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Use of rDNA polymorphism for identification of Heterophyidae infecting freshwater fishes

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ABSTRACT: Infections by trematodes are among the most common fish-borne zoonoses. Metacercariae of the Family Heterophyidae in marine and freshwater fishes are nonfastidious in their choice of definitive hosts, and therefore, cause infections in human and domestic animals. In the present study, species-specific polymerase chain reaction (PCR) assays were developed for identifying and differentiating the various species examined. Sequencing and aligning the 18S (SSU) rDNA revealed interspecific variation for which species-specific DNA oligonucleotides were designed and used for the identification of 6 heterophyid species recovered from piscivorous birds. The oligonucleotides were further used to evaluate the various stages (cercariae recovered from snails, metacercariae recovered from fish and adult trematodes) of the digeneans. By applying this method we elucidated for the first time the life cycle of *Pygidiopsis genata.* The phylogenetic interrelationship among the newly sequenced species of Heterophyidae is outlined.

KEY WORDS: Digenea · Heterophyidae · Birds · Fish · 18S rDNA gene · SSU

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INTRODUCTION

Trematodes of the Family Heterophyidae Odhner, 1914 are significant both as fish pathogens and as agents causing zoonotic infections among humans consuming raw or incompletely cooked fish. Severe infection by the heterophyid *Centrocestus* spp. in the gills (Paperna 1996), *Haplorchis pumilio* in the connective tissue (Sommerville 1982) and more recently heart infections by *Phagicola longa* (R. Dzikowski & I. Paperna unpubl. data) have resulted in massive losses in farmed cichlid fish (Paperna 1995). Because the adult stages of many heterophyids are nonfastidious in their choice of definite hosts, infections with severe clinical consequences have been reported in humans after consuming fish infected with metacercariae (Deardorff & Overstreet 1990, Ko 1995).

Witenberg (1929) described 14 species of the Family Heterophyidae from both naturally and experimentally infected avian and mammalian definitive hosts. While specific determination of larval stages by morphological traits is often difficult and ambiguous, experimental demonstration of the life history is frequently unachievable due to the unidentified nature of the specific intermediate or definitive host. The use of molecular methodologies has allowed links to be elucidated between the various developmental stages, i.e. cercariae, metacercariae and adults of specific trematodes (Cribb et al. 1998, Jousson et al. 1998, Anderson 1999, Bartoli et al. 2000, Levy et al. 2002). Various ribosomal genes, especially the 18S (small subunit, SSU) along with the inter-specific polymorphic regions, include highly conserved regions for which 'universal primers' can be designed for amplification of this gene from a newly studied species (Hillis & Dixon 1991, Littlewood & Olson 2001). PCR methodologies have also enabled amplification of these genes from a single cercaria without DNA extraction (Grevelding et al. 1997).

The objectives of the present study were to develop species-specific oligonucleotides as a diagnostic tool for the identification of heterophyid species recovered from piscivorous birds in Israel. These oligonucleotides

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were then used for identification of cercariae recovered from snails and metacercariae recovered from fish, demonstrating their usefulness as a diagnostic tool important for fish and public health. An effort made to define the entire life history of *Pygidiopsis genata* was successfully carried out. Finally we attempted to examine the phylogenetic links between the Heterophyidae and various other trematodes that were concurrently investigated.

MATERIALS AND METHODS

Sources of parasites and morphological characterization. During 2000 to 2002, adult trematodes were collected from 45 cormorants *Phalacrocorax carbo*, 4 little egrets *Egretta garzetta*, 3 night herons *Nycticorax nycticorax*, 2 white pelicans *Pelecanus onocrotalus* and a catfish *Clarias gariepinus*. Collection permits were obtained from the Office of Nature Conservation, Ministry of Environmental Quality, Israel.

Trematodes were collected from the sedimentation of the intestine content, then removed and placed in clean physiological saline and preserved in 70% ethanol. Several specimens of each species were fixed in 70% ethanol overnight under light pressure, stained with Aceto-Carmine, and whole mounted for morphologic examination.

Metacercariae of *Centrocestus* sp. (gills), *Haplorchis* sp. (subcutaneous) *Phagicola* sp. (heart: conus arteriosus) and *Pygidiopsis* sp. (kidney), and an unidentified metacercaria from the liver, were obtained from *Tilapia zillii* (Cichlidae). The fish were collected from commercial fish ponds and Lake Kinneret. *Clinostomum complanatum* metacercariae (subcutaneous, intermuscular) were collected from *Barbus canis* (Cyprinidae) from Lake Kinneret. All metacercariae were preserved in 70% ethanol.

