of mtDNA sequences from *Fasciola hepatica* and *Fasciola gigantica*. The entire mt genome from a single specimen of *F. magna* was amplified in 5 overlapping fragments, using the primers shown in Additional file 1: Table S1.

PCR reactions were conducted in a total volume of 50 μl, using 25 μl PrimeStar Max DNA polymerase premix (Takara, Dalian, China), 25 pmol of each primer (synthesized in Genewiz, Suzhou, China), 0.5 μl DNA templates, and H2O, in a thermocycler (Biometra, Göttingen, Germany). PCR cycling conditions started with an initial denaturation at 98 °C for 2 min, followed by 22 cycles of denaturation at 92 °C for 18 s, annealing at 52–65 °C for 12 s and extension at 60 °C for 1–5 min, followed by 92 °C denaturation for 2 min, plus 25 cycles of 92 °C for 18 s (denaturation), 50–67 °C for 12 s (annealing) and 66 °C for 3–6 min, with a final extension step for 10 min at 66 °C. A negative control (no DNA) was included in each amplification run. Amplicons (2.5 μl) were electrophoresed in a 2 % agarose gel, stained with Gold View I (Solarbio, Beijing, China) and photographed by GelDoc - It TS™ Imaging System (UVP, USA).

**Assembly, annotation and bioinformatics analysis**

Sequences were assembled manually and aligned against the entire mt genome sequences of *F. hepatica* (GenBank accession No. NC_002546) and *F. gigantica* (NC_024025) using MAFFT 7.122 to infer boundaries for each gene. Amino acid sequences of 12 protein-coding genes were translated using MEGA v.6.06 and NCBI translation Table 21 (Trematode Mitochondrial Code). The tRNA genes were affirmed using the programs tRNAscan-SE [23] and ARWEN (http://130.235.46.10/ARWEN/) or by comparison with those from the *F. hepatica* and *F. gigantica* mt genomes. The two tRNA genes were identified by comparison with those of *F. hepatica* and *F. gigantica*.

A comparative analysis of the nucleotide sequences of each protein-coding gene, the amino acid sequences, two ribosomal RNA genes, 22 tRNA genes as well as non-coding regions (NCRs) among *F. magna*, *F. hepatica* and *F. gigantica* was conducted.

**Phylogenetic analysis**

The concatenated amino acid sequences of *F. magna* mt genome, conceptually translated from individual genes of each mt genome, were aligned with those of published mt genomes from selected trematodes, including *Opisthorchis felineus* (GenBank accession No. EU_921260) and *Clonorchis sinensis* (FJ_381664) (Opisthorchidae); *Metagonimus yokogawai* (KC_330755) and *Haplorchis taichui* (KF_214770) (Heterophyidae); *Paragonimus westermani* Japanese isolate (AF219379) and *Paragonimus westermani* Indian isolate (NC_027673) (Paragonimidae); *F. hepatica*, *Fasciola* sp. (KF_543343) and *F. gigantica* (Fasciolidae); *Hypoderaeum* sp. (KMI111525) (Echinostomatidae); *Paramphistomum*
leydeni (KP341657) and Fiscoederius elongatus (KM397348) (Paramphistomatidae); Diplostomum spathaceum (KR269763) and Diplostomum pseudopathaceum (KR269764) (Diplostomidae); Ogmodoctyle sikae (KR006934) (Notocotylidae); Eurytrema pancreaticum (KP241855) (Dicrocoeliidae); Schistosoma turkestanicum (HQ_283100) and Schistosoma japonicum (HM_120842) (Schistosomatidae). The sequence for the monogenean Gyrodactylus derjavinoides (NC_010976) (Gyrodactylidae), was included as the outgroup.

All inferred amino acid sequences were aligned using MAFFT 7.122. Poorly aligned sites and divergent regions of the alignment were eliminated using Gblocks Server v. 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) using default settings, selecting the option of less strict conservation of flanking positions. The alignment was then converted into nexus format using Clustal X1.83 and subjected to phylogenetic analysis using Bayesian inference (BI). A mixed model was used in BI analysis using MrBayes 3.1.1 [24], because the most suitable amino acid evolution model JTT + G + F, selected by ProtTest 3.4 based on the Akaike information criterion (AIC) [25], was not available in the current MrBayes version. Four independent Markov chain were run for 10,000,000 metropolis-coupled MCMC generations, sampling trees every 1,000 generations. The first 2,500 trees (25 %) were discarded as ‘burn-in,’ and the remaining trees were used for calculating Bayesian posterior probabilities. The analysis was regarded as completed when the potential scale reduction factor was close to 1, and the average standard deviation of split frequencies was below 0.01. Phylograms were prepared using FigTree v. 1.42 [26].

