Redescription and phylogenetic relationships of *Euparyphium capitaneum* Dietz, 1909, the type-species of *Euparyphium* Dietz, 1909 (Digenea: Echinostomatidae)

Olena Kudlai · Vasyl V. Tkach · Eric E. Pulis · Aneta Kostadinova

Abstract *Euparyphium capitaneum* Dietz, 1909, the type-species of the genus *Euparyphium* Dietz, 1909, is described on the basis of material collected from the type-host *Anhinga anhinga* (L.) from Pascagoula River, which drains into the northern coast of the Gulf of Mexico. Combination of light and scanning electron microscopy observations of freshly collected and properly fixed specimens in our study has allowed us to provide novel information on the morphology and topology of the reproductive systems and other morphological features of the species. A Bayesian inference analysis based on the newly-obtained partial sequence of the nuclear 28S rRNA gene for *E. capitaneum* and 24 previously published sequences from the superfamily Echinostomatoidea Looss, 1899 provided evidence supporting the distinct status of the genera *Euparyphium* and *Isthmiophora* Lühe, 1909.

Introduction

Euparyphium Dietz, 1909 is a small genus of the Echinostomatidae Looss, 1899 including intestinal parasites of birds and mammals (Kostadinova, 2005). The species composition of *Euparyphium* has been unstable and a subject of several taxonomic revisions since the erection of the genus by Dietz (1909); this partially reflects the peculiar morphology of the type-species *Euparyphium capitaneum* Dietz, 1909. Some of the species that at different times belonged to *Euparyphium* were moved between several genera, most notably *Isthmiophora* Lühe, 1909 and *Echinocirrus* Mendheim, 1943. Skrjabin & Bashkirova (1956) synonymised *Echinocirrus* and *Isthmiophora* with *Euparyphium* and erroneously listed *Euparyphium melis* (Schrank, 1788) as the type-species of *Euparyphium*; this has been followed by some other authors. Kostadinova & Gibson (2002) have corrected the situation and separated the genera *Euparyphium* (type-species *E. capitaneum*) and *Isthmiophora* (type-species *I. melis*). The detailed account of these systematic and nomenclatural changes has been provided by Kostadinova & Gibson (2002). According to

O. Kudlai
Laboratory of Parasitology, Nature Research Centre,
Akademijos 2, 08412 Vilnius, Lithuania

O. Kudlai
Department of Parasitology, Institute of Zoology,
B. Khmelnytskogo 15, Kyiv 01601, Ukraine

V. V. Tkach (✉)
Department of Biology, University of North Dakota,
10 Cornell Street, Grand Forks, ND 58202, USA
e-mail: vasyl.tkach@email.und.edu

E. E. Pulis
Institute for Marine Mammal Studies, 10801 Dolphin
Lane, Gulfport, MS 39502, USA

A. Kostadinova
Institute of Parasitology, Biology Centre of the Academy
of Sciences of the Czech Republic, Branišovská 31,
370 05 České Budějovice, Czech Republic

this most recent revision of *Euparyphium*, the genus is currently represented by four nominal species: *E. capitaneum* (syn. *E. anhingae*), *E. murinum* Tubangui, 1931, *E. guerreroi* Tubangui, 1931 and *E. albuferensis* Esteban, Toledo, Sanchez & Munoz-Antoli, 1997. Except for the type-species, *Euparyphium* spp. were originally described from the mammalian host *Rattus norvegicus* (Berkenhout).

The type-species, *E. capitaneum* Dietz, 1909, was briefly described based on material from *Anhinga anhinga* (L.) in Brazil (Dietz, 1909, 1910). For more than a century this species was recorded twice. Pérez Vigueras (1944) reported *E. capitaneum* from *A. anhinga* in Cuba and Premvati (1968) described *E. anhingae* Premvati, 1968 from *A. anhinga* in Florida; this species has been later synonymised with *E. capitaneum* by Kostadinova & Gibson (2002). Unfortunately, the description of Premvati was based on clearly misshapen specimens that could not be later located, lacks many details of the organisation of the reproductive organs and shows inconsistencies with respect to the description of the collar spines. Thus to date, no confirmation of the unusual morphology of the type-material of *E. capitaneum* examined by Dietz (1909, 1910) that resulted in a somewhat composite diagnosis of *Euparyphium* (see Kostadinova, 2005) exists.