Cercariae were collected from 3 species of freshwater snails (*Melanoides tuberculata*, *Melanopsis costata* and *Bythinia hawaderiana*) from Lake Kinneret. Data (snail host, preliminary identifications, etc.) for the cercariae were recorded and individual specimens were frozen at –70°C or fixed in 70% ethanol.

Gene amplification. Individual worms, metacercariae, and cercariae were washed overnight in buffer (10 mM Tris-EDTA). Following another 2 washes (1 h each), a single metacercariae or cercariae was placed into a 0.2 ml PCR tube and gently crushed prior to DNA extractions or direct amplification. DNA was extracted from single adult worms using a DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. Polymerase chain reactions were carried out in 50 µl volumes using 10× buffer, 2.5 U *Taq* polymerase (HotStarTaq, Qiagen), 10 mM of each deoxynucleotide triphosphate and 100 ng of each primer. Amplification of the 18S (SSU) and internal transcribed spacer (ITS) of the rDNA gene were carried out as described in Levy et al. (2002) using universal trematodes primer sets. These primer sets could not amplify the rDNA of the majority of Heterophyidae spp. found in the present study. Therefore, different primers designated for conserved regions were used to amplify the 18S regions of *Phagicola longa*, *Haplorchis pumilio*, *H. taichui*, *Centrocestus* sp., the unidentified liver metacercaria (Het 18S F/R) and *Pygidiopsis genata* (Uni 18S F/R) (Table 1). Different primers were also used to amplify the ITS regions of *H. pumilio*, *H. taichui*, *Centrocestus* sp., *P. genata* (19 F/A2 R) and *P. longa* (412 F/204a R) (Table 1). PCR amplicons were subjected to electrophoresis in 1.5% agarose gels stained with ethidium bromide and visualized under ultraviolet light.

PCR products of all species, except the 18S of *Haplorchis pumilio*, *H. taichui*, *Centrocestus* sp. and *Pygidiopsis genata* were purified using a Qiagene QIAquick PCR purification kit and sequenced directly on an ABI377 DNA sequencer using the Deoxy^{TM} Terminator Cycle sequencing kit (Applied Biosystems AB^{TM}) following the manufacturer's instructions.

PCR products of the 18S amplifications of *Haplorchis pumilio*, *H. taichui*, *Centrocestus* sp. and *Pygidiopsis genata* contained extraneous amplicons. The amplicons of interest were cut out of the gels and further purified using a Qiagene gel Extraction Kit and cloned into a pGEM-T Easy Vector system, utilizing blue/white selection (Sambrook et al. 1989). Plasmids from white colonies were screened for inserts using PCR amplification. Positive colonies were incubated overnight and then purified using the High Pure Purification Kit (Roche), quantified by spectrophotometry and sequenced.

Sequencing of the 18S regions was done using the internal primers 18S 360F and 18S 1533R (Levy et al. 2002). The sequencing of the ITS of *Haplorchis pumilio Pygidiopsis genata* and *Centrocestus* sp. was done using the internal primer ITS 455 F (Table 1). Sequencing the 3′ of the 18S of *Centrocestus* sp., *P. genata*, *H. taichui* and *H. pumilio* required another PCR amplification using the reverse primers Cnt ITS 154 R, Pyg ITS 140 R, tai ITS 140 R and pum ITS 140 R, respectively, with Het 1824 F (annealing temperature 58°C and 30 s at 72°C). Sequence analyses were done following the same procedures described previously in Levy et al. (2002). Species-specific oligonucleotide primer sets were designed for the 6 heterophyid species: *Centrocestus* sp., *H. pumilio*, *H. taichui*, *Dexiogonimus ciureanus*, *Phagicola longa* and *P. genata* using the specific forward primers Cnt 1463 F, pum 1463 F, tai 1463 F, Dex 1463 F, Pha 1463 F and Pyg 1463 F,

Table 1. Oligonucleotide primers used to sequence and assay rDNA 18S and ITS by polymerase chain reaction (PCR)

