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FISH PARASITOLOGY - ORIGINAL PAPER

A new species of *Clinostomum* Leidy, 1856 in East Asia based on genomic and morphological data

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Abstract

Metacercariae of *Clinostomum* Leidy, 1856 are frequently encountered in freshwater fish. In 2015, a provisional species of *Clinostomum* in People's Republic of China (PRC) was distinguished from *C. complanatum* (Rudolphi, 1819) in Europe based on divergent cytochrome *c* oxidase I (CO1). However, in subsequent studies in East Asia, the same divergent CO1 genotype was identified as *C. complanatum*. These matching sequences suggest that either the provisional East Asian species was incorrectly distinguished from *C. complanatum* in 2015 or that *C. complanatum* in East Asia was misidentified in later studies. We tested these alternatives by sequencing the mitochondrial genome of *C. complanatum* in Italy, which was 5.7% divergent from a previously published sequence from *Clinostomum* in PRC, including differences in 80 of 3390 (2.4%) translated amino acids. Partial CO1 sequences of specimens from PRC and those from Italy, Romania, and Turkey also each formed reciprocally monophyletic clades. Partial CO1 from the East Asian clade varied by mean 3.6% (range 2.4–4.8%) from *C. complanatum* from Italy, Romania, and Turkey; mean intra-clade CO1 variation was 0.3% (range 0–1.9%). Metacercariae from Europe and East Asia display significant morphometric variation, and data from the literature suggest morphological differences in the genital complex of adults. Although sequences of nuclear rDNA did not differ between isolates from the west and East Asia, taken together, these results lead us to describe a new species of *Clinostomum*.

Keywords Yellow grub · Halzoun · Helminth · Fish parasite · Molecular prospecting · DNA barcode · Species identification

Sean A. Locke and Monica Caffara contributed equally to this work.

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Introduction

Clinostomum complanatum (Platyhelminthes, Digenea) was described as *Distoma complanatum* in Berlin by Rudolphi (1819) and has since been reported from all non-polar regions. The wide distribution and mobility of the parasite's main definitive hosts, the Ardeidae, make plausible this broad geographic range, but many records of *C. complanatum* have long been questioned (Matthews and Cribb 1998). In the first molecular study of the cosmopolitan distribution of *C. complanatum*, genetic differences in North American isolates suggested *C. complanatum* was limited to the Old World (Dzikowski et al. 2004). This result was supported in nine subsequent studies in which *C. complanatum* in Europe was genetically distinct from species of *Clinostomum* sequenced in Africa, the Americas, and Asia (Locke et al. 2015a and references therein). Most relevant here are metacercariae of *Clinostomum* from *Carassius auratus* in Hubei, People's Republic of China (PRC), from which we obtained DNA sequences leading us to designate the material as *Clinostomum* sp. 8, an unidentified species closely related to, but distinct from, *C. complanatum* (Locke et al. 2015a).

Four recent studies call into question the distinction between *Clinostomum* sp. 8 and *C. complanatum*. The first was the publication of the mitochondrial genome of a metacercaria of *Clinostomum* from *Ca. auratus* from Hubei, which Chen et al. (2016) identified as *C. complanatum*. Wang et al. (2017) reported 18S sequences from cercariae and metacercariae of *C. complanatum* in Taiwan. Li et al. (2018) reported cytochrome *c* oxidase I (CO1) mtDNA and internal transcribed spacer (ITS) rDNA sequences from metacercariae of *C. complanatum* from Sichuan, PRC. Finally, Iwaki et al. (2018) sequenced CO1 in an adult of *C. complanatum* from *Phalacrocorax carbo* in Aichi, Japan. The CO1 sequences of both Chen et al. (2016) and Li et al. (2018) match those of *Clinostomum* sp. 8. The sequence generated by Iwaki et al. (2018), from a different portion of the CO1 gene, matches the mitochondrial genome of Chen et al. (2016). These matching CO1 sequences suggest that either *C. complanatum* was misidentified by these authors, or that *Clinostomum* sp. 8 was incorrectly distinguished from *C. complanatum* by Locke et al. (2015a). Our aim in this study was to test these alternatives.

Chen et al. (2016)'s identification of *C. complanatum* was based on the identity of an unpublished sequence from the second of the two internal transcribed spacers in the rDNA array to that of *C. complanatum* from Europe (Caffara et al. 2011), and comparison to a morphological description of *C. complanatum* from newts by Caffara et al. (2014) was mentioned. In Taiwan, Wang et al. (2017) identified *C. complanatum* based on qualitative morphological comparison of cercariae and metacercariae to Kim and Nagasawa (1996) and Shini et al. (2015) and near identity (403/404 identical nucleotides) of partial 18S sequence with that of a metacercariae from Italy. In Sichuan, PRC, Li et al. (2018) identified *C. complanatum* based on phylogenetic analysis of CO1 and ITS sequences, excluding data from *Clinostomum* sp. 8, and qualitative and quantitative morphological comparison of metacercariae with published descriptions. Iwaki et al. (2018) identified an adult from *P. carbo* in Japan as *C. complanatum* based on matching CO1 (876/879 identical nucleotides) to the sequence from Chen et al. (2016) and through comparison to descriptions of adults by Kagei et al. (1988), Matthews and Cribb (1998), and Caffara et al. (2011). None of these studies mentioned *Clinostomum* sp. 8 or the possibility of an identification other than *C. complanatum*. Several of the morphological references relied upon by these recent studies followed Liao (1992), who studied the life cycle, particularly the development of eggs and cercariae, of what was identified as *C. complanatum* in Guangdong, PRC, based on personal communications with regional authors. We are aware of no critical evaluation of the possibility that *Clinostomum* in East Asia is represented by species other than *C. complanatum*. To determine whether these historical and more recent records represent *C. complanatum* sensu stricto or a distinct species of *Clinostomum*, we obtained comparable molecular and morphological data from isolates in both the region where *C. complanatum* was described (Europe) and in East Asia.

Materials and methods

A metacercaria of *C. complanatum* from *Squalius cephalus* from Santerno River (44.279, 11.586), Italy, was used to generate data for comparison to the mitochondrial genome sequence (KM923964) that Chen et al. (2016) obtained from a specimen from *Ca. auratus* in Hubei, PRC. Extracted DNA from the Italian specimen was shotgun sequenced in a tenth of a lane on an Illumina HiSeq 4000, and 150-bp paired-end libraries were built with Nextera adapters at Genewiz (NJ). To assemble Illumina reads into a mitochondrial genome, a partial CO1 (JF718591) sequence from *C. complanatum* collected in Italy was used to seed iterative assemblies in Geneious V9. The mitochondrial genome was also separately assembled using the sequence (KM923964) of Chen et al. (2016) as a scaffold. The preceding two assemblies both employed default, medium–low sensitivity parameters (≥ 50-bp overlap for extension, ≤ 20% mismatches, maximum ambiguity 4, and ≤ 10% gaps). A third assembly was attempted using strict parameters (≥ 75-bp overlap for extension, 0 mismatches, maximum ambiguity 1, 0 gaps) to map reads to the sequence (KM923964) of Chen et al. (2016), to detect highly similar, heteroplasmic mtDNA in the specimen from Italy. The assembled mitochondrial genome of the Italian specimen was annotated in MITOS (Bernt et al. 2013) and by

comparison to the sequence (KM923964) of Chen et al. (2016) and those of Diplostomoidea (Brabec et al. 2015, KR269763-4; Locke et al. 2018, MH536507-13). The rDNA operon of the Italian specimen of *C. complanatum* was assembled using iterative extension of the sequence of ITS1-5.8S-ITS2 (JF718629) from *C. complanatum* from *Barbus barbus* collected in Italy and default Geneious map-to-reference parameters; annotation was performed by comparison to sequences from the Diplostomoidea (Brabec et al. 2015, KR269765-6; Locke et al. 2018, MH521246-52).