Findings
Genome content and organization
The complete mt genome sequence of F. magna (GenBank accession no. KR006934) is 14,047 bp in length (Fig. 1) and contains 36 genes that are transcribed in the same direction, including 12 protein-coding genes (nad1-6, nad4L, cox1-3, atp6 and cyt b), 22 tRNA genes and two rRNA genes (rrnL and rrnS), lacking the atp8 gene (Table 1), consistent with those of selected trematode species available on GenBank [17–19, 27, 28]. There is only one NCR in F. magna mt genome, whereas the mt genomes of Fasciola flukes have two non-coding regions [17, 27].

The arrangement of genes in the F. magna mt genome is similar to that of Fasciola spp. [17], except that only one non-coding region (NCR) in F. magna mt genome is located between trnE (13,355–13,422) and cox3 (1–645) (Table 1). The gene order of F. magna mt genes is similar to that in species of the Paramphistomatidae, Notocotylidae, Echinostomatidae, Heterophyidae and Opisthorchiidae, but is distinct from some flukes of the Schistosomatidae (S. mansoni, S. spindale and S. haematobium) [29].

The nucleotide composition of F. magna mt genome is obviously biased towards A and T. The value of total A + T content for F. magna mtDNA is 61.42 %, within the range recognized in other trematode mt genomes (54.38 % in Paragonimus westerni Indian isolates [30], 72.71 % in Schistosoma spindale [29]). The content of C is low (10.3 %) and that of T is high (44.0 %). The A + T content for each gene or region of F. magna mt genome ranged from 48.48 % (trnL2) to 68.18 % (trnG) (nad3, 64.43 %; cox2, 59.7 %). All 12 protein-coding genes of F. magna mtDNA possess a lower A + T percentage than those of Fa. hepatica and Fa. gigantica [17, 27], except for nad5 (Additional file 2: Table S2).

Annotation of F. magna mt genome
In the mt genome of F. magna, the protein-coding genes had ATG or GTG as start codons and TAG or TAA as stop codons (Table 1). Half of the protein-coding genes of F. magna were initiated with GTG (nad4L, nad4, nad1, cox1, nad6 and nad5). Incomplete codons were not detected in the mt genome of F. magna.

The 22 tRNA genes of F. magna mt genome ranged from 57 to 69 bp in length. The structure of all tRNA sequences is similar to those of Fa. hepatica and Fa. gigantica [17, 27]. The large ribosomal RNA gene (rrnL) and the adjacent small ribosomal RNA gene (rrnS) are located between trnT and cox2, and separated by trnC.
The length of the *rrn* RNA genes is 984 bp and 765 bp, respectively. The only NCR of *F. magna* mt genome is of 520 bp in length, and is located between *trnE* and *cox3*. It contains two complete direct repeats: six copies of a 23 nt - repeat A (AGA TAG GAT AGG CAT CTG GTA TA) and five copies of a 37 nt - repeat B (GGT GCC CCC GGT GAA GGG GGA AAA GGA AGG TTG TAA G). There are five AB repeats, with one A at the end (located at positions 13,620–13,642).

### Comparative analysis among mt genomes of *F. magna, Fa. hepatica* and *Fa. gigantica*

The difference between complete mt genomes of *F. magna* and *Fa. hepatica* was 22.66 % (3,290 nt), which is