Primer type	Designation	Species	Sequence 5′ – 3′	Annealing temperature (°C)
Heterophyidae 18S	Het 18S F	Liver metacercaria *Haplorchis pumillo* *H. taichui* *Centrocestus* sp.	TCA TAT GCT TGT CTC AGA	53
	Het 18S R	*Pagicola longa*	ACG GAA ACC TTG TTA CGA	
Trematode universal 18S	Uni 18S F	*Pygidiopsis genata*	GCT TGT CTC AGA GAT TAA GCC	50
	Uni 18S R		ACG GAA ACC TTG TTA CGA C	
Heterophyidae ITS	19 F	*H. pumilio* *H. taichui* *Centrocestus* sp.	CGA GTC GTA ACA AGG TTT CCG	64
	A2 R	*P. genata*	TAT GCT TAA GTT CAG CGG GT	
	412 F	*P. longa*	TAA CAG GTC TGT GAT	53
	204 Ra		ATA TGC TTA AAT TCA GCG GGT	
ITS sequencing (internal)	ITS 455 F	*H. pumilio* *P. genata* *Centrocestus* sp. Unidentified Plagiorchiidae sp.	CTC TTC ATC GAC ACA CGA GC	50
Heterophydiae 18S 3′ sequencing	Cnt ITS 154 R	*Centrocestus* sp.	CAC CGT AGG CAG ACA AGG CT	58
	Pyg ITS 140 R	*P. genata*	TCC GAT ATC GAC AGC AAA CG	
	tai ITS 140 R	*H. taichui*	ACG GAA CTG GCG TCG ATC CG	
	pum ITS 140 R	*H. pumilio*	GTG ATC CCG GAG TTC ACT AT	
	Het 1824 F	All 4 above	ACC GCC CGT CGC TAC TAC CG	
Species specific primers	Cnt 1463 F	*Centrocestus* sp.	ACC CGT GCG GGT GGC GGT GAT CA	72
	pum 1463 F	*H. pumilio*	ACT CGT GCA GGT AGC GGT GGT CG	72
	tai 1463 F	*H. taichui*	GCC TGT GCA GGT AGC GGT GCT CG	72
	Dex 1463 F	*D. ciureanus*	GCT CGT GCA GGT GGC GGT GCT CG	72
	Pha 1463 F	*P. longa*	ACT CGT GCG GGT GGC GGT ATT CT	69
	Pyg 1463 F	*P. genata*	ATC CGT GCA GGT GGC GGT TAT CT	69
	Het 1824 R	All 6 above	AAT CGG TAG TAG CGA CGG GCG GT	69–72

respectively, with Het 1824 R. The cycling conditions were as described in Levy et al. (2002) with few exceptions; the optimal conditions required 34 cycles with annealing temperature set at 69 to 72°C (see Table 1) for 45 and 30 s at 72°C.

Endonuclease restriction digest. We employed the PCR-RFLP procedure in order to distinguish between *Haplorchis pu- milio*, *H. taichui* and *Dexiogonimus ciureanus*. A 5 µl aliquot of the rDNA 18S PCR product (1.5 µg) of each species was digested with *Hin f1* (Promega) according to the manufacturer's specifications, at 37°C for 4 h. Electrophoresis of the resulting fragments was done on a 1.5% agarose gel and visualized with ethidium bromide.

Phylogeny analysis. The rDNA 18S (~1800 bp) nucleotide sequences were aligned using the aligning tool supplied by the MacOSX ARB phylogenetic program package (34). Phylogenetic trees were generated with the neighbor-joining and maximum likelihood methods with the ARB program package using the Felsenstein correction method, applying a 50% cutoff filter (34). Branching order was supported by both methods.

RESULTS

Thirteen trematode species were recovered from birds: *Haplorchis pumilio*, *H. taichui*, *Centrocestus* sp., *Pygidiopsis genata*, *Phagicola longa*, *Dexiogonimus ciureanus*, *Clinostomum complanatum*, an unidentified species of Strigeidae, *Bolbophorus levantinus*, *Bolbophorus confusus*, *Paryphostomum radiatum*, *Petasiger phalacrocoracis* and *Holostephanus dubinini.* An unidentified plagiorchiid and an unidentified metacercaria from the liver were recovered from fish (Table 2).

The rDNA 18S and the ITS gene sequences of the 14 adult trematode species as well as that of the unidentified metacercaria (liver), were obtained and submitted to GenBank (Table 2). Based on the alignment of the 18S sequences of the subject heterophyid species, there is only 1 polymorphic site between all 6 heterophyid species (starting at 1463 bp of the 5′ end) to which species-specific oligonucleotide primer sets have been designed (Fig. 1).

