Can avian flyways reflect dispersal barriers of clinostomid parasites? First evidence from the mitogenome of Clinostomum complanatum

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Can avian flyways reflect dispersal barriers of clinostomid parasites? First evidence from the mitogenome of *Clinostomum complanatum*

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Abstract

Clinostomum complanatum (Rudolphi, 1814) is an economically important parasitic flatworm (Trematoda, Digenea), yet little is known on the population structure of these animals. We characterise a new mitochondrial genome for *C. complanatum*, derived from an Iranian specimen. The newly obtained sequence is used to position the species in the digenean tree of life. The first-ever intraspecific comparison at mitogenome scale within *C. complanatum* revealed a high degree of similarity to the previously sequenced mitogenome of a distant (Italian) population. Avian migratory routes mirror phylogenetic clustering, and hence we suggest that infection of a flying host enables genetic exchange between parasites across large geographic distances. Comparative mitogenomic work in *Clinostomum* spp. at both the intra- (*C. complanatum*) and interspecific (*C. complanatum*–*C. sinensis*) level further shows that usage of new and/or additional mitochondrial markers is preferred over single-gene methods for high-resolution diagnostics and population biology.

Keywords

flatworm, Trematoda, mitochondrion, genome, phylogenetics

1. Introduction

Clinostomum complanatum (Rudolphi, 1814) is a parasitic flatworm (Digenea, Platyhelminthes) exhibiting a typical multi-host trematode life cycle (Sukhedo 2012). Adult flukes attach to the throat or mouth cavity of piscivorous birds, reptiles or, occasionally, mammals (Sutili, Gressler et al. 2014). Humans can also become accidental hosts through the consumption of infected raw fish products (Park, Kim et al. 2009; Hara, Miyauchi et al. 2014; Lee, Park et al. 2017; Kim, Cho et al. 2019) and infections of metacercariae ('yellow grub') in cultivated fish can produce severe pathological effects (Lo, Huber et al. 1981; Dias, Eiras et al. 2003; Shareef and Abidi 2012) and incur considerable economic losses in aquaculture (Li, Liu et al. 2018).

The systematic complexity of *Clinostomum* Leidy, 1856 renders parasite identification difficult: many species are morphologically similar, and studies are often based solely on metacercariae, without an established link to adult life stages. This has resulted in a convoluted taxonomic history of the genus (Sereno-Uribe, Pinacho-Pinacho et al. 2013 and references therein). This systematic predicament, combined with the complex, often uncharted life cycle of clinostomids, vastly muddles species' distribution patterns. As a consequence, the biogeography of clinostomids remains poorly understood, although a deep Old–New World division has been suggested (Locke, Caffara et al. 2015).

To overcome these challenges, large-scale sequencing efforts of ITS and *cox1* markers have been directed towards identification (Gustinelli, Caffara et al. 2010; Rosser, Baumgartner et al. 2018), species delineation (e.g., Sereno-Uribe, Pinacho-Pinacho et al. 2013; Pérez-Ponce de Léon, García-Varela et al. 2016) and taxonomic revisions of the genus (Dzikowski, Levy et al. 2004; Caffara, Locke et al. 2019).

However, while such studies have yielded influential systematic insights, the phylogenetic resolution provided by these markers stagnates in studies of evolutionarily close organisms. Indeed, inferred ITS/*cox1* topologies often contain many unresolved or weakly supported nodes (e.g., partial *cox1* trees in Caffara, Locke et al. 2019; Zimik, Sharma et al. 2019). Exploring particularly close interrelationships,

e.g., on parasite population structure, presses the need for other or additional genetic markers.

The mitochondrial (mt) genome is a promising resource to search for suitable, fast-evolving markers, and the first mt genomes of *Clinostomum* spp. were recently characterized (Chen, Feng et al. 2016; Locke, Caffara et al. 2019). These sequences were derived from metacercariae of *C. complanatum* infecting European chub (*Squalius cephalus* (Linnaeus, 1758), Cyprinidae) in Italy, and of *Clinostomum sinensis* Locke *et al.*, 2019, found in muscle tissue of an infected goldfish (*Carassius auratus* Linnaeus, 1758, Cyprinidae) in Hubei, China (note that the latter sequence was originally deposited as *C. complanatum*, but later transferred to *C. sinensis* by Locke et al., 2019).

In this study, we re-examine the mitochondrial genome of *Clinostomum* spp. starting with a new sequencing effort of *C. complanatum*. For this, we target a Middle East population of *C. complanatum*, hence allowing us to conduct both an intraspecific and interspecific comparison at the mitogenomic level. Recent advances in genomics of non-model helminths allowed the discovery of contrasting patterns when making comparisons either at the level of conspecific populations or closely related species (e.g., Kmentová, Hahn et al. 2021), which has important implications for the interpretation of population genomic or molecular diagnostic analyses. Newly generated sequence data are used to position the studied organisms in the mitogenomic tree of digeneans and the current *cox1*-based tree of *Clinostomum*. In addition to this phylogenomic/phylogeographic reconstruction, we aimed to identify the most divergent regions in the mitogenome of *Clinostomum*, which may provide more resolution in population or evolutionary studies compared to *cox1*.

2. Material and methods

2.1 Sampling

Adult parasites were collected on 9 August 2008 from a roadkill young male Night heron (*Nycticorax nycticorax* (Linnaeus, 1758)) found in Tonekabon, Mazandaran Province, Iran. The intensity of infection was 1218 worms in the mouth and entrance to the oesophagus. However, as the bird was roadkill, not all parasites were of sufficient quality for downstream work. In the same summer, metacercariae were

retrieved from the abdominal cavity and gill chamber of *Capoeta capoeta* (Güldenstädt, 1773) (Cyprinidae) in Isfahan city, Iran. All worms were cleaned in saline solution and subsequently fixed in ethanol 70%. In the field, specimens were identified *as Clinostomum* sp., based on colour, overall shape, and site of collection. Specimens were then transported to the lab, where genus identity was verified under a microscope. Awaiting molecular verification, specimens were provisionally labelled as *Clinostomum complanatum*.