In the course of helminthological investigation of aquatic birds in Mississippi between 2007 and 2012 we found numerous specimens of *E. capitaneum* in several *A. anhinga*. This new, well-fixed material enabled us to re-describe this species and provide previously unknown details of its anatomy based on light and scanning electron microscopy observations. Furthermore, the DNA sequence obtained from this material was used to estimate the phylogenetic relationships of *E. capitaneum* and other members of the Echinostomatoidea Looss, 1899 with available sequences.

Materials and methods

Numerous specimens of *E. capitaneum* were found in five *A. anhinga* collected by EEP and VVT from oxbow lakes in the Pascagoula River drainage area (Mississippi, USA) in December of 2007, August of 2010, and March of 2012. Details of the localities are provided below. Digeneans were removed from host intestines,

rinsed in saline, heat-killed with hot water and preserved in 70% ethanol. The specimens for light microscopy examination were stained with aqueous alum carmine, dehydrated in a graded ethanol series, cleared in clove oil and mounted permanently in Damar gum. Drawings were made on a DIC-equipped compound Olympus BX51 microscope with the aid of a drawing tube. All measurements in the text and tables are in micrometres. The following abbreviations were used in the tables: BL, body length; BW, maximum body width; CL, collar length; CW, collar width; OSL, oral sucker length; OSW, oral sucker width; PL, prepharynx length; PHL, pharynx length; PHW, pharynx width; OL, oesophagus length; CSL, cirrus-sac length; CSW, maximum cirrus-sac width; VSL, ventral sucker length; VSW, ventral sucker width; ATL, anterior testis length; ATW, anterior testis width; PTL, posterior testis length; PTW, posterior testis width; OVL, ovary length; OVW, ovary width; MEL, Mehlis' gland length; MEW, Mehlis' gland width; EL, egg-length; EW, egg-width; FORE, forebody length; UFL, uterine field length (distance between the ovary and posterior margin of the ventral sucker); PTFL, post-testicular field length (distance between the posterior margin of the posterior testis and posterior extremity of body). In addition to the standard measurements the following relative proportions were calculated after Kostadinova (2005): BW(%), maximum body width as a proportion of body length; FO(%), length of the forebody as a proportion of body length; U(%), length of the uterine field posterior to ventral sucker (used as an approximation for the uterine length) as a proportion of body length; T(%), length of the post-testicular field as a proportion of body length. The specimens studied are deposited in the collection of the Harold W. Manter Laboratory of Parasitology at the University of Nebraska, Lincoln (HWML).

The specimens observed under scanning electron microscopy (SEM) were fixed in 70% ethanol, dehydrated in a graded ethanol series and dried with hexamethyldisilazane (Ted Pella Inc., Redding, California) as a transition fluid. The specimens were mounted on an aluminum stub using conductive double-sided tape, coated with gold-palladium, and examined with the use of a Hitachi 4700 scanning electron microscope (Hitachi U.S.A., Mountain View, California) at an accelerating voltage of 5–10 kV.

Genomic DNA was extracted from five specimens of *E. capitaneum* according to the protocol described by