We used the *Centrocestus* sp. (from Israel) specific primer (Cnt 1463 F, Het 1824 R) to link the *Centro-*

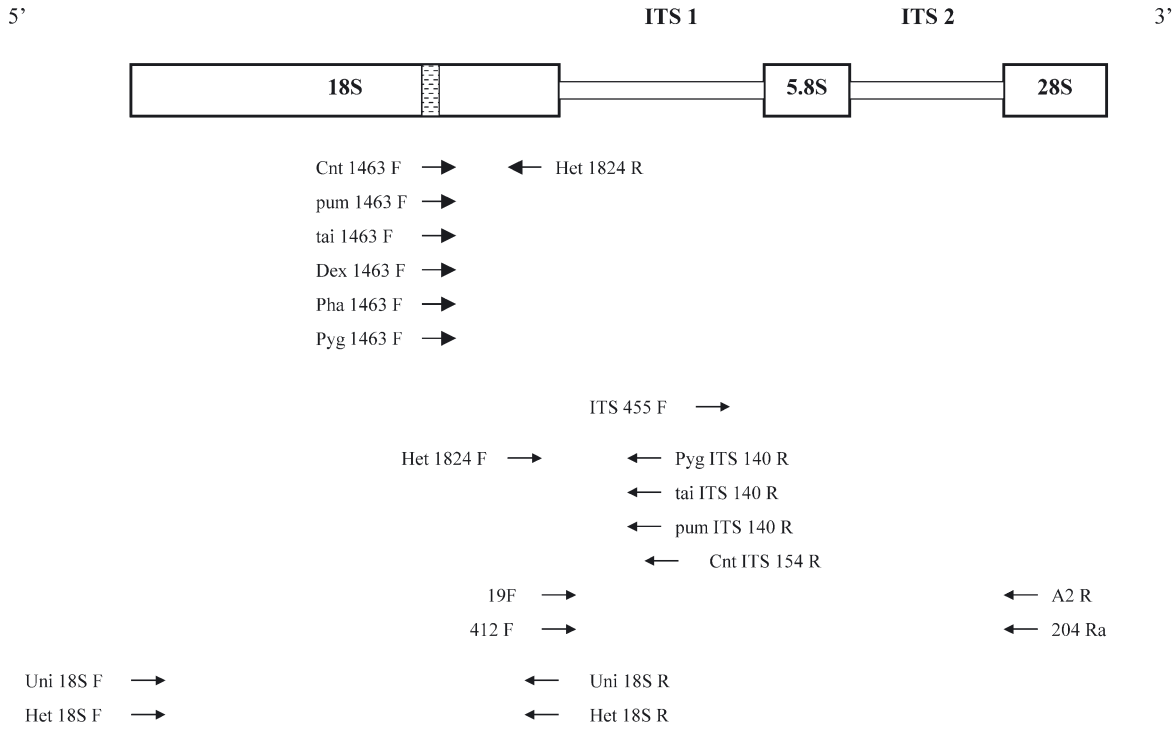

Fig. 1. Map of the PCR primers used for the amplification and sequencing (small arrows) of the 18S and ITS rDNA regions of the digeneans investigated and heterophyids' species-specific primers (big arrows). Arrows indicate priming sense. ▒: Heterophyids' polymorphic region

cestus-type pleurolophocercous cercariae, which emerged from the snail *Melanoides tuberculata*, the *Centrocestus* sp. metacercaria from the gills of *Tilapia zillii* and the adult *Centrocestus* sp. from the cormorant (Fig. 2A). Using *Pygidiopsis genata* specific primers (Pyg 1463 F, Het 1824 R), we have found a specific genetic link between the pleurolophocercous cercariae that emerged from the snail *Melanopsis costata*, the *Pygidiopsis* sp. metacercaria found in the kidney of *T. zillii* and the adult *P. genata* found in the cormorant (Fig. 2B). By the same methodology, *Phagicola longa* specific primers (Pha 1463 F, Het 1824 R) have enabled us to link the *Phagicola* sp. metacercaria from the heart of *T. zillii* to the adult *P. longa* from the cormorant. The snail host remains unknown, as infected snails were not found (Fig. 2C). Similarly, using the specific primer set (pum 1463 F, Het 1824 R), we have established the genetic association between all the stages of *Haplorchis pumilio*, including the cercariae in *M. tuberculata*, the metacercariae in *T. zilli* and the adult worms in cormorants (Fig. 2D).

Table 2. Trematode species, recovered from piscivorous birds in Israel during 2000 to 2002, listed with their rDNA 18S-ITS GenBank accession number