2.2 Sequencing

The DNeasy Blood and Tissue Kit (Qiagen) was used for DNA extraction following the manufacturer's instructions (metacercariae C11 and C12, from *C. capoeta*; **Supplementary file 1**). Polymerase Chain Reaction (PCR) was performed in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) using Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare), adding 1 µL of each primer (20 µM) (Sigma Aldrich), 2 µL of template DNA, and 21 µL of double distilled, autoclaved and filter-sterilized water, to a total reaction volume of 25 µL. Two degenerate primers with an M13 tail attached were used to amplify the barcode region of the mitochondrial gene coding for the first subunit of cytochrome *c* oxidase (*cox1*). These primer sequences, MplatCOX1dF (5'-TGTAAAACGACGGCCAGTTTWCITTRGATCATAAG-3') and MplatCOX1dR (5'-CAGGAAACAGCTATGACTGAAAYAAYAIIGGATCICCACC-3') (Moszczynska, Locke et al. 2009) allow the amplification of 30 nucleotides more in addition to a sequence amplified with the aid of the Folmer, Black et al. (1994) barcoding primers.

After an initial denaturation of 2 min at 94 °C, samples were subjected to 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 60 s at 72 °C. After a final elongation of 10 min at 72 °C, samples were cooled to 4 °C. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol. We sequenced both strands with the same primers as in the initial PCR amplification; products were run on an in-house (Natural History Museum, London) Applied Biosystems 3730 DNA analyser with BigDye version 1.1. Sequences were validated in MEGA6 (Tamura, Stecher et al. 2013) and consensus sequences assembled using Clustal W (Thompson, Higgins et al. 1994) also implemented in MEGA.

In order to obtain the complete mitochondrial genome of *Clinostomum complanatum*, DNA was extracted from a single adult specimen (C16, from *N. nycticorax*; **Supplementary file 1**) using the Genomic II Isolate Kit (Bioline) following the manufacturer's instructions. Double-stranded DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen) and 200 ng was prepared for sequencing using a TruSeq Nano Library Preparation Kit (Illumina). Paired-end sequencing was performed on an Illumina MiSeq Sequencing platform on approximately 1/6th of a 600 cycle V3 kit, at the University of Cambridge, Departments of Biochemistry, DNA Sequencing Facility. All obtained sequence data are publicly available under GenBank numbers [xxxxxx].

2.3 Assembly

The complete mitochondrial genome of *C. complanatum* was assembled using Geneious R8.1.7 (Biomatters, http://www.geneious.com) (Kearse, Moir et al. 2012). First, low-quality bases were removed using the Trim Ends function with the following parameters: Error Probability Limit = 0.1, Maximum ambiguities = 0, Maximum low-quality bases = 0, and ensuring both 5' and 3' ends were selected. Trimmed reads were subsequently assembled to available partial *cox1* reference sequences, GenBank accessions JF718588–95. Unassembled reads were then iteratively mapped and reassembled to the putative mitochondrial genome sequence until the resulting contig could be circularised. Nucleotide composition was calculated in Geneious Pro v11.15. Coding strand asymmetry was manually calculated following the formulas of Perna and Kocher (1995).

2.4 Annotation

Protein-coding genes (PCGs) and ribosomal RNA genes (rRNAs) were identified on the MITOS web server (Bernt, Donath et al. 2013), employing the echinoderm and flatworm mitochondrial code (GenBank transl_table=9). Annotations were matched to open reading frames (ORFs) predicted in Geneious. Transfer RNAs (tRNAs) were detected in MiTFi as implemented in MITOS (Jühling, Pütz et al. 2011), ARWEN v1.2 (Laslett and Canbäck 2008) and tRNAscan-SE (Lowe and Eddy 1997; Lowe and Chen 2016). When conflicting results were reported, the solution proposing the lowest energy structure was chosen using RNAeval (Hofacker, Fontana et al. 1994; Lorenz,

Bernhart et al. 2011; Lorenz, Hofacker et al. 2016), under an estimated environment temperature of 40 °C. This temperature was chosen to approach the body temperature of the avian host of the obtained specimen (Midtgård 1983). Secondary structures were drawn in RNAplot v2.4.13 (Hofacker, Fontana et al. 1994; Lorenz, Bernhart et al. 2011; Lorenz, Hofacker et al. 2016). Repeats were detected using the online versions of Tandem Repeats Finder (Benson 1999) and YASS (Noe and Kucherov 2005), with a cut-off of E < 0.01. The annotated mitogenome was visualised in OGDRAW v1.3.1 (Lohse, Drechsel et al. 2007; Greiner, Lehwark et al. 2019).

2.5 Molecular identification and *cox1* phylogeny

As the diagnostic value of morphological characters has been questioned for *Clinostomum* spp., we verified our identification by BLASTing (Altschul, Madden et al. 1997) the newly obtained *cox1* sequences on the NCBI website (ncbi.nlm.nih.gov). In addition, all available *cox1* sequences of *Clinostomum complanatum* and one sequence of each congener were 'mined' from GenBank (Benson, Karsch-Mizrachi et al. 2012). *Euclinostomum heterostomum* (Rudolphi, 1809) and *Ithyoclinostomum* sp. Witenberg, 1925 were also included in this dataset as outgroups. An overview of all included sequences is provided in **Supplementary file 1**. A translational alignment was built using MUSCLE v3.8.425 (Edgar 2004) in Geneious (transl_table 9) (Kearse, Moir et al. 2012). Ambiguously aligned regions were removed from the alignment via the Gblocks server (Castresana 2000), employing settings for a less stringent selection.

A maximum-likelihood (ML) based model selection was performed in IQ-TREE v2.0.3 (Nguyen, Schmidt et al. 2015). The best-fitting model was used to calculate genetic distances between *cox1* sequences using PAUP v4.0a (Swofford 2003). ML tree inference was conducted under this model in IQ-TREE. 1000 ultrafast bootstraps (UFboot; Hoang, Chernomor et al. 2017) and 1000 SH-aLRT (Guindon, Dufayard et al. 2010) branch test replicas were performed to assess clade support. Bayesian inference (BI) was carried out in MrBayes v3.2.7a (Ronquist, Teslenko et al. 2012). One cold chain and four hot chains were run for 10 million generations on the CIPRES science gateway (Miller, Pfeiffer et al. 2010). Trees were sampled every 1000th generation, the first 25% being discarded as burn-in. Convergence was assessed by

the average standard deviation of split frequencies dropping below 0.01, the potential scale reduction factor approaching 1.0, and log-likelihoods reaching a stationary distribution. A majority-rule consensus tree was constructed from all retained topologies.