| Gene   | Coding position (5′–3′) | Length (bp) | Start/Stop codons | No. of amino acids | Intergenic nucleotides |
|--------|-------------------------|-------------|-------------------|-------------------|------------------------|
| *cox3* | 1–645                   | 645         | ATG/TAA           | 215               | 4                      |
| *trnH* | 650–713                 | 64          |                   | 1                 |                        |
| *cytb* | 715–1827                | 1113        | ATG/TAG           | 371               | 7                      |
| *nad4L*| 1835–2107               | 273         | GTG/TAG           | 91                | -40                    |
| *nad4* | 2068–3348               | 1281        | GTG/TAG           | 427               | 1                      |
| *trnQ* | 3350–3412               | 63          |                   | 11                |                        |
| *trnF* | 3424–3486               | 63          |                   | 14                |                        |
| *trnM* | 3501–3566               | 66          |                   | 0                 |                        |
| *atp6* | 3567–4085               | 516         | ATG/TAA           | 172               | 4                      |
| *nad2* | 4090–4959               | 870         | ATG/TAG           | 290               | 2                      |
| *trnV* | 4962–5023               | 62          |                   | 7                 |                        |
| *trnA* | 5031–5092               | 62          |                   | 6                 |                        |
| *trnD* | 5099–5160               | 62          |                   | 1                 |                        |
| *nad1* | 5162–6064               | 903         | GTG/TAG           | 301               | 7                      |
| *tmN*  | 6072–6137               | 66          |                   | 4                 |                        |
| *tmP*  | 6142–6210               | 69          |                   | 0                 |                        |
| *tmI*  | 6211–6273               | 63          |                   | 5                 |                        |
| *tmK*  | 6279–6343               | 65          |                   | 0                 |                        |
| *nad3* | 6344–6700               | 357         | ATG/TAA           | 119               | 4                      |
| *tmS1* | 6705–6763               | 59          |                   | 8                 |                        |
| *tmW*  | 6772–6836               | 65          |                   | 3                 |                        |
| *cox1* | 6840–8384               | 1545        | GTG/TAG           | 515               | 23                     |
| *tm T* | 8408–8469               | 62          |                   | 0                 |                        |
| *rrnL* | 8470–9453               | 984         |                   | 2                 |                        |
| *tmC*  | 9456–9518               | 63          |                   | -2                |                        |
| *rrnS* | 9517–10281              | 765         |                   | 2                 |                        |
| *cox2* | 10284–10886             | 603         | ATG/TAG           | 201               | 32                     |
| *nad6* | 10919–11371             | 453         | GTG/TAG           | 151               | 0                      |
| *tmY*  | 11372–11428             | 57          |                   | 12                |                        |
| *tmL1* | 11441–11504             | 64          |                   | 2                 |                        |
| *tmS2* | 11506–11566             | 60          |                   | 10                |                        |
| *tmL2* | 11577–11642             | 66          |                   | -3                |                        |
| *tmR*  | 11640–11705             | 66          |                   | -2                |                        |
| *nad5* | 11704–13272             | 1569        | GTG/TAG           | 523               | 10                     |
| *tmG*  | 13283–13348             | 66          |                   | 6                 |                        |
| *tmE*  | 13355–13422             | 68          |                   | 0                 |                        |
| *NCR*  | 13423–14047             | 520         |                   | 0                 |                        |

Abbreviation: NCR Non-coding region
close to that between *F. magna* and *Fa. gigantica* (22.65 %, 3,297 nt) (Table 2). Considering the 12 protein-coding genes, different nucleotides were present at 18.80 % of positions (1,897 nt) between *F. magna* and *Fa. hepatica*, and at 18.62 % of positions (1,879 nt) between *F. magna* and *Fa. gigantica*. At the inferred amino acid level, there were 605 substitutions (17.97 %) of amino acids between *F. magna* and *Fa. hepatica*, and 614 substitutions (18.24 %) between *F. magna* and *Fa. gigantica* (Table 2).

At the nucleotide level, sequence differences in protein-coding genes ranged from 13.1 to 24.2 % (between *F. magna* and *Fa. hepatica*) and from 12.8 to 26.2 % (between *F. magna* and *Fa. gigantica*), with *cox1*, *nad4L* and *cytb* being the most conserved genes, and *nad6*, *nad5* and *nad2* being the least conserved genes among those three species. At the amino acid level, sequence differences ranged from 9.2 to 25.4 % between *F. magna* and *Fa. hepatica*, and from 8.4 to 27.8 % between *F. magna* and *Fa. gigantica*: *cox1*, *cytb*, *nad4L* and *nad1* were the most conserved protein-coding genes, while *nad6*, *nad2* and *nad5* were the least conserved.

Comparisons between the mt genomes of *F. magna* and *Fasciola* spp., at both nucleotide and amino acid levels, indicate that the most conserved and the least conserved gene in the Fasciolidae are *cox1* and *nad6*, respectively. Besides, the *nad5* is highly variable, and genes of *nad4L* and *cytb* are rather conserved. These characteristics are in accordance with flukes of the families Paramphistomatidae and Notocotylidae [18, 28].

Nucleotide differences were also found in ribosomal RNA genes: between *F. magna* and *Fa. hepatica* (*rrnL*, 18.3 %; *rrnS*, 22.2 %) and between *F. magna* and *Fa. gigantica* (*rrnL*, 16.6 %; *rrnS*, 21.4 %) as well as in tRNA genes (16.3 % between *F. magna* and *Fa. hepatica* and 16.0 % between *F. magna* and *Fa. gigantica*). Meaningful sequence