Trematode species	GenBank accession number	Host
Centrocestus sp. (Looss, 1989)	AY245699	*Egretta garzetta*
Haplorchis pumilio (Looss, 1896)	AY245706	*Phalacrocorax carbo*
Haplorchis taichui (Nishigori, 1924)	AY245705	*P. carbo*
Phagicola longa (Ransom, 1920)	AY245703	*P. carbo*
Pygidiopsis genata Looss, 1907	AY245710	*P. carbo*
Dexiogonimus ciureanus Witenberg, 1928	AY245702	*P. carbo*
Bolbophorus levantinus (Dubois, 1970)	AF490576	*Nycticorax nycticorax*
Bolbophorus confusus (Krause, 1914)	AY242851	*Pelecanus onocrotalus*
Paryphostomum radiatum (Dujardin, 1845)	AY245708	*P. carbo*
Petasiger phalacrocoracis (Yamaguti, 1939)	AY245709	*P. carbo*
Holostephanus dubinini Vojtek et Vojtkova, 1968	AY245707	*P. carbo*
Clinostomum complanatum (Rudolphi, 1819)	AY245701	*E. garzetta*
Unidentified Strigeidae sp. Railliet, 1919	AY245711	*E. garzetta*
Unidentified Plagiorchiidae sp.	AY245700	*Clarias gariepinus*
Unidentified metacercaria (Liver)	AY245704	*Tilapia zillii*

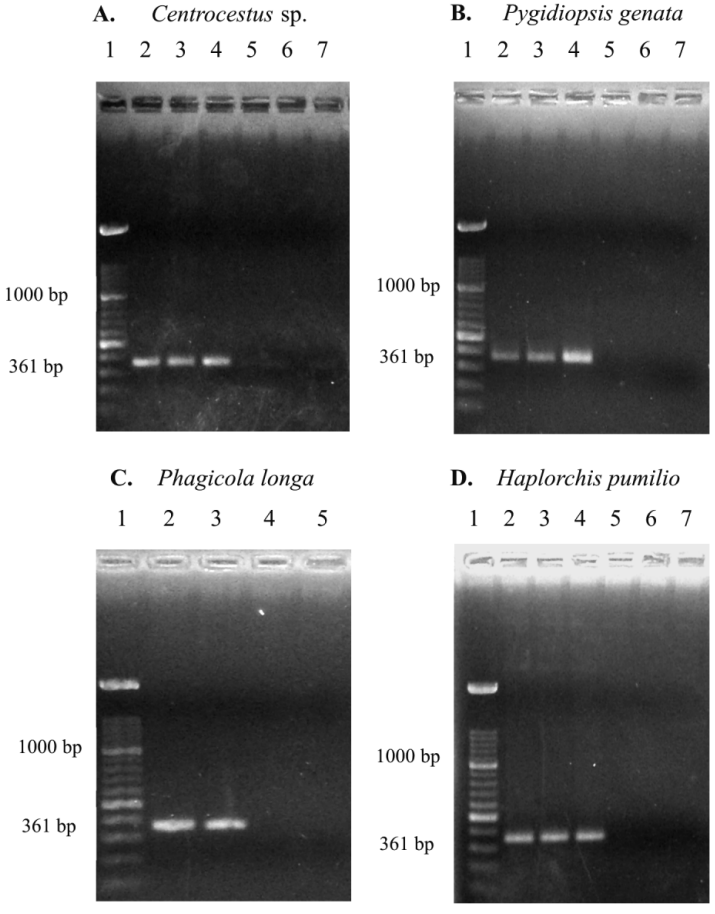

Fig. 2. Agarose gels showing PCR products of heterophyid trematodes using species-specific oligonucleotide primer sets. Lanes are as follows: (A) *Centrocestus* sp.: 1, 100 bp DNA marker; 2, single adult; 3, single metacercaria; 4, single cercaria; 5, *Tilapia zillii*; 6, *Melanoides tuberculata*; 7, H_2O. (B) *Pygidiopsis genata*: 1, 100 bp DNA marker; 2, single adult; 3, single metacercaria; 4, single cercaria; 5, *T. zillii*; 6, M. costata; 7, H_2O. (C) *Phagicola longa*: 1, 100 bp DNA marker; 2, single adult; 3, single metacercaria; 4, *T. zillii*; 5, H_2O. (D) *Haplorchis pumilio*: 1, 100 bp DNA marker; 2, single adult; 3, single metacercaria; 4, single cercaria; 5, *T. zillii*; 6, *M. tuberculata*; 7, H_2O

None of the above primer sets cross-reacted with DNA from the host fish, the host snails or any other heterophyid species investigated. Faint bands were obtained, however, when using *Haplorchis pumilio* and *H. taichui* specific primer sets with the DNA of *Dexiogonimus ciureanus*. In order to avoid misidentification between these species, endonuclease restriction digest was applied. The digestion of the complete rDNA 18S PCR product, using *Hin f1* restriction enzyme, yielded restriction fragments length polymorphisms among the 3 species (Fig. 3).