Obtained trees were visualised in FigTree v1.4.4 (Rambaut 2006–2021) and weakly supported clades (SH-aLRT < 80 and/or UFBoot < 95 and/or pp < 0.95; IQ-TREE developer's recommendations, section 15.3 in manual) were collapsed. Building on the obtained topologies, we ventured to explore the biogeography of clinostomids in light of their avian hosts. To do so, we mined the localities recorded for all included sequences from GenBank and tagged these according to their occurrence on any of the migratory routes listed by BirdLife International (2018) (see their figure p.9—'Generalised global flyways for migratory landbirds and waterbirds'). These localities were then converted into colour codes, and manually mapped on the inferred *cox1* phylogeny.

2.6 Multimarker mitochondrial phylogeny

Fourteen datasets (12 PCGs and both rRNA genes) were compiled, representing the newly assembled and all previously published digenean mitochondrial genomes available on GenBank. Four cestode sequences were included as outgroups. Accession numbers of included sequences are provided in **Supplementary file 2**. PCGs were individually aligned using Muscle in Geneious (transl_table 9). Both ribosomal datasets were aligned on the MAFFT online server (Kuraku, Zmasek et al. 2013; Katoh, Rozewicki et al. 2019), using the Q-INS-i algorithm, accounting for secondary structures in the alignments. Problematic regions were removed in Gblocks. Individual alignments were concatenated in arbitrary (alphabetical) order in Geneious.

An initial partitioning scheme was manually constructed for the complete dataset, subdividing the concatenated alignment in genes and, when applicable, codon positions. The alignment and the corresponding partition file were then fed into the ModelFinder tool of the IQ-TREE suite (Trifinopoulos, Nguyen et al. 2016). Default settings were used and partition merging was enabled (Chernomor, von Haeseler et al. 2016). The latter feature determines the best-fit partitioning scheme for a particular

dataset, while also calculating the best-fitting evolutionary models for each selected subset. A second iteration was performed, restricting the outcomes to models supported by MrBayes (-m TEST -mset mrbayes).

Following the output of IQ-tree, a new partitioning scheme was specified for the ML and BI analyses (**Supplementary file 3**). An ML tree search was performed on the IQ-TREE web server, calculating 1000 UFBoot and 1000 SH-aLRT replicas as support values. Bayesian analyses were conducted in MrBayes on the CIPRES web server, using the same parameters as described above for the *cox1* dataset. The resulting trees were visualised as above.

3. Results

3.1 Interrelationships, distances, and identification based on *cox1*

A BLAST search of the newly obtained *cox1* sequence resulted in a best-scoring hit (E-value 0.0, percentage identity 99.7%) with the previously published mitogenome of *C. complanatum* (MK814187). The best-fitting substitution models for *cox1* alignments were inferred to be TN+F+I+G4 (first codon position) and GTR+F+I+G4 (second and third positions). Model-based ML genetic distances between the newly assembled and previously published sequences of *C. complanatum* (0.000–0.017; green in **Supplementary file 4**) are much smaller compared to congeneric distances to the new sequences (0.048–1.263; yellow in **Supplementary file 4**). Phylogenetic ML/BI inference resulted in *cox1* topologies with many weakly supported clades: the uncollapsed ML tree is provided in **Fig. 1a** and weakly supported clades are highlighted on each branch according to either support value dropping below threshold. The newly generated *cox1* sequences cluster strongly with other sequences of *C. complanatum* (SH-aLRT/UFboot/pp 87/92/1), yet most resolution within this clade is lost after collapsing problematic nodes (**Fig. 1b**).