Additional European and East Asian isolates of *Clinostomum* were collected to obtain further molecular and morphological data. In the east, four metacercariae were collected from *Opsariichthys pachycephalus* and 11 from *Candidia barbata* from River Daja near Taichung City (Taiwan) (24.1149, 120.4449) while 2 were taken from *Candidia barbata* from a stream near Meizihliao, Pingliin District, New Taipei City (Taiwan) (24.5745, 121.4614). In the west, two metacercariae of *C. complanatum* were collected in Romania from *Scardinius erythropthalmus* and *Perca fluviatilis* in channel 36 near Tulcea City, Danube delta (45.1331, 28.5313), and Rosu Lake Danube (45.39, 29.3411), Danube delta, respectively. Total lengths of metacercariae were measured before a small piece of the posterior end was removed for extraction of DNA. Morphometrics of these hologenophores (sensu Pleijel et al. 2008) were taken after clarification with Amman's lactophenol and staining by Malzacher's method (Pritchard and Kruse 1982). Line drawings were made with the aid of a drawing tube, and measurements are given in micrometers following Matthews and Cribb (1998). DNA was extracted from hologenophore subsamples using a PureLink Genomic DNA Kit (Invitrogen) following the manufacturer's protocol. Internal transcribed spacer 1, 5.8S, and internal transcribed spacer 2 rDNA (ITS) was amplified with the protocols and primers of Gustinelli et al. (2010), while partial CO1 mtDNA was amplified and sequenced with those of Moszczynska et al. (2009). The products were purified (NucleoSpin Gel and PCR Cleanup, Mackerey-Nagel) and sequenced (StarSEQ GmbH, Mainz, Germany). Contigs were assembled with Vector NTI AdvanceTM 11 software (Invitrogen). Sequences are published in GenBank (accessions: ITS – MK796826-30 and partial CO1 – MK801711-19) and were aligned with those of prior studies in MEGA and subjected to maximum likelihood (using RAXML, Stamatakis 2014) and Bayesian (Ronquist et al. 2012) phylogenetic analysis, the latter with *Ithyoclinostomum* set as outgroup.

Morphometric variation was visualized using principal component analyses (PCA) of original measurements of metacercariae of *C. complanatum* from fish (Caffara et al. 2011) and amphibians (Caffara et al. 2014) as well as data newly obtained in the present study from metacercariae from fish collected in Romania and Taiwan. To test for multivariate differences in morphometrics in metacercariae from East Asia and Europe, the data were subjected to ANOSIMs of normalized Euclidean morphometric distances. Mean values in individual morphometrics were compared using *t* tests after pooling means, standard deviations, and degrees of freedom (Hays 1994) from the present study with data from Simsek et al. (2018) and Li et al. (2018).

Results

Molecular results

Mitochondrial DNA

The DNA of a metacercaria of *C. complanatum* from *S. cephalus* from Santerno River, Italy, yielded 90,981,552 paired-end 150-bp reads. Identical results were obtained from an iterative assembly of reads seeded with CO1 (JF718591) from *C. complanatum* from *B. barbus* collected in Italy, and medium–low sensitivity mapping to KM923964 from *Ca. auratus* from Hubei (i.e., Geneious default parameters). At least 842,902 reads (0.93% of read pool) were assembled into a contig by mapping to KM923964 or using iterative extension of the small CO1 scaffold (JF718591), with mean 9242.4, range 3148–30,306 read-depth per site along the 13,727-bp contig, described below. With strict assembly parameters, however, only 2596 reads (2.9×10^{-5} % of read pool) were mapped to the mitochondrial genome sequence (KM923964) of Chen et al. (2016), with mean coverage of 29 (range 0–1830) reads per site. Most of the mitochondrial genome sequence (KM923964) of Chen et al. (2016) found no matches in the read pool using the strict assembly method; reads mapped strictly only to three discontinuous regions that together represent 5% of the 13,796-bp length of KM923964: 538 reads assembled to a 228-bp span covering the 3′ end of cox3, tRNA-His, and the 5′ region of cytb; 8 reads assembled to a 157-bp region at the 5′ end of cox-1; and 2050 reads assembled to a 317-bp region at the 3′ end of 16S rRNA. In other words, deep sequencing of the European specimen revealed no copies of the mitochondrial haplotype of the material from Hubei sequenced by Chen et al. (2016).

The 13,727-bp mitochondrial genome of *C. complanatum* from Italy (MK814187) was 94.3% similar to the 13,796-bp sequence (KM923964) that Chen et al. (2016) obtained from material collected in Hubei, PRC. The orders of protein-coding and ribosomal genes were identical, and lengths were identical or similar in the two sequences (Table 1). Similarity was highest among RNA genes (tRNA mean similarity 97.3%, range 88.5–100%; rrnL similarity 97.4%, rrnS similarity 98.1%), followed by protein-coding genes (mean similarity 94.6%, range 93.0–96.5%) (Fig. 1). Translated amino acids varied in all protein-coding genes except ND4L. Eighty

Table 1 Organization of the mitochondrial genome of *Clinostomum complanatum* (Rudolphi 1819) sampled from *Squalius cephalus* collected in Santerno River, Italy (MK814187), including the positions and lengths of genes and their similarity to a mitochondrial genome of *Clinostomum sinensis* n. sp. originating in Hubei, People's Republic of China (KM923964, Chen et al. 2016).

Gene	Start	Stop	Length	Similarity (%) to *Clinostomum sinensis* n. sp., KM923964
COX3	1	643	643	94.4
tRNA-His	660	724	65	100
CYTB	725	1849	1125	95.73
ND4L	1821	2108	288	96.53
ND4	2069	3348	1280	93.75
tRNA-Gln	3353	3415	63	98.41
tRNA-Phe	3420	3484	65	95.38
tRNA-Met	3494	3560	67	98.51
ATP6	3562	4074	513	92.98
ND2	4075	4959	885	93.22
tRNA-Val	4959	5023	65	98.46
tRNA-Ala	5026	5090	65 (63)[a]	93.85
tRNA-Asp	5095	5158	64	98.44
ND1	5154	6056	903	95.57
tRNA-Asn	6070	6130	61	100
tRNA-Pro	6136	6200	65	96.92
tRNA-Ile	6204	6267	64	100
tRNA-Lys	6268	6332	65	98.46
ND3	6333	6689	357	95.24
tRNA-Ser	6707	6765	59 (61)[a]	88.52
tRNA-Trp	6766	6830	65	96.92
COX1	6832	8374	1543	95.01
tRNA-Thr	8390	8455	66	93.94
rrnL	8448	9437	990 (992)[a]	97.38
tRNA-Cys	9436	9501	66	95.45
rrnS	9499	10,250	752	98.14
COX2	10,248	10,842	595	95.63
ND6	10,852	11,298	447	93.96
tRNA-Tyr	11,307	11,369	63	96.83
tRNA-Leu	11,373	11,438	66 (67)[a]	97.01
tRNA-Ser	11,433	11,498	66	96.97
tRNA-Leu	11,505	11,568	64 (65)[a]	98.46
tRNA-Arg	11,575	11,639	65	100
ND5	11,640	13,229	1590	92.96
tRNA-Glu	13,234	13,297	64	98.44
AT-loop	13,298	13,659	362	78.31
tRNA-Gly	13,660	13,724	65	100

[a] Values in parenthesis are lengths in KM923964

of 3390 (2.4%) translated amino acids of the concatenated genes varied between the Italian and Chinese isolates. Other than ND4L, translations of individual genes varied at 1–5% of their amino acids. The two genomes were least similar (76.1%) in non-protein-coding, non-RNA regions, which totaled 566 bp in length.

Seventy-five overlapping partial CO1 sequences are available from *Clinostomum* in Eastern and Western Eurasia. Sixteen sequences from the eastern region (7 newly generated in the present study—MK801711-17, and 9 from Locke et al. 2015a—KP110579-87) were used, plus 30 from Locke et al. (2015a), Chen et al. (2016), and Li et al. (2018); three sequences were obtained from Romania and Italy in the present study (Romania KM801718-19; Italy MK814187), and 24 were obtained from samples in Italy and Turkey (Caffara et al. 2011, 2014; Gaglio et al. 2016; Simsek et al. 2018). High levels of variation that we believe probably represent base-calling errors or other artifacts occur in 5′ or 3′ ends in eight CO1 sequences published by Li et al. (2018) (MF741740-4, MF741746, MF741757-8). Excluding these data, uncorrected CO1 p-distances from East Asian samples varied by mean 3.57% (range 2.38–4.77%) from *C. complanatum* from the West (Italy, Romania, and Turkey), and within eastern and western groups, variation was mean 0.33% (range 0–1.85%). Including all data from Li et al. (2018), CO1 p-distances between eastern and western samples were mean 3.74% (range 2.38–7.45%) and within-region p-distances were mean 0.57% (range 0–3.44%) (Fig. 2).

In phylogenetic analyses (excluding the eight aforementioned CO1 sequences), CO1 sequences from western samples of *C. complanatum* (Romania, Italy, Turkey), and those from *Clinostomum* from eastern Asia, formed reciprocally monophyletic lineages (Fig. 3), although the eastern clade lacked strong statistical support, and the topology of the tree presented differences with some recent analyses (e.g., alliance of *Odhneriotrema incommodum* with *Clinostomum album*; see Woodyard et al. 2017).