Tkach & Pawlowski ([1999](#)). About 1,350 bp long fragment at the 5′ end of the 28S rRNA gene was amplified from three of these specimens by polymerase chain reaction on Eppendorf EP gradient thermal cycler using the forward primer dig12 (5′-AAG CAT ATC ACT AAG CGG-3′) and the reverse primer 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach et al., [2003](#)). About 2,600 bp long fragment spanning the 3′ end of the 18S gene, internal transcribed spacer region (ITS1 + 5.8S gene + ITS2) and partial 28S gene were amplified from the remaining two specimens using the forward primer ITSF (5′-CGC CCG TCG CTA CTA CCG ATT G-3′) and the reverse primer 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach et al., [2003](#)). PCR reactions were prepared using One-Taq quick load PCR mix (New England Biolabs). The PCR products were purified using QIAquick PCR purification Kit (Qiagen, Germany). PCR primers and an additional internal forward primer 300F (5′- CAA GTA CCG TGA GGG AAA GTT G-3′) and reverse primers 300R (5′-CAA CTT TCC CTC ACG GTA CTT G-3′) and diglr (5′-CCG CTT AGT GAT ATG CTT-3′) were used in the sequencing reactions. PCR products were cycle-sequenced directly using ABI BigDye™ (Applied Biosystems, Foster City, California) chemistry, alcohol-precipitated, and run on an ABI Prism 3100™ automated capillary sequencer (Applied Biosystems). Contiguous sequences were assembled using Sequencher (GeneCodes Corp., ver. 4.2), and submitted to GenBank under accession numbers KP009616–KP009620.

The newly-generated sequences for *E. capitaneum* and matching sequences of the 28S rRNA gene of other echinostomatoidean digeneans available on GenBank (see Table [3](#) for details) were used in the phylogenetic analysis. A sequence of *Notocotylus attenuatus* (Rudolphi, 1809) (AF184259) was used as an outgroup based on the topologies in the phylogenetic trees of the Digenea published by Olson et al. ([2003](#)). For phylogenetic analyses the sequences were aligned using ClustalX as implemented in the BioEdit program, version 7.0.1 (Hall, [1999](#)). The alignment was then trimmed to the length of the shortest sequence, manually refined using BioEdit, saved in FASTA format and imported into the MacClade ver. 4.02 software (http://macclade.org/macclade.html). Upon selection of the exclusion sets the alignments were saved in NEXUS format for subsequent analyses.

Positions with ambiguous alignment were excluded from the analysis.

Phylogenetic analysis was carried out using Bayesian inference (BI) as implemented in the MrBayes software (ver. 3.1) (Huelsenbeck & Ronquist, [2001](#)). The Bayesian analyses were run with the following nucleotide substitution model settings: lset nst = 6, rates = invgamma, samplefreq = 100, ncat = 4, shape = estimate, inferrates = yes and basefreq = empirical, that correspond to a general time reversible (GTR) model including estimates of the proportion of invariant sites (I) and gamma (G) distributed among-site rate variation. The nucleotide substitution model was determined using MrModel-Test 2.3 software (Nylander, [2004](#)). Markov chain Monte Carlo (MCMC) chains were run for 3,000,000 generations, log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees by setting the ''burnin'' parameter at 750. This number of generations was considered sufficient because the standard deviation dropped well below 0.01 at the end of the run. Trees were visualised using the FigTree ver. 1.4 software (Rambaut, [2012](#)).

Family Echinostomatidae Looss, 1899
Genus *Euparyphium* Dietz, 1909

Euparyphium capitaneum Dietz, 1909

Host: *Anhinga anhinga* (Linnaeus) (Suliformes: Anhingidae).
Localities: Three lakes in the drainage area of the Pascagoula River in Jackson County (30°45′22″N, 88°39′14″W and 30°37′05″N, 88°38′14″W) and George County (30°53′41″N, 88°44′42″W), Mississippi, USA.
Site in host: Intestine.
Prevalence and abundance: All five birds examined were infected with several to several dozen *E. capitaneum*.
Voucher material: Deposited in HWML, accession numbers HWML 75112–75114 (15 specimens).
Representative DNA sequences: KP009616–KP009617 (partial 18S; complete ITS1, 5.8S and ITS2; partial 28S sRNA gene); KP009618–KP009620 (partial 28S rRNA gene).