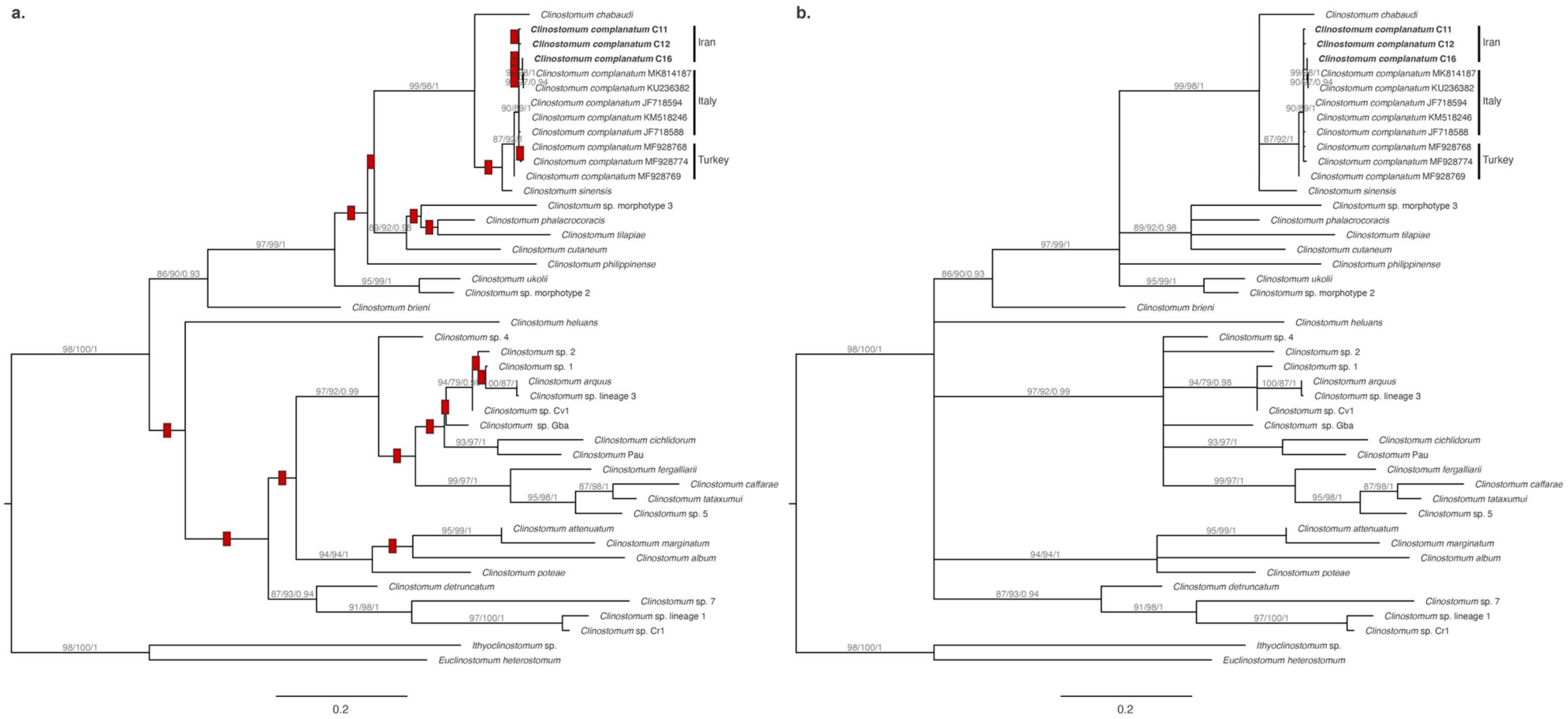

Fig. 1 Maximum likelihood (ML) *cox1* tree of *Clinostomum* spp., obtained in IQ-TREE v2.0.3 (Nguyen, Schmidt et al. 2015). Newly obtained sequences are indicated in bold. Branch lengths denote the number of expected nucleotide substitutions per site. **a.** Uncollapsed ML tree. Support values above each branch respectively represent SH-aLRT values, ultrafast bootstraps (UFboot), and posterior probabilities (pp). A red rectangle indicates a weakly supported clade (SH-aLRT < 80 and/or UFBoot < 95 and/or pp < 0.95). **b.** The same tree, after collapse of weakly supported clades. Topology is identical to the Bayesian tree

3.2 Architecture of the mitochondrial genome of *Clinostomum complanatum*

Illumina MiSeq produced 12,475,228 reads total (6,237,614 paired). A single contig of 14,395 bp was assembled: 71,784 reads (0,58%) mapped to the assembly with an average of 934x coverage. The mitogenome is AT-rich (64.0%), with a positive GC skew (0.39) and negative AT skew (-0.34). The annotated mitochondrial genome is visualised in **Fig. 2**. Transfer RNAs with corresponding free energy levels are given in **Supplementary file 5**. All expected 12 PCGs, 2 rRNAs and 22 tRNAs were annotated (**Supplementary file 6**) (Wey-Fabrizius, Podsiadlowski et al. 2013). All genes are transcribed from the same, positive strand. A single non-coding, AT-rich stretch (1,027 bp) occurs between *trnE* and *trnG*. Several repeats occur in this segment (**Fig. 3**).