The mitochondrial data suggest recently separated species of *Clinostomum* in the western and eastern Palearctic. These data also show that the eastern species is distinct from other named and unnamed species of *Clinostomum*, including *C. complanatum* (Figs. 1, 2, and 3).

Nuclear ribosomal DNA

The iterative assembly of reads yielded a contig with approximately 250,550 reads (0.28% of read pool) and mean 6569.3, range 5021–7905 read-depth per site along the 7211-bp contig (length excluding external transcribed spacers) (GenBank accession MK811210). In phylogenetic analysis of ITS1-5.8S-ITS2, sequences from Italy + Turkey and East Asia did not form reciprocally monophyletic clades (not shown). ITS distances within the East Asian samples and within the European and Turkish samples (mean = 0.15%, range 0–1.08%) did not differ from those between these regions (mean = 0.18%, range 0–1.82%). This lack of regional monophyly and distance-

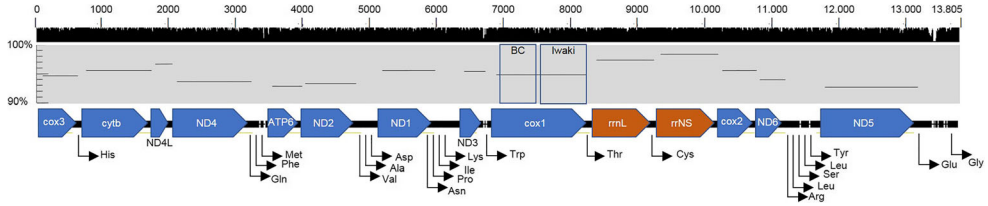

Fig. 1 Schematic of linearized mitochondrial genome of *Clinostomum complanatum* (Rudolphi 1819) sampled from *Squalius cephalus* from Santerno River, Italy (MK814187), and alignment with sequence from *Clinostomum sinensis* n. sp. originating in Hubei, People's Republic of China (KM923964, syn. *C. complanatum* of Chen et al. 2016). Shaded shapes along bottom point in direction of transcription of protein-coding and rRNA genes; hairline arrows indicate position of tRNA genes. Gaps in solid horizontal black line between shapes show indels in alignment with KM923964. Central gray band shows total sequence similarity of protein-coding genes to KM923964. Site nucleotide identities with KM923964 are shown at top (sliding window size = 10 bp). Boxes indicate the barcode region of cytochrome *c* oxidase I analyzed in Figs. 2 and 3 and the region sequenced by Iwaki et al. (2018).

based separation is evident from the alignment of 62 ITS sequences (Fig. 4), in which characters unique to either the eastern or western groups of samples are absent.

While no clear rDNA variations emerged between the species, one transition in ITS1 occurred in 17/17 samples from Italy but only 6/36 samples from China. An insertion at the 3′ end of ITS2 sequences was found in all 27 sequences from Sichuan of Li et al. (2018) but not in any samples from Italy or Turkey. Re-inspection of electropherograms from samples from Hubei (KP110579-83, KP110585-6, available on BOLD; Locke et al. 2015a) revealed a secondary thymine or guanine peak at this point (Fig. 4), not observed in the sequence records, that corresponds to the insertion in the sequences of Li et al. (2018). This secondary peak was not observed in electropherograms from samples (JF718624, JF718629, KM518258-59) from Italy on BOLD, and there was no variation at this position in the specimen subject to shotgun sequencing in the present study in > 99% (6006 of 6060) Illumina reads. It therefore appears possible that at least one fixed difference may occur in ITS2 sequences of the eastern and western species, although its detection in Sanger sequences may be dependent on electropherogram quality and intra-individual variation in ITS. The contiguous 18S portion of the assembly differed at one position from the 18S sequence (KU994881) of Wang et al. (2017).

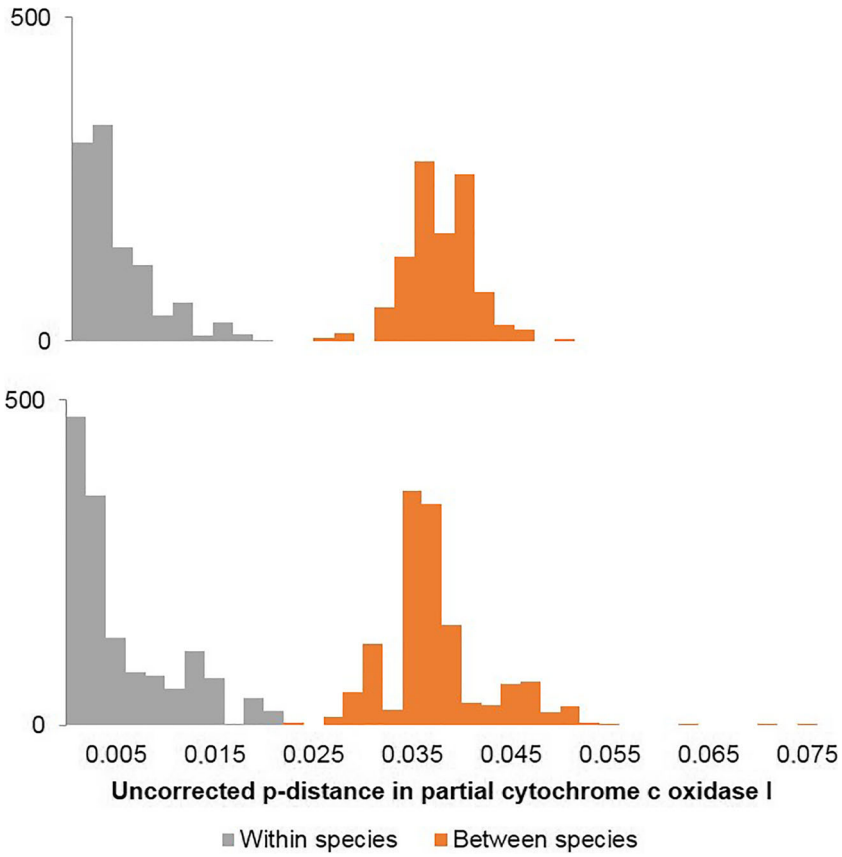

Fig. 2 Frequency of p-distances among partial sequences of cytochrome *c* oxidase 1 from up to 75 specimens of *Clinostomum* in eastern (People's Republic of China) and western (Romania, Italy, and Turkey) regions. Data from the present study and Caffara et al. (2011, 2014), Locke et al. (2015a), Chen et al. (2016), Gaglio et al. (2016), Simsek et al. (2018), and Li et al. (2018). The upper panel shows distances among all sequences except eight (MF741740-4, MF741746, MF741757-8) with unusually high levels of variation at 5′ or 3′ ends, indicating possible base-calling errors. The lower panel shows distances among all 75 available sequences.

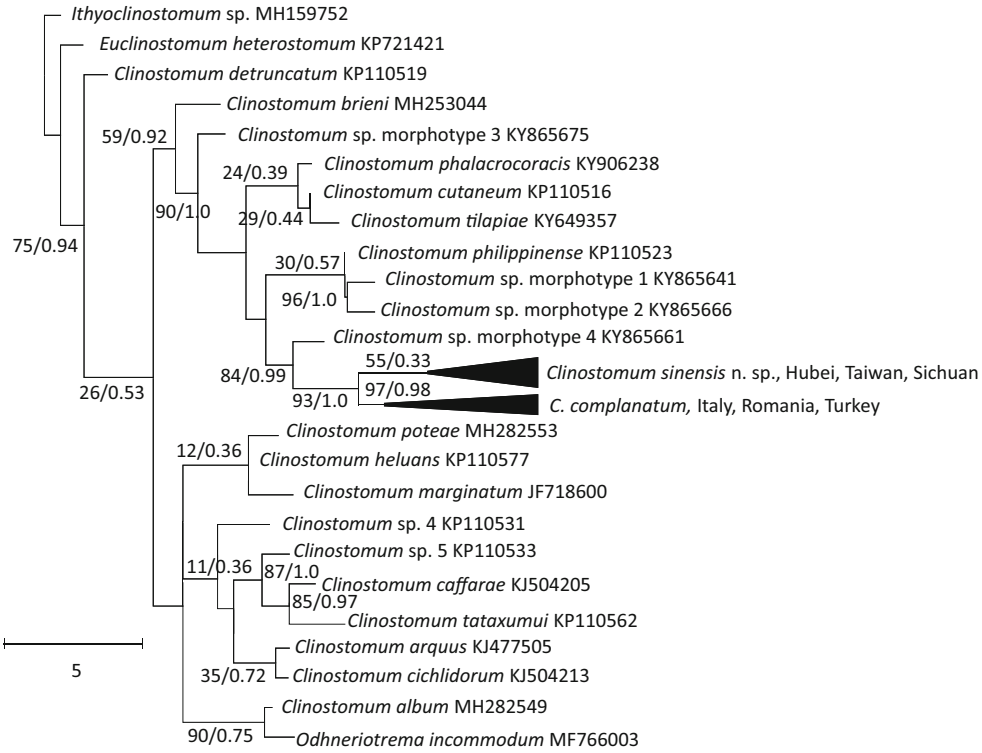

Fig. 3 Phylogenetic analysis of 44 partial sequences of cytochrome *c* oxidase I of *Clinostomum*, including 12 non-redundant sequences from material from Sichuan and Hubei, People's Republic of China, and Taiwan (of 46 total sequences, but excluding MF741740-4, MF741746, MF741757-8; see "Results"), and eight from Italy, Turkey, and Romania (of 28 total sequences). The 378-bp alignment (see supplementary file S1) was analyzed using GTR+G+I in ML and with Nst = 2 rates = invgamma ngammacat = 5 in BI. Nodes are annotated with frequency of clades in 1000 bootstrap ML replicates/posterior probability in Bayesian analysis (8252 topologies).