The phylogenetic analysis (Fig. 4) placed all species of the Heterophyidae (Order Opisthorchiida) in 1 cluster. The heterophyid cluster segregated into 3 groups. The first group included *Phagicola longa*, *Pygidiopsis genata* and a *Phagicola*-like sp. from the USA, with the latter 2 being closely related. The second group including *Haplorchis pumilio* and *H. taichui*, both with 1 testis (i.e. Haplorchinae; sensu Witenberg 1929) and *Dexiogonimus ciureanus* (Heterophyinae; sensu Witenberg 1929), which have 2 testes. The final group included *Centrocestus* sp. from Israel and the cercariae of *Centrocestus* sp. infecting *Melanoides tuberculata* from the southern USA. There was, however, sufficient molecular evidence for the segregation of these 2 samples of *Centrocestus* into distinct species. The clustering of the remaining studied trematodes corresponded with their established taxonomic division into the orders Strigeidida, Echinostomida and Plagiorchiida (Fig. 4).

DISCUSSION

Species-specific PCR assays, based on rDNA 18S sequences as well as RFLP analysis, have proven useful in demonstrating genetic links between cercariae, metacercariae and adult worms of the Heterophyidae species. These tools may be used for early diagnosis as they were shown to be sensitive in the identification of

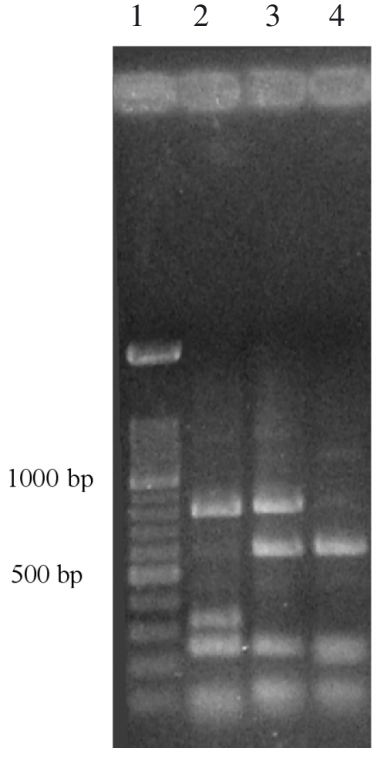

Fig. 3. Polymerase chain reaction amplified rDNA 18S digested with restriction enzyme Hin f1. Lanes 1 to 4: 1, 100 bp DNA marker; 2, *Haplorchis pumilio*; 3, *H. taichui*; 4, *Dexiogonimus ciureanus*