### Table 2: Comparison of nucleotides and predicted amino acids sequences among *Fascioloides magna* (Fm), *Fasciola hepatica* (Fh) and *Fasciola gigantica* (Fg)

| Gene   | nt difference (%) | aa difference (%) |
|-------|------------------|------------------|
|       | Fm/Fh            | Fm/Fg            | Fh/Fg            | Fm/Fh            | Fm/Fg            | Fh/Fg            |
| cox3  | 19.8             | 18.7             | 13.4             | 20.1             | 22.4             | 13.1             |
| cyt b | 16.1             | 16.4             | 8.3              | 11.6             | 13.2             | 6.5              |
| nad4L | 15.3             | 16.1             | 8.4              | 12.1             | 15.4             | 5.5              |
| nad4  | 21.4             | 20.9             | 13.5             | 21.7             | 20.8             | 10.6             |
| atp6  | 21.3             | 21.2             | 13.8             | 22.0             | 20.2             | 13.3             |
| nad2  | 21.1             | 22.8             | 11.6             | 25.4             | 24.7             | 11.4             |
| nad1  | 15.0             | 14.9             | 8.4              | 13.0             | 14.3             | 6.6              |
| nad3  | 19.0             | 19.0             | 10.6             | 13.4             | 15.1             | 7.6              |
| cox1  | 13.1             | 12.8             | 9.1              | 9.2              | 8.4              | 5.5              |
| cox2  | 19.2             | 19.2             | 11.6             | 14.4             | 15.4             | 6.5              |
| nad6  | 24.2             | 26.2             | 16.3             | 22.5             | 27.8             | 13.2             |
| nad5  | 21.5             | 19.8             | 13.7             | 23.6             | 22.8             | 12.3             |
| rrnL  | 18.3             | 16.6             | 10.6             |                  |                  |                  |
| rrnS  | 22.2             | 21.4             | 11.1             |                  |                  |                  |
| 22 tRNAs | 16.3           | 16.0             | 9.9              |                  |                  |                  |
| Overall | 22.7             | 22.7             | 12.2             | 17.97            | 18.24            | 9.4              |

**Table 2** Comparison of nucleotides and predicted amino acids sequences among *Fascioloides magna* (Fm), *Fasciola hepatica* (Fh) and *Fasciola gigantica* (Fg).

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**Fig. 2** Phylogenetic relationships of *Fascioloides magna* and other trematodes. Tree inferred from the concatenated amino acid sequence dataset for 12 protein-coding genes from 19 trematodes was performed by Bayesian inference (BI). *Gyrodactylus derjavinioides* (NC_010976) was chosen as the outgroup.
comparisons of NCRs in mt genomes of the three fascioloid trematodes is not possible, because there is only one NCR present in *F. magna* mt genome, while in both *Fa. hepatica* and *Fa. gigantica* there are two NCRs.

**Phylogenetic analysis**

In the phylogenetic tree inferred from the concatenated amino acid sequence dataset of all 12 mt proteins (Fig. 2) *F. magna* clustered with three other *Fasciola* species with strong support (Bpp = 1). The closest family to the Fasciolidae is Echinostomatidae, represented by *Hypoderaeum* sp. The taxonomic relationships of the selected trematodes are in concordance with results of previous studies [17–19, 28]. Each node received the maximum possible nodal support (Bpp = 1).

In several recent phylogenetic studies, the *F. magna* was characterized only based on partial 28S rDNA [31] and combined ITS1, ITS2 and *nad1* sequences [32]. The relationship between the genera *Fasciola* and *Fasciolopsis* was considered as being very close and the genetic relationship between *F. magna* and *Fasciola jacksoni* (or *Fascioloides jacksoni*) is disputable [31–33]. Further studies are warranted to determine the mt genome of *Fa. jacksoni* and solve this controversy in the family Fasciolidae.

**Conclusions**

The present study determined the complete mt genome sequence of the pathogenic liver fluke *F. magna* and revealed its close relationship with the species of *Fasciola*. The complete mt genome data of *F. magna* provides a resource for further investigations of the phylogeny, epidemiology, biology and population genetics of the family Fasciolidae and other trematodes.

**Additional files**

**Additional file 1: Table S1.** Sequences of primers used to amplify fragments of *Fascioloides magna* mitochondrial genome. (DOCX 13 kb)

**Additional file 2: Table S2.** Comparison of A + T content of mitochondrial genomes of *Fascioloides magna* (Fm), *Fasciola hepatica* (Fh) and *Fasciola gigantica* (Fg). (DOCX 20 kb)

**Abbreviations**

mt, mitochondrial; mtDNA, mitochondrial DNA; rDNA, ribosomal DNA; BI, Bayesian inference; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; ITS, internal transcribed spacer; NCR, non-coding region

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**Availability of data and material**

The datasets supporting the conclusions of this article are included within the article and its additional files. The complete mt genome sequence of *F. magna* is deposited in the GenBank database under the accession number KR006934.

**Authors’ contributions**

XQZ and MK designed this study and critically revised the manuscript. JM and JIH performed the experiments, analysed data and drafted the manuscript. GHL and RL participated in sample collection and manuscript revision. All the authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

This study was approved by the Animal Ethics Committee of Charles University, Czech Republic.

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