Thus, unlike the mitochondrial data, rDNA sequences do not show clear differences between *Clinostomum* of western and eastern Palearctic origin. However, given the greater number of variable characters in the mitochondrial data, as well as the overlap in intra- and interspecific variation recorded in ITS in *Clinostomum* and other digeneans (Vilas et al. 2005; Locke et al. 2015a, b; Rosser et al. 2018), as a whole, the molecular data indicate separate, closely related species. As a result, the isolates studied by Chen et al. (2016) and Li et al. (2018) were considered to belong to the East Asian species. Iwaki et al. (2018) sequenced a different region of CO1 that matched (876/879 identical nucleotides) the sequence from Chen et al. (2016) (Fig. 1) and is also considered to belong to the East Asian species distinct from *C. complanatum*. The

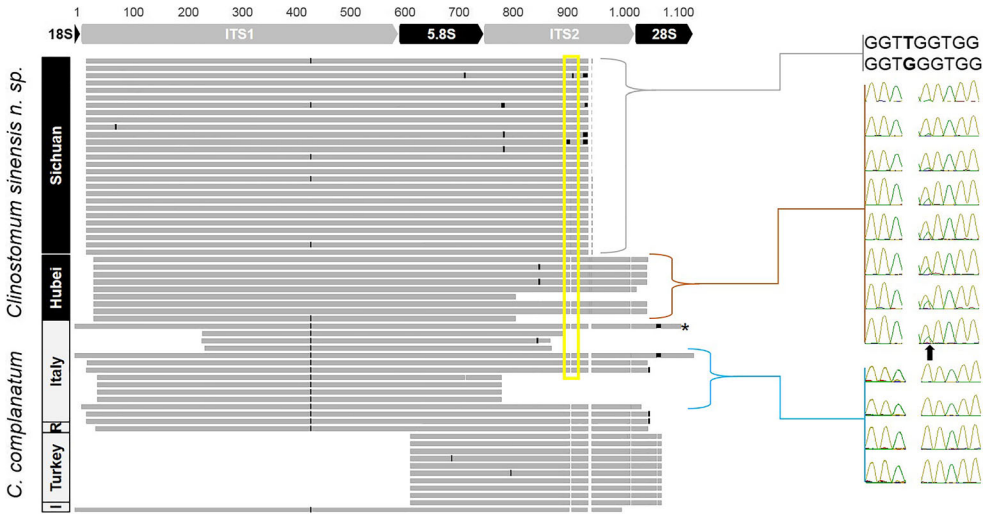

Fig. 4 Schematic of alignment of 62 sequences of rDNA internal transcribed spacer regions from *Clinostomum* from the People's Republic of China, Europe, Turkey, and Israel. Gray bars indicate identical sequences; black marks are mutations with respect to a majority consensus sequence (not shown); white gaps are deletions. Labels at left indicate geographic provenance (R = Romania, I = Israel). Portion of rDNA operon assembled from shotgun sequences of *C. complanatum* in present study indicated by asterisk. Region in yellow box indicates a double peak discussed in results. Data are from the present study and Dzikowski et al. (2004), Caffara et al. (2011, 2014), Simsek et al. (2018), and Li et al. (2018).

metacercariae in Turkey in which Simsek et al. (2018) sequenced the barcode region of CO1 were considered *C. complanatum* sensu stricto (Fig. 3).

Morphological and taxonomic results

Clinostomum sinensis n. sp.

Synonyms: *Clinostomum complanatum* of Chen et al. (2016), Li et al. (2018), Iwaki et al. (2018), and probably of Liao (1992); *Clinostomum* sp. 8 of Locke et al. (2015a)

Type host: *Candidia barbata* (second intermediate host)

Type locality: River Daja near Taichung City, Taiwan

Other hosts: *Opsariichthys pachycephalus*, *Myxocyprinus asiaticus*, *Carassius auratus*, *Ctenopharyngodon idella* (second intermediate hosts), *Phalacrocorax carbo* (definitive host)

Representative DNA sequences: ITS - MK796826-28; CO1 - MK801711-17

Type specimen, hologenophores, and paragenophores deposited in the Museum of Southwestern Biology, Division of Parasites, University of New Mexico (Accessions MSB: Para: 29096–29097).

Etymology: *Clinostomum sinensis* n. sp. is named for its geographic origin.

Other localities: Hubei, Guangdong, People's Republic of China; stream near Meizihliao, Pingliin District, New Taipei City, Taiwan; Aichi, Japan

Morphological features of the metacercariae from *O. pachycephalus* and *C. barbata* from Taiwan (Table 2, Figs. 5 and 6): body stout, linguiform, elongated with flattened posterior end. Oral sucker surrounded by well evident oral collar. Pre-pharynx not visible. Pharynx visible in most specimens, muscular. Intestinal bifurcation anterior to ventral sucker. Ceca lateral to ventral sucker extending to posterior end of body. Ventral sucker muscular, well developed, larger than oral sucker. Ceca with small lateral sacculations from level of posterior testis to end of body. Testes two, tandem,

Table 2 Measurements of metacercariae [given as range (mean ± SD, *n*), in μm], of *Clinostomum* sampled in the present study (*n* = 11, *C. sinensis* n. sp.; *n* = 2, *C. complanatum*) and in Caffara et al. (2011) (*n* = 10, *C. complanatum*), Caffara et al. (2014) (*n* = 11, *C. complanatum*), Simsek et al. (2018) (*n* = 12, *C. complanatum*), and Li et al. (2018) (*n* = 27, *C. sinensis* n. sp.). Data are broken down by source in Supplementary Table 1.

	Clinostomum complanatum	*Clinostomum sinensis* n. sp.	*t*	*P*
OCW	616–1030 (792 ± 88, 23)	519–708 (606 ± 61, 10)	6.081	< 0.0005
BL	2637–7874 (4806 ± 1077, 35)	2470–4772 (3246 ± 615, 38)	7.670	< 0.0005
BW	1104–2434 (1611 ± 324, 35)	791–1507 (1232 ± 158, 38)	6.432	< 0.0005
BL/BW	2.2–4.37 (3.01 ± 0.420, 35)	1.85–4.19 (2.68 ± 0.65, 38)	2.533	0.014
OSL	154–337 (260 ± 159, 35)	156–295 (218 ± 33, 38)	1.594	0.115
OSW	231–507 (338 ± 64, 35)	230–391 (288 ± 42, 38)	3.929	< 0.0005
OSW/BW	0.16–0.28 (0.22 ± 0.03, 23)	0.16–0.35 (0.23 ± 0.05, 38)	8.329	< 0.0005
VSL	461–910 (713 ± 85, 35)	385–795 (529 ± 131, 37)	7.024	< 0.0005
VSW	482–952 (748 ± 93, 35)	430–849 (594 ± 103, 37)	6.606	< 0.0005
VSW/OSW	1.78–2.69 (2.26 ± 0.23, 35)	1.33–2.79 (2.08 ± 0.36, 37)	2.503	0.015
VSW/BW	0.39–0.63 (0.489 ± 0.074, 23)	0.31–0.72 (0.49 ± 0.12, 37)	0.138	0.172
Db OS–VS	222–1115 (757 ± 311, 35)	513–954 (685 ± 108, 37)	1.322	0.191
ATL	168–957 (390 ± 156, 35)	212–438 (292 ± 42, 38)	3.717	< 0.0005
ATW	203–559 (358 ± 84, 35)	176–363 (253 ± 48, 38)	6.596	< 0.0005
ATW/ ATL	0.46–1.48 (0.996 ± 0.255, 35)	0.61–1.21 (0.87 ± 0.12, 38)	2.757	0.007
PTL	141–441 (296 ± 72, 35)	178–374 (247 ± 51, 38)	3.357	0.001
PTW	212–602 (409 ± 98, 35)	225–441 (286 ± 57, 38)	6.573	< 0.0005
PTW/PTL	0.54–2.2 (1.43 ± 0.33, 35)	0.7–1.61 (1.19 ± 0.26, 38)	3.475	0.001
Db AT–PT	211–527 (315 ± 63, 35)	111–370 (304 ± 53, 38)	0.835	0.407
OL	57–164 (123 ± 31, 34)	57–143 (86 ± 21, 38)	5.914	< 0.0005
OW	76–178 (111 ± 20, 34)	70–114 (87 ± 9, 38)	6.414	< 0.0005
OW/OL	0.59–1.58 (0.95 ± 0.24, 34)	0.67–1.53 (1.08 ± 0.28, 38)	2.133	0.036
CSL	207–405 (272 ± 43, 35)	132–326 (204 ± 53, 38)	6.073	< 0.0005
CSW	105–197 (142 ± 22, 35)	117–205 (145 ± 25, 38)	0.480	0.632
CSL/BL	0.03–0.09 (0.058 ± 0.013, 35)	0.04–0.09 (0.06 ± 0.01, 38)	1.852	0.068