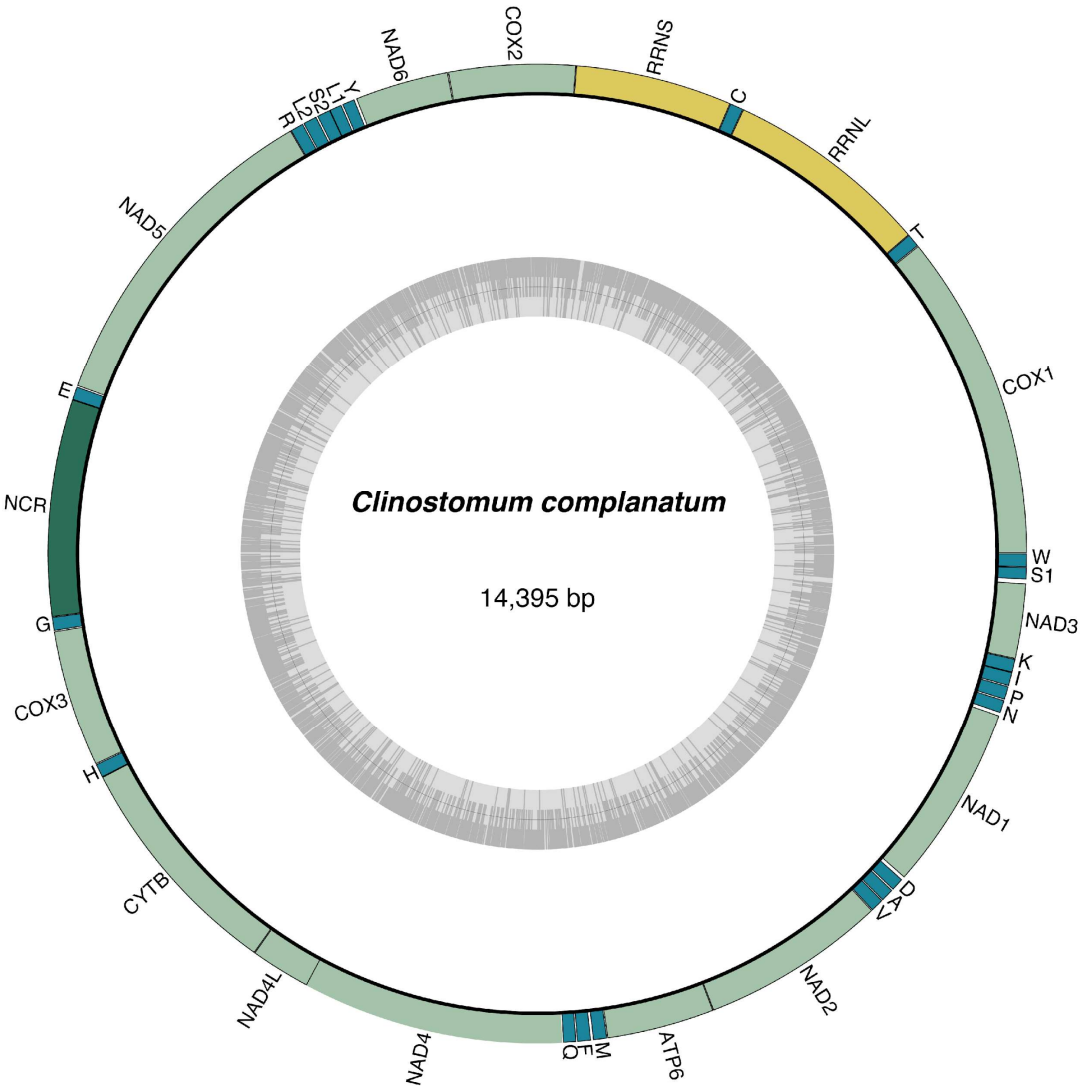

Fig. 2 Gene map of the newly assembled mitochondrial genome of *Clinostomum complanatum*, visualised in OGDRAW v1.3.1 (Lohse, Drechsel et al. 2007; Greiner, Lehwark et al. 2019). The inner ring shows a graph of the nucleotide composition

throughout the sequence: the dark grey and light grey plots represent the GC and AT content respectively

Fig. 3 Repetitive segments in the non-coding region of the newly assembled mitochondrial genome of *Clinostomum complanatum*, as inferred in YASS (Noe and Kucherov 2005) at E < 0.01. Tandem Repeats (TRF) were detected in Tandem Repeats Finder (Benson 1999). Repeats are numbered according to period size, with each repetitive copy carrying the same number. A green rectangle denotes a direct repeat, while blue labels indicate inverted repeats

3.3 Mitogenomic comparison within *Clinostomum complanatum* and between *C. complanatum* and *C. sinensis*

Gene order is identical between all three currently available mitogenomes of *Clinostomum* spp. Mitochondrial genome length seemingly varies substantially between sequences, yet this is largely due to length variations in the non-coding region (NCR; **Table 1**). Nucleotide statistics are largely similar between sequences (**Table 1**). Intraspecific and interspecific pairwise distances between mitochondrial PCGs of *Clinostomum* spp. are detailed in **Table 2** and visualised in **Fig. 4**.

Table 1 Nucleotide statistics of the newly assembled and previously published (MK814187) mitochondrial genomes of *Clinostomum complanatum* and *Clinostomum sinensis* (NC_027082). Length and GC content were inferred in Geneious Pro v11.15 (Kearse, Moir et al. 2012) and skew was calculated manually following the formulas of Perna and Kocher (1995)

	C. complanatum Iran (this study)	*C. complanatum* Italy (MK814187)	*C. sinensis* China (NC_027082)
Contig length (bp)	14,395	13,727	13,796

Contig length (bp) excl. non-coding region	13,366	13,365	13,390
GC content (%)	36.0	35.9	35.7
AT skew	-0.34	-0.35	-0.35
GC skew	0.39	0.39	0.40

Table 2 Number of genetic differences and pairwise identity (Geneious Pro v11.15; Kearse, Moir et al. 2012) between the newly assembled mitochondrial genome of *Clinostomum complanatum* and the previously published sequence (MK814187) (left side, intraspecific), and the mitochondrial genome of *Clinostomum sinensis* (NC_027082) (right side, interspecific). For protein-coding genes, numbers before and after the slash respectively indicate synonymous and non-synonymous substitutions.

	Intraspecific		Interspecific	
	# differences	% identity	# differences	% identity
atp6	3/0	99.4	32/8	93.7
cox1	5/0	99.7	75/5	95.2
cox2	0/0	100	26/3	95.6
cox3	2/0	99.7	34/3	94.7
cytb	1/0	99.9	47/4	95.7
nad1	3/0	99.6	39/4	95.6
nad2	1/0	99.9	59/13	93.3
nad3	2/0	99.4	14/2	96.0
nad4	3/0	99.8	80/10	93.8
nad4l	0/0	100	10/0	96.5
nad5	4/1	99.7	112/24	93.0
nad6	2/0	99.7	29/6	93.6
rrns	1	99.9	15	98.0
rrnl	0	100	26	97.4