Redescription (Figs. 1–3)

[Based on 15 specimens; metrical data in Tables 1, 2.] Body very elongate (BW = 7–14%), with maximum width at level of ventral sucker. Forebody flattened dorsoventrally, long to very long, representing 20–32% of body length; hindbody subcylindrical. Tegument armed with large spines arranged in alternating transverse rows extending from close to posterior margin of collar to level of anterior testis ventrally (Figs. 2A, 3A) and level of ventral sucker dorsally; transverse rows dense in forebody (Fig. 2A, D, E), progressively more widely spaced in hindbody (Figs. 2A, 3A); tegument in ventral median field from mid-level of forebody to ventral sucker, including its anterior margin, devoid of spines (Fig. 2A, D).

Head collar reniform, well developed, with distinct ventral ridge. Collar spines 27; 4 angle spines on each ventral lappet (2 dorsal and 2 ventral), longer than marginal spines, one angle spine distinctly larger (Table 2); lateral spines in single row, first lateral spine smallest (Table 2); dorsal spines in double row (Figs. 1B, 2B–E), aboral spines slightly longer than oral. Oral sucker slightly transversely oval. Ventral sucker large, cup-shaped, with deep cavity and strongly muscular walls, located in first quarter of body; inner rim of ventral sucker with c.20 small sensory receptors (Fig. 2G) in a semicircle on upper half; outer rim with large papillae at base (Fig. 2F, G). Prepharynx distinct. Pharynx large, elongate-oval. Oesophagus long, thick-walled. Intestinal bifurcation just anterior to ventral sucker. Caeca thick-walled, reach close to posterior extremity of body.

Testes 2, tandem, contiguous, elongate-oval, wavy; anterior testis pre-equatorial; posterior testis post-equatorial. Post-testicular field very long, representing 27–50% of body length. Cirrus-sac elongate-oval, anterodorsal to ventral sucker, between intestinal bifurcation and mid-level of ventral sucker. Internal seminal vesicle saccular, elongate-oval, with attenuated distal portion. Pars prostatica moderately developed. Cirrus relatively long, tubular, unspined, with sponge-like surface (Fig. 3B–D) and sensory papillae at base (Fig. 3B). Genital pore small, median, just posterior to intestinal bifurcation.

Ovary elongate-oval, entire, dextral, pre-equatorial. Mehlis' gland large, diffuse, elongate-oval, submedian, contiguous with ovary and anterior testis. Uterine seminal receptacle not observed. Uterine field very short (U = 0–7%), with few intercaecal loops. Metraterm muscular, somewhat shorter than cirrus-sac. Eggs not numerous. Vitellarium follicular; follicles large, distributed in 2 lateral fields between level of ovary and posterior extremity; approach median line in post-testicular field; vitelline reservoir median, dorsal to Mehlis' gland.

Excretory vesicle Y-shaped; pore ventro-subterminal.

Remarks

Although sufficient for the erection of the genus, the original description of *E. capitaneum* is incomplete, with little information provided on the morphology of terminal genitalia. Of particular importance is the lack of data on the presence/absence of spines on the cirrus, one of the major characteristics differentiating the closest genera *Euparyphium* and *Isthmiophora*. The only other description of the species also provided what appears to be erroneous information on the number of collar spines that may be due to either loss of some spines or the orientation of the collars in the mounted specimens that prevented accurate counting of spines (see Premvati, 1968).

The newly-collected material of *E. capitaneum* both agrees well with the original description of the species and confirms its affiliation with *Euparyphium*, especially in relation to the features listed as characteristic of the type-species by Kostadinova (2005) (i.e. collar with 27 spines; contiguous, equatorial, strongly elongate, wavy testes; and very short uterus) as well as of the features of generic importance (i.e. two groups of four angle spines that are longer than the marginal spines; dorsal spines in the aboral row longer than those in the oral row; and unspined cirrus).