Abbreviations: *OCW* oral collar width, *BL* body length, *BW* body, *OSL* oral sucker length, *OSW* oral sucker width, *VSL* ventral sucker length, *VSW* ventral sucker width, *Db* distance between, *ATL* anterior testis length, *ATW* anterior testis width, *PTL* posterior testis length, *PTW* posterior testis width, *OL* ovary length, *OW* ovary width, *CPL* cirrus pouch length, *CPW* cirrus pouch width

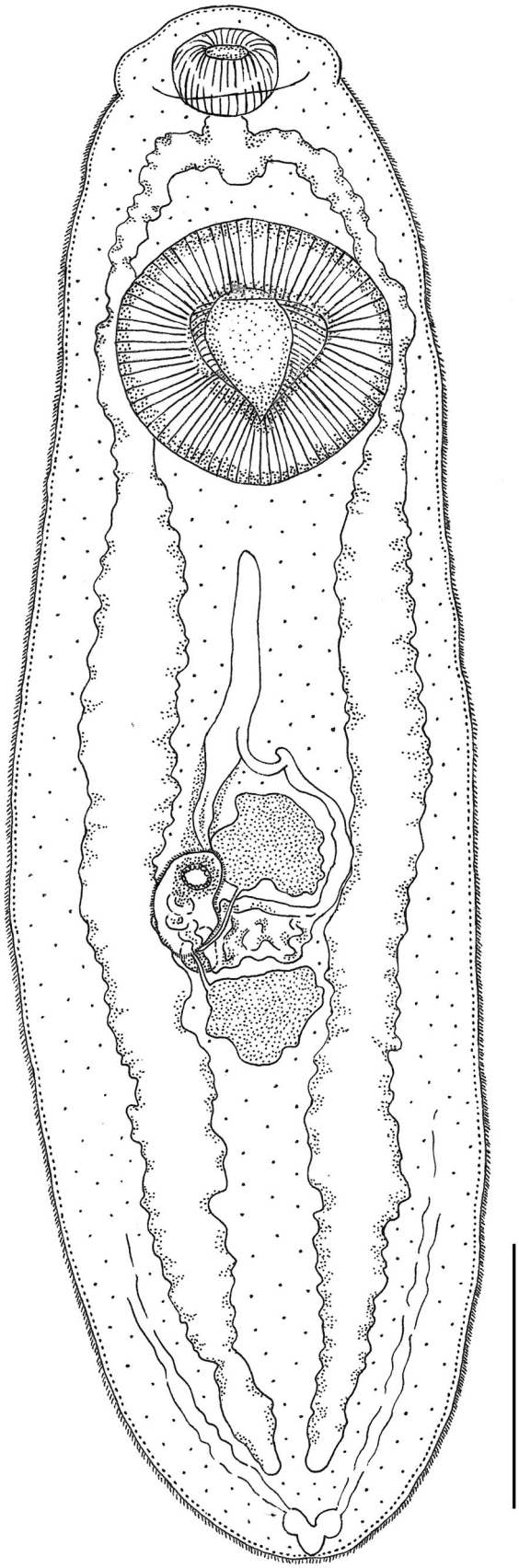

Fig. 5 Line drawing of *Clinostomum sinensis* n. sp. from *Candidia barbata* in Taiwan

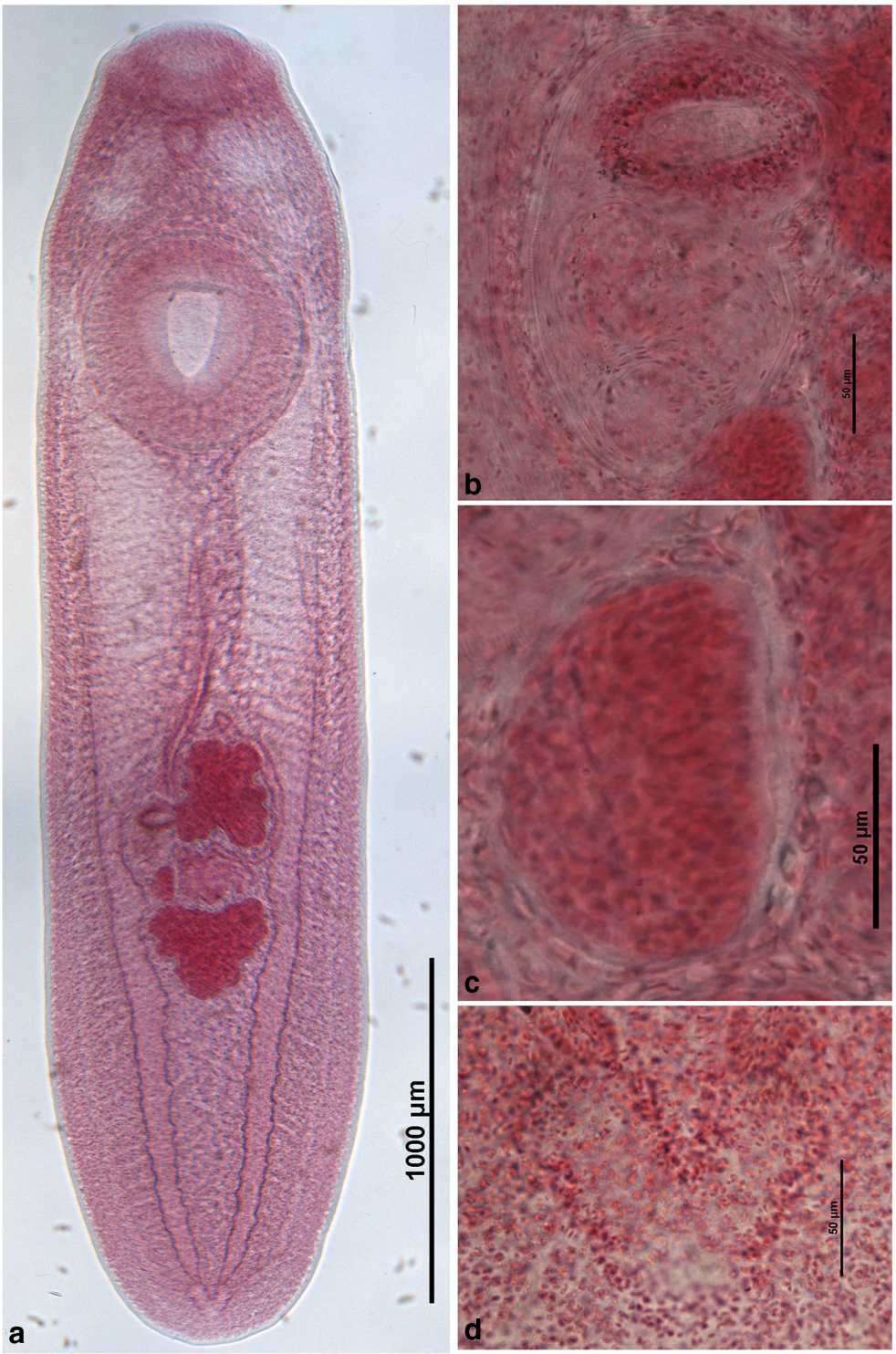

Fig. 6 *Clinostomum sinensis* n. sp. from *Candidia barbata* in Taiwan: whole mount (**a**), genital pore and cirrus sac (**b**), ovary (**c**), excretory bladder (**d**)

intercecal. Anterior testis, roughly triangular with rounded apex, lobed, in posterior end of middle third of body. Posterior testis triangular lobed, in anterior part of the posterior third of body. Efferent ducts from lateral right margin of testes ending in left margin of cirrus pouch. Cirrus pouch bean shaped, thick walled, intertesticular dextral, touching posterior right lobe of anterior testis, opening into genital atrium. Genital atrium with small papillae. Ovary ovoid, intertesticular, touching left posterior margin of cirrus pouch, in some specimens partially covered by cirrus pouch. Uteroduct passing around left margin of anterior testis forming knee-like fold before opening into uterine sac. Uterine sac tubular with metraterm approaching right margin of anterior testis. Tegument covered by thick spines from posterior part of oral collar. Excretory bladder Y-shaped. Excretory pore terminal.