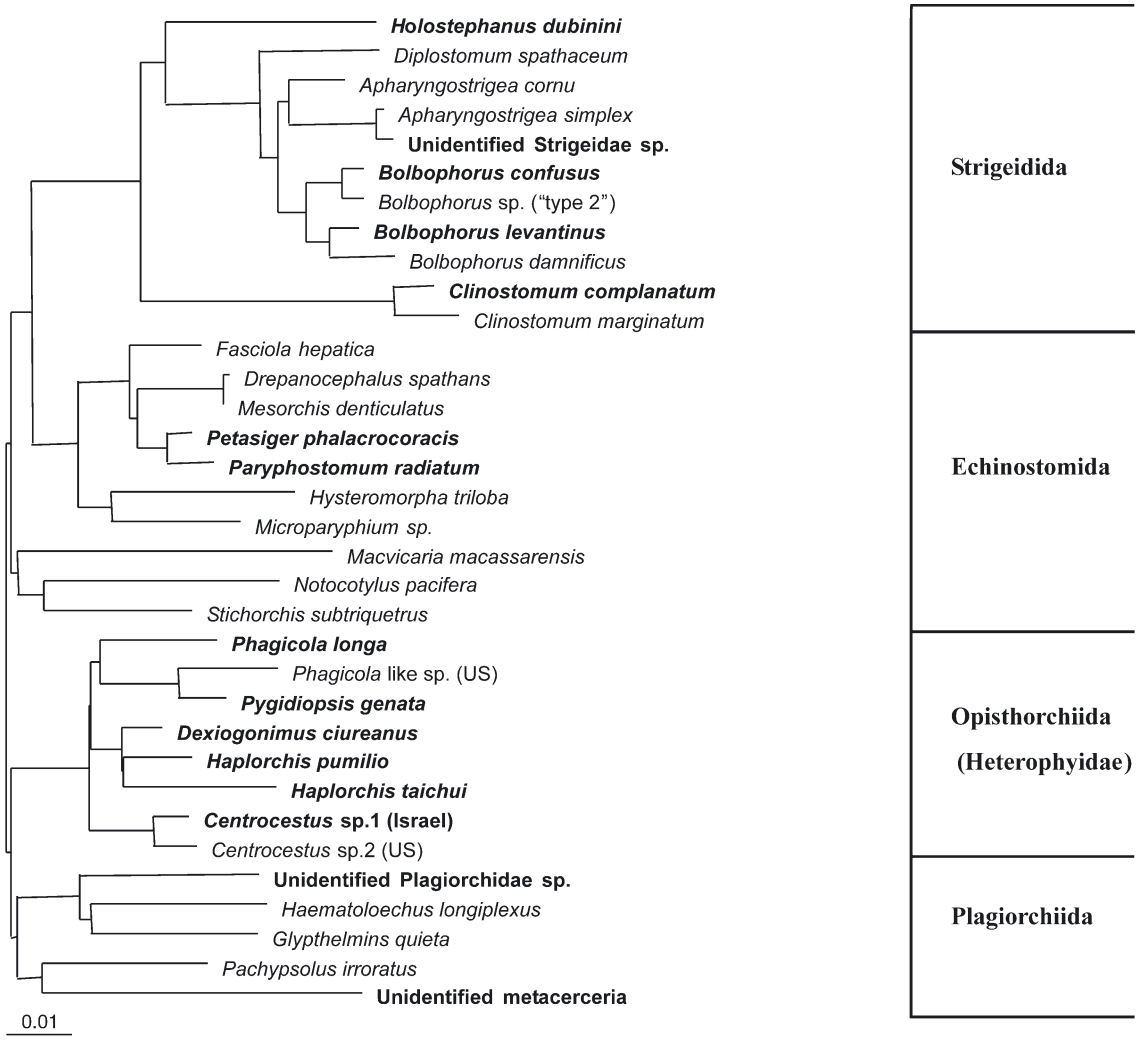

Fig. 4. Phylogenetic analysis of heterophyid species and their relations to other major groups of trematodes based on the alignment of the rDNA 18S genes (species that were sequenced in the present study are in bold). Scale bar = 1% estimate difference in nucleotide sequence positions

early infections in snails even before cercariae were shed (Levy et al. 2002). Their application is significant for both fish and public health as well as for studying trematodes life history. Using this methodology we have elucidated the molluscan and piscine intermediate hosts of *Pygidiopsis genata*. Unveiling all the intermediate hosts of *P. genata* would make experimental infections applicable for the further studying of its life history. We anticipate that further use of *Phagicola longa* specific assay would enable, in the future, the elucidation of its first intermediate host. *Haplorchis pumilio* and *H. taichui* metacercarial and adult infections have been reported in a variety of fish mammals and birds in Israel (Witenberg 1929). However, although both species are transmitted by *Melanoides tuberculata* (Pearson 1964), in the present study we could only demonstrate the genetic links of all the life stages of *H. pumilio*. The reason may be that, as reported by Witenberg (1929), *H. taichui* is less common and therefore none of its cercariae and metacercaria were found.

Where experimental demonstration of the life history is unachievable, molecular methodologies have been employed to unveil the life histories of trematodes. The ITS rDNA region has been utilized for species-specific identifications (Cribb et al. 1998, Jousson et al. 1998, Anderson 1999). However, the validity of using the ITS region as a diagnostic tool has been questioned, since intra-specific variation has been found in the rDNA ITS loci of some echinostomatids and *Paragonimus westermani* (Sorensen et al. 1998, van Herwerden et al. 1999). Consequently, with our primer sets being designed for the variable regions in the 18S, we chose in our study to utilize the rDNA sequences of both the

18S and ITS regions. The ITS regions may still be used for diagnostic confirmation and for the detection of intra- and inter-specific phylogenetic variation among isolates of poorly differentiated groups.

High-sequence similarity (96 to 98% identity) was shown within the 18S rDNA sequence of the 6 representatives of the heterophyids. Because of this similarity we were obliged to design our specific primers to 1 location. Also, RFLP digestion was required in order to confirm differentiation between *Dexiogonimus ciureanus* and *Haplorchis* spp. This is the most remarkable example in our study of the high-genetic similarity of species of different genera, which were conventionally classified into different subfamilies, Heterophyinae and Haplorchinae, based on the presence of 2 testes and 1 testis, respectively (Witenberg 1929). Although a large amount of 18S rDNA data is available for digenean, 18S sequences for only 3 heterophyid were published (Olson et al. 2003) of which only *Galactosomum lacteum*, recovered from a cormorant, was an adult form. Even with the 6 new sequences, the molecular data available on rDNA of the Heterophyidae are still very limited. Therefore, the purpose of the presented tree was to put the newly sequenced species in their phylogenetic context, and it should be considered preliminary until, as was demonstrated for the Schistosomatidae, more data becomes available for comprehensive phylogeny analysis (Lockyer et al. 2003). In view of the discrepancies between the morphology-based classification and that established from genetic interrelations, future classifications should incorporate both morphological and genetic methodologies. Finally, it should be emphasized that additional species may not demonstrate variation at the same location of their 18S sequence. Therefore, until more sequence data of this marginally investigated group becomes available, verification of the gene sequence is recommended when positive results of these assays are achieved.