Importantly, SEM observations of several specimens demonstrated the lack of spines on the cirrus in *E. capitaneum* (Fig. 3B–D), rather the cirrus has a sponge-like surface with rhomboid "cells" stretching in the everted cirrus. The ovary in the present material is elongate-oval as opposed to spherical in the original description. The topology of the cirrus-sac was mentioned and illustrated in the description by Premvati (1968), but no metrical data were provided. Additional morphological features not mentioned or illustrated in either of the previous descriptions and characterised here are the metraterm, Mehlis' gland, vitelline reservoir and armed tegument. The SEM
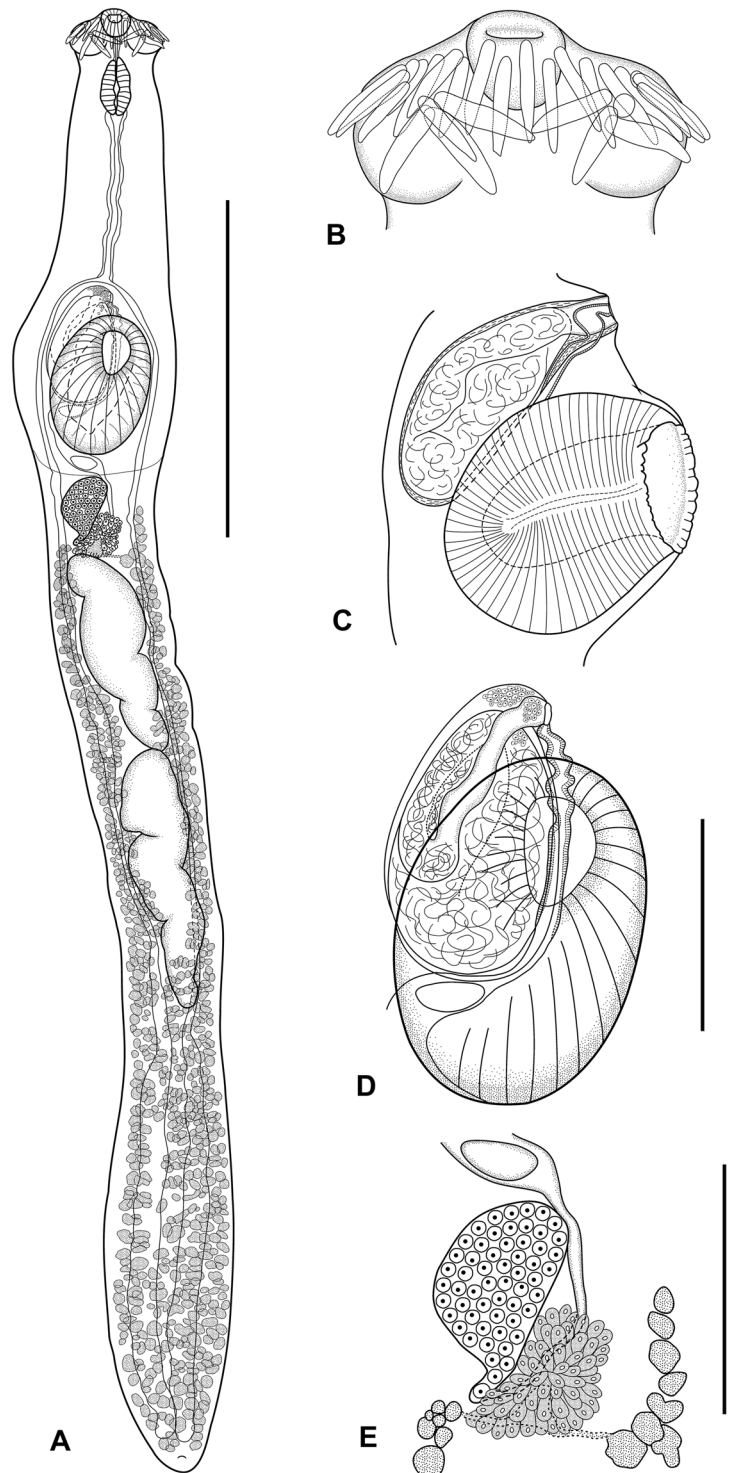
Fig. 1 *Euparyphium capitaneum* Dietz, 1909 ex *Anhinga anhinga*. A, Complete specimen, ventral view; B, Head collar; C, Cirrus-sac and ventral sucker, lateral view; D, Detail of the terminal genitalia, Ventral view; E, Detail of the female reproductive complex. *Scalebars*: A, 1,000 μm; B, 200 μm; C–E, 250 μm

Table 1 Comparative metrical data for *Euparyphium capitaneum* ex *Anhinga anhinga* (L.)