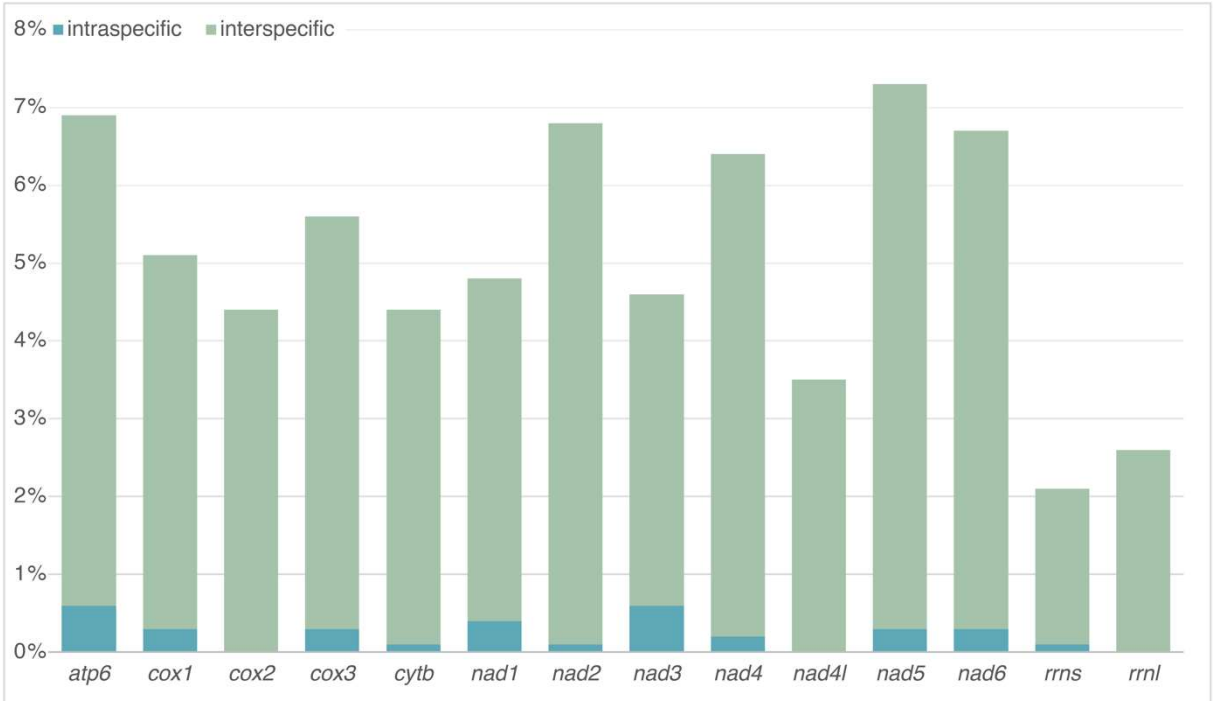

Fig. 4 Relative genetic distances per mitochondrial protein-coding gene between the Iranian and Italian sequence of *Clinostomum complanatum* (blue) and between *C. complanatum* and *Clinostomum sinensis* (green), as calculated in PAUP v4.0a (Swofford 2003)

3.4 Mitochondrial multimarker tree

The resulting topology is visualised in **Fig. 5**. The majority of established digenean families and genera were inferred to be monophyletic in the mitogenomic tree, with few exceptions: *Fasciola* Linnaeus, 1758 and Echinostomatidae Looss, 1899 are resolved as paraphyletic, while Diplostomidae Poirier, 1886 is polyphyletic due to the paraphyletic taxon Strigeidae Railliet, 1919 being nested within this family. The newly obtained mitochondrial sequence unambiguously clusters with the Italian sequence of *Clinostomum complanatum* (SH-aLRT/UFBoot/pp 99.8/100/1). *Clinostomum sinensis* is retrieved as sister to *C. complanatum* (SH-aLRT/UFBoot 100/100/1), rendering both *Clinostomum* and Clinostomidae Lühe, 1901 monophyletic.

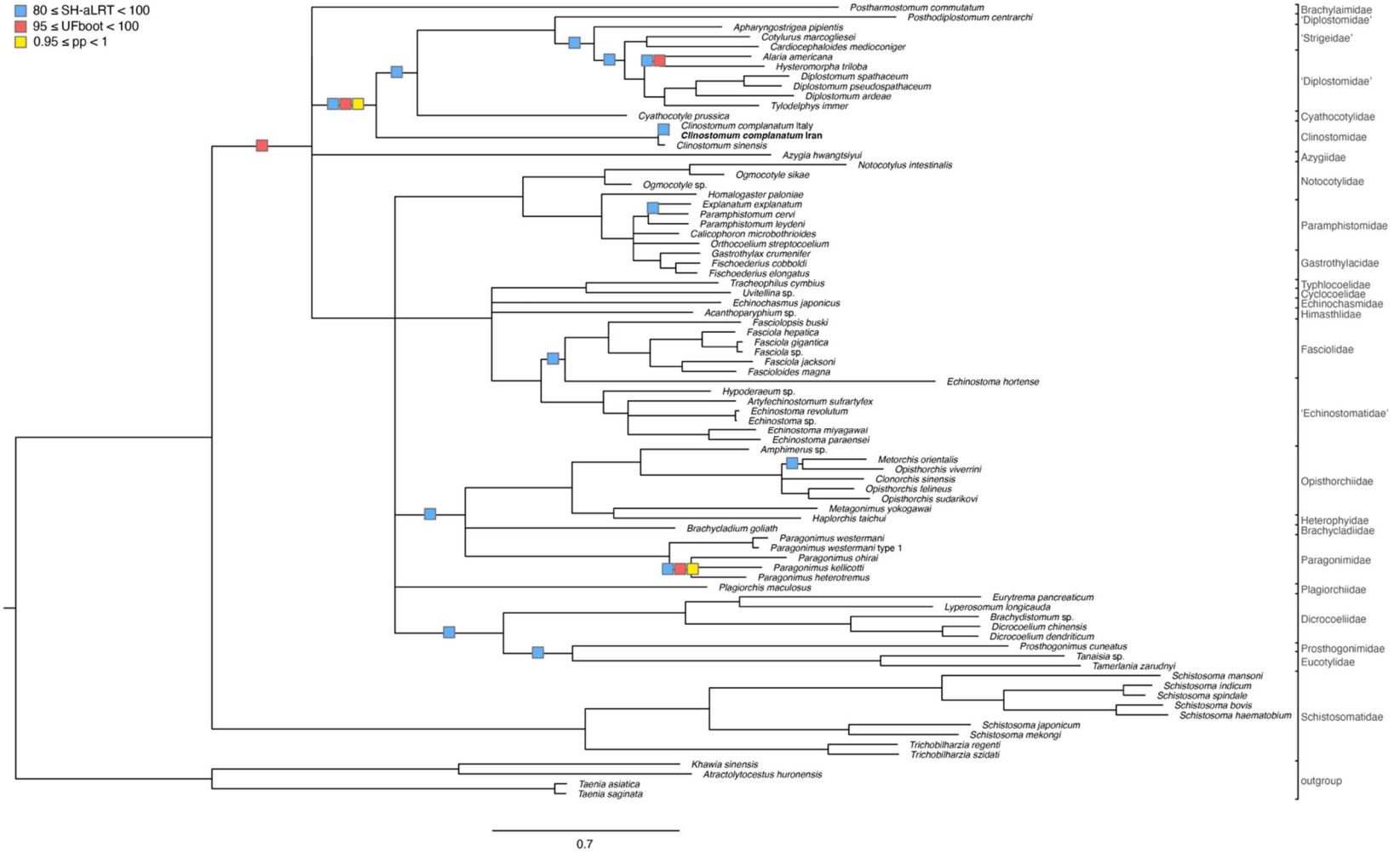

Fig. 5 Maximum likelihood mitochondrial tree of trematodes based on twelve protein-coding and two ribosomal RNA genes. Weakly supported clades (SH-aLRT < 80 and/or UFBoot < 95 and/or pp < 0.95) have been collapsed. The displayed topology is identical to the Bayesian tree. Symbols on branches represent support values: the corresponding legend is displayed in the top left. Branches without symbols are maximally supported. The newly obtained mitogenomic sequence is indicated in bold. Branch lengths denote the number of expected nucleotide substitutions per site