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LITERATURE CITED

Anderson GR (1999) Identification and maturation of the metacercaria of *Indodidymozoon pearsoni*. J Helminthol 73:21–26

Bartoli P, Jousson O, Russell-Pinto F (2000) The life cycle of *Monorchis parvus* (Digenea: Monorchiidae) demonstrated by developmental and molecular data. J Parasitol 86(3): 479–489

Cribb TH, Anderson GR, Adlard RD, Bray RA (1998) A DNA-based demonstration of a three host life-cycle for the Bivesiculidae (Platyhelminthes: Digenea). Int J Parasitol 28:1791–1795

Deardorff TL, Overstreet RM (1990) Sea-food transmitted zoonoses in the United States: the fishes, the dishes, and the worms. In: Ward DR, Hackney C (eds) Microbiology of marine food products. Van Nostrand Reihold, New York, p 211–265

Grevelding CG, Kampkotter A, Kuntz W (1997) *Schistosoma mansoni*: sexing cercariae by PCR without DNA extraction. Exp Parasitol 85:99–100

Hillis DM, Dixon MT (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. Q Rev Biol 66:411–453

Jousson O, Bartoli P, Zaninetti L, Pawlowski J (1998) Use of the ITS rDNA for elucidation of some life cycles of mesometridae (Trematoda, Digenea). Int J Parasitol 28:1403–1411

Ko RC (1995) Fish-borne parasitic zoonoses. In: Woo PTK (ed) Fish diseases and disorders: protozoan and metazoan infections, Vol 1. CAB International, Cambridge, p 631–671

Levy MG, Flowers JR, Poore MF, Khoo L and 5 others (2002) Morphologic, pathologic, and genetic investigations of *Bolbophorus* spp. (Diplostomatida, Trematoda) affecting cultured *Ictalurus punctatus* in the Mississippi Delta. J Aquat Anim Health 14:235–246

Littlewood DTJ, Olson PD (2001) Small subunit rDNA and the Platyhelminthes: signals, noise, conflicts and compromise. In: Littlewood DTJ, Bray RA (ed) Interrelationships of the Platyhelminthes. Taylor & Francis, New York, p 262–278

Lockyer AE, Olson PD, Østergaard P, Rollinson D and 11 others (2003) The phylogeny of the Schistosomatidae based onthree genes with emphasis on the interrelationships of Schistosoma Weinland, 1858. Parasitology 126:203–224

Olson PD, Cribb TH, Tkach VV, Bray RA, Littlewood DTJ (2003) Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). Int J Parasitol 33:733–755

Paperna I (1995) Digenea (Phylum Platyhelminthes). In: Woo PTK (ed) Fish diseases and disorders: protozoan and Metazoan Infections, Vol 1. CAB International, Cambridge, p 329–389

Paperna I (1996) Parasites, infections and diseases of fishes in Africa—an update. Central Institute of Freshwater Aquaculture, Technical Paper no. 31, Food and Agriculture Organization, United Nations, Rome, p 122–142

Pearson JC (1964) A revision of the subfamily Haplorchinae Looss, 1899 (Trematoda: Heterophyidae) I. The *Haplorchis* group. Parasitology 54:601–676

Sambrook J, Fritsch EJ, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring, New York, p 1.53–1.73

Sommervile C (1982) The pathology of *Haplorchis pumilio* (Looss 1986) infections in cultured tilapias. J Fish Dis 5: 243–250

Sorensen RE, Curtis J, Minchella DJ (1998) Intraspecific variation in the rDNA ITS loci of 37 collar-spined echinostomes from North America: implications for sequence-based diagnoses and phylogenetics. J Parasitol 84(5): 992–997

Strunk O, Ludwig W (1998) ARB: a software environment for sequence data. Department of Microbiology, Technische Universität München, Munich, www.mikro.biologie.tu-muenchen.de

van Herwerden L, Blair D, Agatsuma T (1999) Intra- and interindividual variation in ITS1 of *Paragonimus westermani* (Trematoda:Digenea) and related species: implications for phylogenetic studies. Mol Phylogenet Evol 12(1): 67–73

Witenberg G (1929) Studies on the trematode family Heterophyidae. Ann Trop Med Parasitol 23(2):131–239

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