Source	Present study		Dietz (1909, 1910)	Premvati (1968)[a]
Origin	Mississippi (USA)		Brazil	Florida (USA)
	Range	Mean	Range	Range
BL	2,605–5,800	4,322	4,500	2,540–4,830
BW	315–563	399	380–410	540–600[b]
CL	95–185	139	–	–
CW	170–301	248	210–260	–
OSL	53–97	75	–	70–85
OSW	62–112	84	60–100	70–90
PL	0–62	36	70–100	–
PHL	99–176	145	129–143	135–160
PHW	62–119	88	96–116	80–110
OL	321–964	608	385–616	–
CSL	175–453	301	385–400	–
CSW	98–194	142	123–138	–
VSL	313–462	381	370	–
VSW	233–370	308	300–330	340–440
OVL	101–301	212	–	100–260
OVW	62–154	99	136–150	100–120
MEL	92–212	160	–	–
MEW	75–145	102	–	–
ATL	339–761	528	700–1,050	400–580
ATW	119–231	173	154–261	250–340
PTL	449–991	619	as above	370–580
PTW	114–218	171	as above	230–310
EL	63–81	73[c]	67	60–70
EW	33–52	46[c]	49	40–45
FORE	620–1,456	1,074	–	540–810
UFL	0–319	161	–	–
PTFL	832–1,938	1,387	–	870–1,810
BW (%)	6.7–14.4	9.7	–	–
FO (%)	19.8–32.2	25.0	–	–
U (%)	0–6.7	3.4	–	–
T (%)	26.6–49.9	32.7	–	–

[a] As *Euparyphium anhingae*; [b] 610–640 μm in the region of testes (see Premvati, 1968); [c] (n = 29)

examination revealed that tegumental spines only reach to the first third of the hindbody (Figs. 2A, 3A).

Regarding the detailed morphometric characterisation achieved here based on abundant, adequately fixed material, our data extend the range of variation of the metrical data for *E. capitaneum*, i.e. wider range and higher upper limits for the size of the body, collar spines, most organs and eggs, and lower upper limits for the size of the testes; see Table 1). Of particular relevance are the deviations observed in *E. capitaneum* from the states and data in the generic diagnosis of Kostadinova (2005): very elongate body [BW = 7–14% (mean 10%) *vs* 12–18%]; long to very long forebody [FO = 20–32% (mean 25%) *vs* 12–20%]; very long post-testicular field [T = 27–50% (mean 33%) *vs* 20–30%]; and very short uterine field [U = 0–7% (mean 3%) *vs* 3–20%]. The metrical data in the description by Premvati (1968) fall within the range for *E. capitaneum* except for the much larger testes width (Table 1).