4 Discussion

4.1 Identification of *Clinostomum complanatum*

Based on the observed morphological similarity, BLAST results, *cox1* distances, and phylogenetic clustering, we consider the specimens studied here representatives of *Clinostomum complanatum*. Apart from confirming the phylogenetic position of the newly sequenced specimens, the newly obtained mitochondrial topology also re-establishes a number of previously known anomalies in digenean classification, including the non-monophyly of several well-known genera and families (see Saijuntha, Sithithaworn et al. 2011; Heneberg 2013; Liu, Zhang et al. 2016; Hernández-Mena, García-Varela et al. 2017; Locke, Van Dam et al. 2018; Li, Qiu et al. 2019). We here refrain from taxonomic decision-making, as it falls outside the scope of this study. However, our results do confirm a number of previously reported issues on the validity of several well-known families and genera, pressing the need for a thorough systematic revision of digenean trematodes.

4.2 Intraspecific similarity in the mitochondrial genome of *Clinostomum complanatum*

Comparative work showed little evidence for mitogenomic variation between Iranian and Italian populations of *Clinostomum complanatum* in terms of architecture and composition, with most apparent differences being observed in the NCR. Due to the assembly difficulties, inherent to short-read sequencing of repetitive segments (**Fig. 3**; Tørresen, Star et al. 2019), this region is not further considered here. Coding sequences exhibit very few nucleotide differences, with relative similarity estimates scoring well above 99% for all mitochondrial genes (**Table 2**). Such a high degree of genetic similarity implies that gene flow readily occurs between the sampled populations, despite their reported localities being over 3500 km apart.

4.3 Avian flyways mirror phylogenetic clustering in the *cox1* topology

An explanation for this genetic homogeneity may be found in the life cycle of these digeneans. While difficult to fully characterise all hosts involved, it is generally accepted that clinostomids subsequently infect a mollusc, a fish, and finally a bird (or sometimes a reptile or mammal), with varying degrees of host specificity (Dias, Eiras et al. 2003;

Sukhedo 2012). Through the mode of life of the final, flying host, dispersal of adult parasites may occur across vast geographic distances, as such enabling genetic exchange. As a first step to explore this possibility, we mapped the eight currently recognised avian flyways (BirdLife International 2018) onto the ML *cox1* phylogeny (**Fig. 6**).

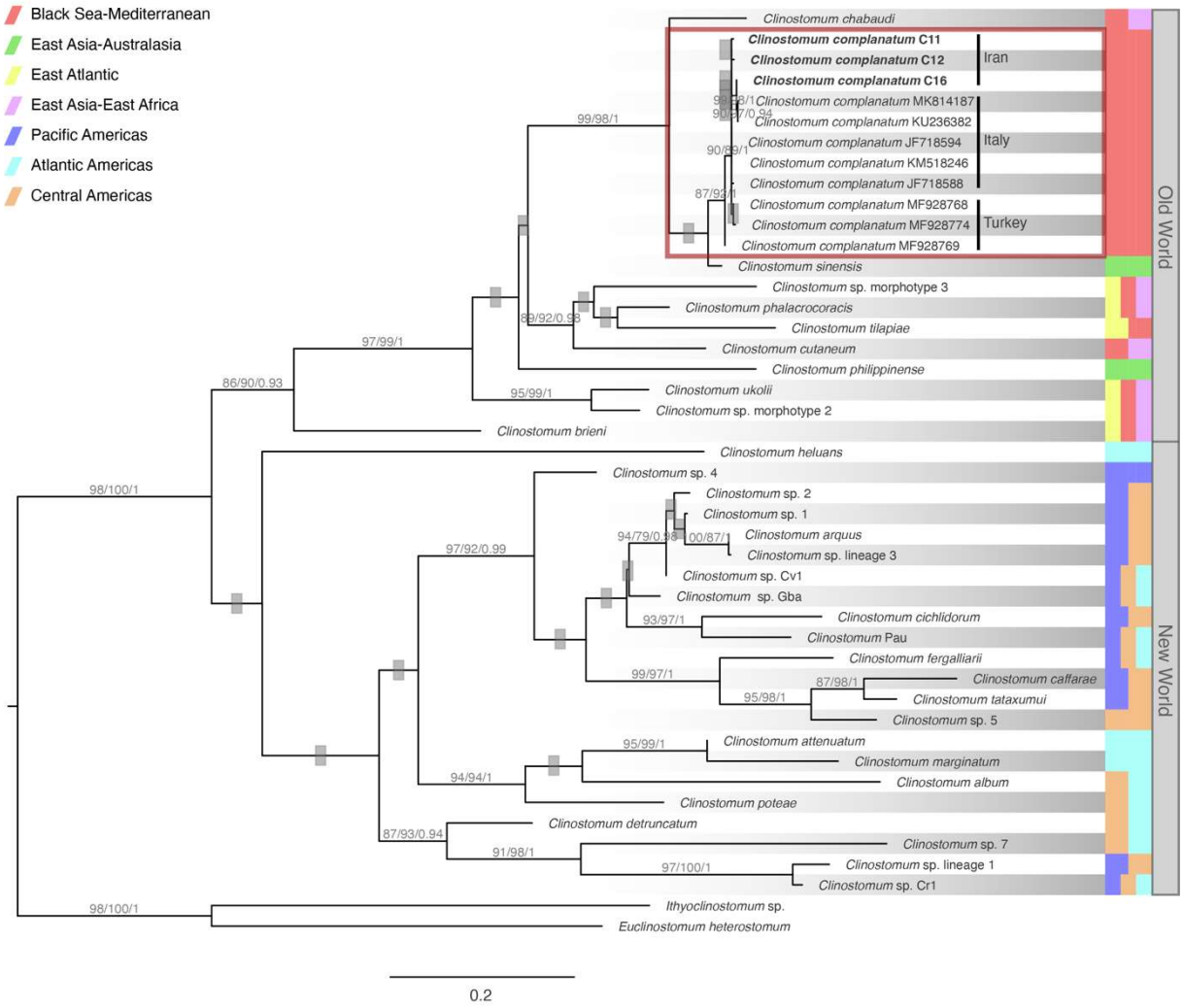

Fig. 6 Uncollapsed maximum likelihood *cox1* phylogeny of *Clinostomum* spp. Coloured boxes represent the respective avian flyways passing the sampling localities of sequenced specimens, the legend of which is displayed in the top left. Localities situated in overlapping flyways are indicated by the usage of multiple colours. For further detailing of these flyways, the reader is referred to the report of BirdLife International (2018, their figure p.9). Support values are identical to those provided in Fig. 1a and accordingly explained in that figure's legend and caption