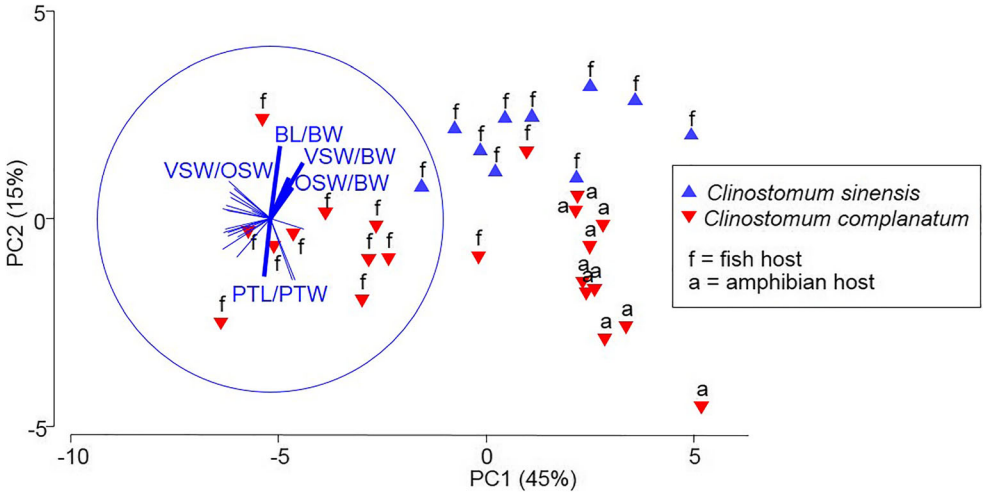

Fig. 7 Principal component analyses of 16 morphometric variables and 8 ratios in metacercariae of *Clinostomum* from Europe and East Asia. Axes are labeled with proportion of total variation explained. Vectors show the strength of morphometric features along the two axes, with the circle indicating vector lengths of perfect correlation. The data are from 10 *Clinostomum sinensis* n. sp. from cyprinid fishes *Opsariichthys pachycephalus* and *Candidia barbata* in Taiwan (present study); 10 *C. complanatum* from cyprinid fishes *Barbus barbus*, *B. meridionalis*, and *Squalius cephalus* in Italy (data from Caffara et al. 2011); 2 *C. complanatum* from *Scardinius erythopthalmus* and *Perca fluviatilis* from Romania (present study); and 10 *C. complanatum* from amphibians, *Lissotriton vulgaris* and *Triturus carnifex* (data from Caffara et al. 2014). See Table 2 for vector label abbreviations

Remarks: In metacercariae of both *C. sinensis* n. sp. and of *C. complanatum*, the genital complex is in middle and posterior third of the body, but the anterior testis in *C. sinensis* n. sp. is more median than in *C. complanatum*, in which it is more strongly left-dislocated by the cirrus pouch and uterine sac. The cirrus pouch in metacercariae of *C. sinensis* n. sp. is intertesticular dextral, almost overlapping the lateral margin of the posterior lobe of anterior testis, while in *C. complanatum*, the cirrus pouch is larger and more distant from the lateral margin of the anterior testis. In *C. sinensis* n. sp., the genital atrium shows small papillae which are absent in *C. complanatum*. The ovary in metacercariae of *C. sinensis* n. sp. is both smaller (Table 2, Fig. 6c) and closer to the cirrus pouch than in *C. complanatum*, in which it is more median.

The dimensions of 13 of 18 structures and five of seven morphometric ratios differed in metacercariae of *C. sinensis* n. sp. and of *C. complanatum* (Table 2). Compared with *C. complanatum*, metacercariae of *C. sinensis* n. sp. were shorter, narrower, with smaller oral collars, smaller oral and ventral suckers, smaller testes and ovaries, and shorter cirrus sacs, although ranges overlap in all of these features. Substantial variation was also attributable to whether worms were obtained from fish or amphibians (Fig. 7). In a two-way test, class of host was a strong source of morphometric variation among metacercariae (ANOSIM $R = 0.710$, $P = 0.001$), but even taking this into account, metacercariae of *C. sinensis* n. sp. and of *C. complanatum s.s.* resemble conspecifics more than heterospecifics (ANOSIM $R = 0.497$, $P = 001$). No individual measurement was strongly discriminating on the first axis in PCA; on the second axis, along which the two species of *Clinostomum* were more separated, morphometric ratios loaded most strongly (loadings 0.3–0.42 in five ratios) (Fig. 7).

The eggs in an adult of *C. sinensis* n. sp. collected by Iwaki et al. (2018) from *P. carbo* are longer than those of *C. complanatum* (Table 3), suggesting that this character may distinguish *C. sinensis* n. sp. from *C. complanatum*. Egg lengths recorded from *C. sinensis* n. sp. by Iwaki et al. (2018) are also larger than those of Old-World or Australasian species from which comparable DNA sequences have not been obtained, including *C. hornum* Nicoll, 1914, *C. australiense* Johnson, 1917, and *C. wilsoni* Matthews and Cribb 1998, in all of which eggs do not exceed 122 μm in length (Matthews and Cribb 1998). Restriction of the vitelline fields posterior to the ventral sucker in the adult of *C. sinensis* n. sp. in (Iwaki et al. 2018) distinguishes it from *C. kassimovi* Vaidova and Feizullaev, 1958. For additional data from adults of *C. sinensis* n. sp., we make the assumption that the specimens studied by Liao (1992) in Guangdong, PRC, are the same species as other isolates in East Asia discussed here, i.e., *C. sinensis* n. sp., even if genetic data to support this are lacking. Liao (1992) obtained eggs by rinsing the oral cavities of infected individuals in three ardeid species. The eggs he obtained were significantly longer than eggs in *C. complanatum* s.s. measured by Caffara et al. (2011) ($t = 2.232$, $df = 1024$, $P = 0.0258$). The line drawings of Liao (1992) resemble *C. sinensis* n. sp. more than *C. complanatum*, especially in some peculiar structures such as the efferent ducts and papillose genital atrium in both metacercaria and adult. Liao (1992) depicted the ovary as crescent shaped in the adult and ovoid in the metacercaria (consistent with metacercariae of *C. sinensis* n. sp. that we

Table 3 Morphometrics from adults of *Clinostomum sinensis* n. sp. and *Clinostomum complanatum* sampled in prior studies, given as range (mean ± SD), in μm unless otherwise noted

	Clinostomum complanatum from *Ardea cinerea*, *Ardea purpurea*, *Egretta egretta*, Italy (n = 5) (Caffara et al. 2011)	*Clinostomum complanatum*; type specimens from *Ardea cinerea*, Berlin, Germany (n = 4) (Braun 1901)	*Clinostomum complanatum* from *Ardea cinerea*, Genoa, Italy (n = 2) (Braun 1901)	*Clinostomum sinensis* n. sp. (syn. *C. complanatum*) from *Phalacrocorax carbo*, Aichi, Japan (n = 1) (Iwaki et al. 2018)	*Clinostomum sinensis* n. sp. (syn. *C. complanatum*) from *Ardeola bacchus*, *Egretta garzeta*, *Nycticorax nycticorax*, Guangdong, PRC (Liao 1992)
Body length (mm)	3.4–6.3 (4.9 ± 0.9)	3.5–4.3	6.1–9.5[a]	4.92	
Body width (mm)	1.5–2.7 (1.9 ± 0.3)		2.6	1.78	
Oral sucker length	190–570 (422 ± 121)	160		340	
Oral sucker width	320–850 (557 ± 161)	290		361	
Ventral sucker length	600–900 (760 ± 78)	500	700[a]	817	
Ventral sucker width	620–900 (737 ± 72)	500	800[a]	892	
Anterior testis length	550–750 (694 ± 67)			500	
Anterior testis width	360–600 (456 ± 82)			693	
Posterior testis length	600–940 (791 ± 99)			363	
Posterior testis width	300–510 (410 ± 81)			775	
Ovary length	220–310 (256 ± 32)			249	
Ovary width	140–300 (213 ± 62)			254	
Egg length	100–125 (114 ± 6)	120	104	135–140	102–140 (121 ± 7, n = 1021)[b]
Egg width	65–90 (74 ± 5)	70	62	71–75	63–81 (75 ± 3, n = 1021)[b]

[a] Braun (1901) noted distortion caused by excessive flattening

[b] Eggs from rinse water of oral cavities of avian hosts

examined). In contrast, adults of *C. complanatum* possess a round or oval ovary.