This visual exercise revealed a large degree of correspondence between flatworm tree topology and bird migratory routes. A clear division between Old and New World clinostomid clades had previously been reported and has been suggested to be linked to avian migratory routes (Caffara, Locke et al. 2011; Locke, Caffara et al. 2015; Pérez-Ponce de Léon, García-Varela et al. 2016; Caffara, Locke et al. 2017; Rosser, Alberson et al. 2017; Montes, Barneche et al. 2021). Indeed, this pattern is mirrored by distinct host flyways, *i.e.*, the Pacific-Central-Atlantic Americas versus all other routes. Also on lower taxonomic levels, this host/geographic signal is apparent. Specifically, all sequences of *C. complanatum* were obtained from specimens sampled in the Black Sea-Mediterranean route (red box in **Fig. 6**), and this corroborates the possibility that gene flow is established through displacement of adult clinostomids via avian hosts. Correspondingly, the more distinct mitogenome of *C. sinensis* is derived from a locality in the non-overlapping East Asia-Australasia flyway.

The life cycle of digeneans is complex and includes subsequent infection of multiple hosts. The host range and specificity of *Clinostomum* spp. is not yet fully characterised, and specimens in intermediate hosts are rarely linked to adult life stages. However, most digeneans are assumed to require a snail to complete their life cycle and a suitable intermediate host must be in place throughout the parasites' range. Certainly, molluscs have limited capability to move outside their immediate habitat compared to birds (e.g., Aubry, Labaune et al. 2006). The first intermediate host of *C. complanatum* in Iran remains unknown to date. To explain our observation of apparent connectivity between distant populations, we hypothesize that *C. complanatum* must either infect a relatively broad range of molluscs [as e.g., *C. marginatum* (Calhoun, Leslie et al. 2019)], or the same intermediate host must occur on both sampling localities [as e.g., *Radix auricularia* (Linnaeus, 1758); GBIF (2021)], perhaps being transported by the final host itself (van Leeuwen, van der Velde et al. 2012).

4.4 Taking a step back: A word of caution for interpretation of *cox1* results

From a more pragmatic point of view, such a large degree of genetic homogeneity between populations underlines the need to target new and fast-evolving markers for future researchers to explore interrelationships at the intraspecific level. Indeed, while it is enticing to explore these biogeographical patterns, care must be taken not to overinterpret the phylogenetic framework obtainable by partial *cox1* gene sequences.

Until now, this gene has been the leading marker of choice in *Clinostomum* spp. sequencing. While much progress has been made using this marker, the resolving power of *cox1* caps at low taxonomic levels, as demonstrated by the low resolution in **Fig. 1b**. While the best-scoring (ML) tree (**Fig. 6**) was discussed in the section above, many clades are weakly supported and a re-assessment including more and/or faster-evolving markers would make for a much more robust case.

Complete (mitochondrial) genomes constitute a powerful resource to discover such regions yet, to date, such sequencing efforts have been minimal for *Clinostomum* spp. With the newly generated mitogenome presented in this contribution, we can now conduct a first exploration of new promising molecular markers, with greater inter-/intraspecific resolving powers, by comparing mitogenomes within and between species (**Table 2**, **Fig. 4**). This is rarely performed in helminths, as intraspecific genetic diversity in these animals is typically not assessed, and even less often compared between species (as in e.g., Perrot-Minnot, Špakulová et al. 2018 and Geraerts, Huyse et al. 2022). Similarly, inter- and intraspecific patterns of mitogenomic diversity are seldom compared (Kmentová, Hahn et al. 2021). With the now available dataset, we are able to for the first time explore this in *Clinostomum*.

From **Fig. 4**, it already appears that relative genetic distances differ between the inter- and intraspecific level. Notably, the popular *cox1* marker is not distinct from other mitochondrial markers in terms of genetic variability. Rather, *atp6*, *nad2*, *nad4*, *nad5*, and *nad6* were inferred to be the most variable between species, while *atp6*, *nad1*, and *nad3* vary more at the intraspecific level. The ribosomal genes *rrns* and *rrnl* appear most conserved both within and between species. This pattern of intrageneric variability per gene roughly corresponds to the situation in other digenean genera (based on pairwise comparisons per genetic alignment; individual matrices not shown). Given the confusing and unresolved state of molecular systematics in *Clinostomum*, and the wide host and geographic range of several species, sampling and sequencing efforts should reflect both intra- and interspecific diversity. Here we demonstrate that this is best done using several mitochondrial markers instead of relying solely on *cox1*.

5. Conclusions

We have assembled and annotated a new mitochondrial genome for *Clinostomum complanatum*. This represents the second-ever mitogenome sequence of this flatworm species, and the first molecular characterisation of an Iranian population of this parasite. Multimarker maximum likelihood and Bayesian analyses confirm the position of the species in the current mitochondrial topology of Digenea. Comparing the newly obtained sequence to the single, previously characterised mitogenome of the species indicates a strong genetic similarity between two distant populations. Infection of a final, flying host may enable gene flow across vast geographical distances. In *Clinostomum* spp., the standard *cox1* marker is not distinct from other mitochondrial genes in terms of variability. Therefore, and as we demonstrated relative genetic distances to differ between the intra- and interspecific level, we recommend future researchers to focus on multiple (mitochondrial) genes rather than only *cox1*.

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