Discussion

Molecular and morphological data gathered here revealed *C. sinensis* n. sp., a previously unrecognized species of *Clinostomum* in East Asia. This conclusion rests partly on the 5.7% divergence of mitochondrial genomes of *C. sinensis* n. sp. and *C. complanatum*. This exceeds the 4.6% variation between *Taenia saginata* and *T. asiatica* (Jeon et al. 2007), which are sister species used a benchmark for comparisons of closely related platyhelminths (Nakao et al. 2007) and is an order of magnitude greater than the divergence upon which other animal species have been separated (Morin et al. 2010). *Clinostomum sinensis* n. sp. is also distinguished from *C. complanatum* based on the divergence (Fig. 2) and reciprocal monophyly (Fig. 3) of partial CO1 sequences from isolates in East Asia and Europe and Turkey, and morphometric variation (Fig. 7, Table 2) and morphological differences in the genital complex.

Conventionally, species of *Clinostomum* are erected based on adult morphology, but most data from *C. sinensis* n. sp. are from metacercariae. Descriptions by Liao (1992) and Iwaki et al. (2018) suggest that adults of *C. sinensis* n. sp. can be distinguished from *C. complanatum* by egg size and the presence of papules on the genital pore. However, examination of similarly fixed specimens from equivalent host species is needed to verify this and further characterize adult morphology in *C. sinensis* n. sp. One advantage of studying adults of *Clinostomum* is that eggs constitute a definitive indicator of maturity, whereas developmental milestones are unknown for metacercariae. Nonetheless, we believe that the systematics of clinostomids can be usefully advanced by considering metacercariae in addition to adults, as herein. Our view follows Ukoli (1966), who argued against the obligatory use of adults for taxonomic purposes in *Clinostomum*, because key morphological aspects of the adult can be discerned in the metacercaria, in some cases with greater clarity. Structures such as the metraterm and ootype become obscured by eggs and may be better visualized in metacercariae than adults. In adults, the larger testes impede observation of the cirrus pouch and ovary, and other parts of the genital complex can be hidden by the vitellarium.

Sequences of DNA should be linked to established or newly erected species with care, particularly in initial studies of less known taxa or regions, as these identifications will serve as a foundation for future work (e.g., Nielsen et al. 2014; Caffara et al. 2016). In *Clinostomum*, Chen et al. (2016) stressed the need for accurate identification

due to the potential impacts of this parasite on aquaculture and human health. Chen et al. (2016) did not consider *C. sinensis* n. sp. (*Clinostomum* sp. 8), and their identification of *C. complanatum* was accepted by later authors (Wang et al. 2017; Iwaki et al. 2018; Li et al. 2018). Collectively, these works relied heavily on local descriptions and records of *C. complanatum* and comparisons to morphological descriptions from the type region were questionable or lacking in detail. For example, Li et al. (2018) found 19 of 24 structures in metacercariae from Sichuan were significantly different from those published from metacercariae in Italy, much as here (Table 2), but still concluded the Sichuan material to be *C. complanatum*. A metacercaria photographed by Wang et al. (2017) appears to be 9 × 3 mm, which is much larger than *C. complanatum* (Table 2). Consequently, we cannot comment on the status of this unusually large species, and the 18S data of Wang et al. (2017), who did not respond to email, do not allow further conclusions.

In correspondence following their publication, Fang R. (pers. comm., 2016, 2017) maintained the identification of *C. complanatum* in Chen et al. (2016), supporting the morphological identification with reference to Zhou (2008), Gu et al. (2015), and Wu (2015). However, these sources assume a cosmopolitan distribution for *C. complanatum* and do not critically evaluate other possibilities. Fang R. (pers. comm., 2016, 2017) also defended the identification based on matching ITS2 in Hubei and Italian isolates, citing Liu et al. (2014) and Ma et al. (2016a, b) in support of this practice. However, these studies do not address the ambiguous levels of variation in ITS that sometimes occurs between closely related digeneans, including *Clinostomum* (Vilas et al. 2005; Locke et al. 2015a, b; Rosser et al. 2018). Indeed, in our reading of Liu et al. (2014), ITS alone did not resolve the status of *Fasciola* sp.

We believe the data show the material studied by ourselves, Liao (1992), Locke et al. (2015a), Chen et al. (2016), Iwaki et al. (2018), and Li et al. (2018) to be *C. sinensis* n. sp., which is closely related to, but genetically and morphologically distinct from, *C. complanatum* and other species in the genus *Clinostomum*.

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Compliance with ethical standards

All applicable international, national, and institutional guidelines for the care and use of animals were followed (MNSDG000000034589 issued by Rezervația Biosferei Delta Dunării in Tulcea, Romania).

Conflict of interest The authors declare that they have no conflict of interest.

References

Bernt M, Donath A, Jühling F, Externbrink F, Florentz C, Fritzsch G, Pütz J, Middendorf M, Stadler PF (2013) MITOS: improved de novo metazoan mitochondrial genome annotation. Mol Phylogenet Evol 69:313–319. https://doi.org/10.1016/j.ympev.2012.08.023

Brabec J, Kostadinova A, Scholz T, Littlewood DTJ (2015) Complete mitochondrial genomes and nuclear ribosomal RNA operons of two species of *Diplostomum* (Platyhelminthes: Trematoda): a molecular resource for taxonomy and molecular epidemiology of important fish pathogens. Parasit Vectors 8:1–11. https://doi.org/10.1186/s13071-015-0949-4

Braun M (1901) Die arten der gattung *Clinostomum* Leidy. Zoologische Jahrbücher Abteilung für Systematik, Geographie und Biologie der Tiere 14:1–48

Caffara M, Locke SA, Gustinelli A, Marcogliese DJ, Fioravanti ML (2011) Morphological and molecular differentiation of *Clinostomum complanatum* and *Clinostomum marginatum* (Digenea: Clinostomidae) metacercariae and adults. J Parasitol 97:884–891. https://doi.org/10.1645/GE-2781.1

Caffara M, Bruni G, Paoletti C, Gustinelli A, Fioravanti ML (2014) Metacercariae of *Clinostomum complanatum* (Trematoda: Digenea) in European newts *Triturus carnifex* and *Lissotriton vulgaris* (Caudata: Salamandridae). J Helminthol 88:278–285. https://doi.org/10.1017/S0022149X13000151

Caffara M, Locke SA, Cristanini C, Davidovich N, Markovich MP, Fioravanti ML (2016) A combined morphometric and molecular approach to identifying metacercariae of *Euclinostomum heterostomum* (Digenea: Clinostomidae). J Parasitol 102:239–248. https://doi.org/10.1645/15-823

Chen L, Feng Y, Chen H-M, Wang LX, Feng HL, Yang X, Mughal MN, Fang R (2016) Complete mitochondrial genome analysis of *Clinostomum complanatum* and its comparison with selected digeneans. Parasitol Res 115:3249–3256. https://doi.org/10.1007/s00436-016-5102-6

Dzikowski R, Levy M, Poore M et al (2004) *Clinostomum complanatum* and *Clinostomum marginatum* (Rudolphi, 1819) (Digenea: Clinostomidae) are separate species based on differences in ribosomal DNA. J Parasitol 90:413–414. https://doi.org/10.1645/GE-159R

Gaglio G, Reina V, Caffara M, Gjurcevic E, Iaria C, Marino F (2016) Risk of introduction of *Clinostomum complanatum* (Digenea: Clinostomidae) to Sicily through use of *Cobitis bilineata* (Canestrini, 1865) as live baits. Bull Eur Assoc Fish Pathol 36:105–110

Gu J, Zhuang G, Wang X, Wang Y (2015) The epidemiology and prevention of *Clinostomum complanatum*. Sci Fish Farm:88–88

Gustinelli A, Caffara M, Florio D, Otachi EO, Wathuta EM, Fioravanti ML (2010) First description of the adult stage of *Clinostomum cutaneum* Paperna, 1964 (Digenea: Clinostomidae) from grey herons *Ardea cinerea* L. and a redescription of the metacercaria from the Nile tilapia *Oreochromis niloticus niloticus* (L.) in Kenya. Syst Parasitol 76:39–51. https://doi.org/10.1007/s11230-010-9231-5

Hays WL (1994) Statistics, Fifth Edition. Harcourt Brace College Publishers, Fort Worth

Iwaki T, Waki T, Arakawa J, Ogawa K (2018) The Digenean *Clinostomum complanatum* found from Great Cormorant *Phalacrocorax carbo* in Japan. Fish Pathol 53:132–135. https://doi.org/10.3147/jsfp.53.132

Jeon H-K, Kim K-H, Eom KS (2007) Complete sequence of the mitochondrial genome of *Taenia saginata*: comparison with *T. solium*

and *T. asiatica*. Parasitol Int 56:243–246. https://doi.org/10.1016/j.parint.2007.04.001

Kagei N, Yanohara Y, Uchikawa R, Sato A (1988) Natural infection with *Clinostomum complanatum* (Rud., 1819) in the birds of southern Japan. Jpn J Parasitol 37:254–257

Kim Y-G, Nagasawa K (1996) Infection of *Clinostomum complanatum* (Rudolphi, 1814) (Trematoda: Digenea) metacercaria in goldfish (*Carassius auratus*) cultured in Korea. J Fish Pathol 9:1–19

Li BF, Liu X-H, Ge H-L et al (2018) The discovery of *Clinostomum complanatum* metacercariae in farmed Chinese sucker, *Myxocyprinus asiaticus*. Aquaculture 495:273–280. https://doi.org/10.1016/j.aquaculture.2018.05.052

Liao X-H (1992) The biology of *Clinostomum complanatum* (Rud.) I. Life history. Wuyi Sci J 9:99–133

Liu G-H, Gasser RB, Young ND, Song HQ, Ai L, Zhu XQ (2014) Complete mitochondrial genomes of the 'intermediate form' of *Fasciola* and *Fasciola gigantica*, and their comparison with *F. hepatica*. Parasit Vectors 7:150. https://doi.org/10.1186/1756-3305-7-150

Locke SA, Caffara M, Marcogliese DJ, Fioravanti ML (2015a) A large-scale molecular survey of *Clinostomum* (Digenea, Clinostomidae). Zool Scr 44:203–217. https://doi.org/10.1111/zsc.12096

Locke SA, Al-Nasiri FS, Caffara M et al (2015b) Diversity, specificity and speciation in larval Diplostomidae (Platyhelminthes: Digenea) in the eyes of freshwater fish, as revealed by DNA barcodes. Int J Parasitol 45:841–855. https://doi.org/10.1016/j.ijpara.2015.07.001

Locke SA, Van Dam A, Caffara M et al (2018) Validity of the Diplostomoidea and Diplostomida (Digenea, Platyhelminthes) upheld in phylogenomic analysis. Int J Parasitol 48:1043–1059. https://doi.org/10.1016/j.ijpara.2018.07.001

Ma J, He J-J, Liu G-H et al (2016a) Mitochondrial genome of *Ogmocotyle sikae* and implications for phylogenetic studies of the Notocotylidae trematodes. Infect Genet Evol 37:208–214. https://doi.org/10.1016/j.meegid.2015.11.018

Ma J, He J-J, Liu G-H, Leontovyč R, Kašný M, Zhu XQ (2016b) Complete mitochondrial genome of the giant liver fluke *Fascioloides magna* (Digenea: Fasciolidae) and its comparison with selected trematodes. Parasit Vectors 9:429–427. https://doi.org/10.1186/s13071-016-1699-7

Matthews D, Cribb TH (1998) Digenetic trematodes of the genus *Clinostomum* Leidy, 1856 (Digenea: Clinostomidae) from birds of Queensland, Australia, including *C. wilsoni* n. sp. from *Egretta intermedia*. Syst Parasitol 39:199–208. https://doi.org/10.1023/A:1005982530560

Morin PA, Archer FI, Foote AD, Vilstrup J, Allen EE, Wade P, Durban J, Parsons K, Pitman R, Li L, Bouffard P, Abel Nielsen SC, Rasmussen M, Willerslev E, Gilbert MT, Harkins T (2010) Complete mitochondrial genome phylogeographic analysis of killer whales (*Orcinus orca*) indicates multiple species. Genome Res 20:908–916. https://doi.org/10.1101/gr.102954.109

Moszczynska A, Locke SA, McLaughlin JD, Marcogliese DJ, Crease TJ (2009) Development of primers for the mitochondrial cytochrome *c* oxidase I gene in digenetic trematodes illustrates the challenge of barcoding parasitic helminths. Mol Ecol Resour 9:75–82. https://doi.org/10.1111/j.1755-0998.2009.02634.x

Nakao M, McManus DP, Schantz PM, Craig PS, Ito A (2007) A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. Parasitology 134:713–722. https://doi.org/10.1017/S0031182006001934

Nielsen MK, Wang J, Davis R, Bellaw JL, Lyons ET, Lear TL, Goday C (2014) *Parascaris univalens* - a victim of large-scale misidentification? Parasitol Res 113:4485–4490. https://doi.org/10.1007/s00436-014-4135-y

Pleijel F, Jondelius U, Norlinder E, Nygren A, Oxelman B, Schander C, Sundberg P, Thollesson M (2008) Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. Mol Phyl Evol 48:369-371https://doi.org/10.1016/j.ympev.2008.03.024

Pritchard MH, Kruse G (1982) The collection and preservation of animal parasites. University of Nebraska Press, Lincoln

Ronquist F, Teslenko M, Van Der Mark P et al (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61:539–542. https://doi.org/10.1093/sysbio/sys029

Rosser TG, Baumgartner WA, Alberson NR, Noto TW, Woodyard ET, Tommy King D, Wise DJ, Griffin MJ (2018) *Clinostomum poteae* n. sp. (Digenea: Clinostomidae), in the trachea of a double-crested cormorant *Phalacrocorax auritus* Lesson, 1831 and molecular data linking the life-cycle stages of *Clinostomum album* Rosser, Alberson, Woodyard, Cunningham, Pote & Griffin, 2017 in Mississippi, USA. Syst Parasitol 95:543–566. https://doi.org/10.1007/s11230-018-9801-5

Rudolphi C (1819) Entozoorum synopsis cui accedunt mantissa duplex et indices locupletissimi. August Rücker, Berlin

Shini KK, Preethakumari VM, Ramitha UC, Vasandakumar MV (2015) Helminth parasitic fauna of a cyprinid fish *Devario malabaricus* (Jerdon). Int J Fish Aquat Stud 2:175–179

Simsek E, Yildirim A, Yilmaz E, Inci A, Duzlu O, Onder Z, Ciloglu A, Yetismis G, Pekmezci GZ (2018) Occurrence and molecular characterization of *Clinostomum complanatum* (Trematoda: Clinostomidae) in freshwater fishes caught from Turkey. Parasitol Res 117:2117–2124. https://doi.org/10.1007/s00436-018-5898-3

Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Ukoli F (1966) On the life history, growth and development from the metacercarial stage to adulthood, of *Clinostomum tilapiae* Ukoli, 1966. J Helminthol 40:215–226

Vilas R, Criscione C, Blouin M (2005) A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites. Parasitology 131:839–846. https://doi.org/10.1017/S0031182005008437

Wang M-L, Chen H-Y, Shih H-H (2017) Occurrence and distribution of yellow grub trematodes (*Clinostomum complanatum*) infection in Taiwan. Parasitol Res 116:1761–1771. https://doi.org/10.1007/s00436-017-5457-3

Woodyard ET, Rosser TG, Rush SA (2017) Alligator wrestling: morphological, molecular, and phylogenetic data on *Odhneriotrema incommodum* (Leidy, 1856) (Digenea: Clinostomidae) from *Alligator mississippiensis* Daudin, 1801 in Mississippi, USA. Parasitol Res 116:2981–2993. https://doi.org/10.1007/s00436-017-5607-7

Wu S (2015) Aquatic animal parasites. China Agriculture Press, Beijing

Zhou G, Xiang S-L, Shi L (2008) The occurrence and control of the disease caused by *Clinostomum complanatum*. Fish Sci Technol Inf 35:301-303