Phylogenetic analysis

Five sequenced specimens of *E. capitaneum* showed no intraspecific sequence variability. Our phylogenetic analysis included representatives of four families belonging to the superfamily Echinostomatoidea according to the latest systematic revision by Kostadinova (2005). The partial 28S rRNA gene sequence (1,243 bp) of *E. capitaneum* was included in the phylogenetic analysis together with 24 sequences of the Echinostomatoidea available on GenBank (Table 3). A sequence of *Cyclocoelum mutabile* Zeder, 1800 was also included for consistency with the results of the phylogenetic analysis by Olson et al. (2003). The final alignment was 1,161 bp long including several introduced gaps. Thirty-two ambiguously aligned sites were excluded from the analysis.
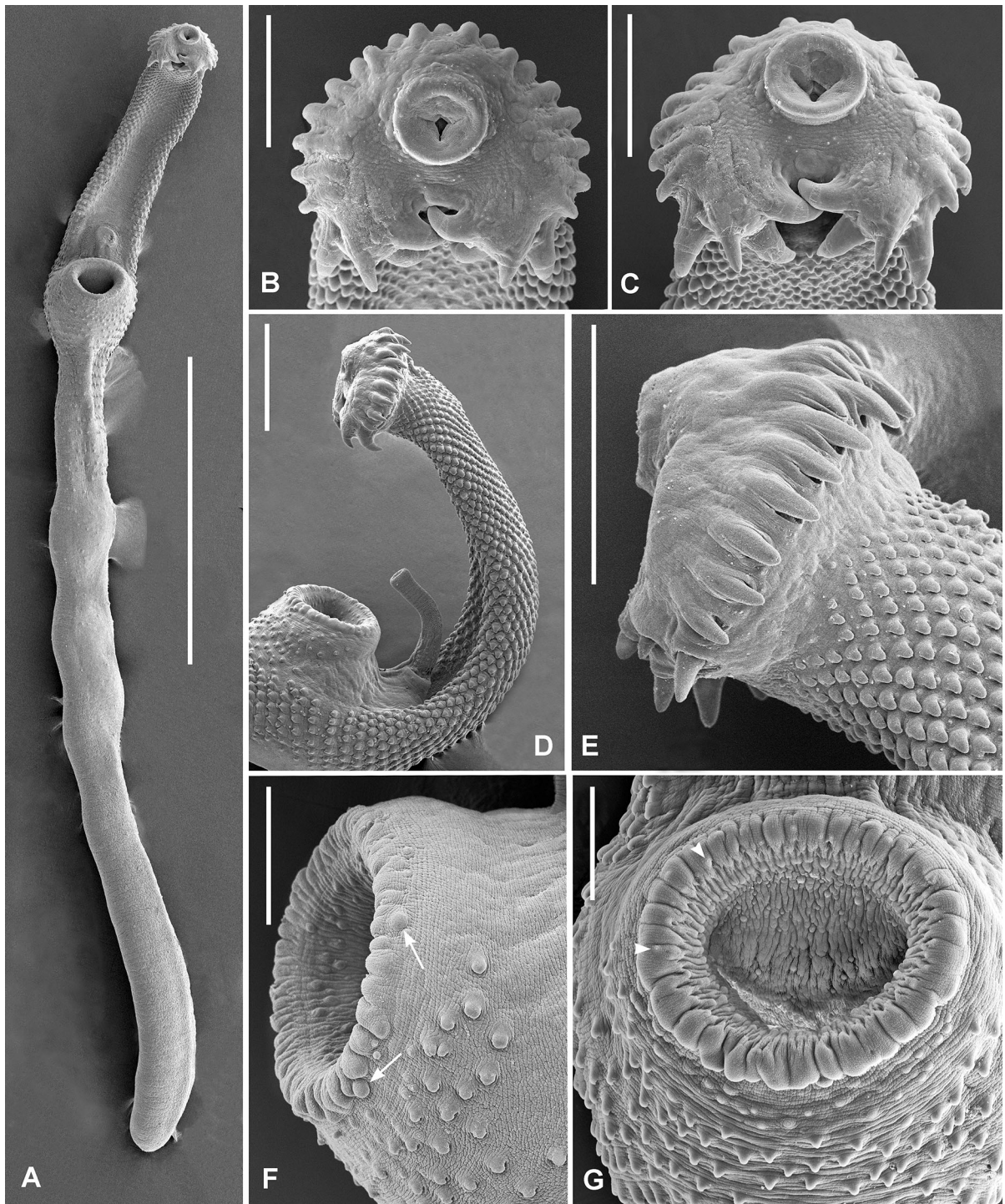
Table 2 Measurements of collar spines of *Euparyphium capitaneum*

Source	Present study	Dietz (1910)	Premvati (1968)[a]
Angle spines	90–114 × 13–17 (n = 17) (103–114 × 15–16) (n = 4)[b]	86–89 × 19 (96–108 × 22)[b]	90–110 × 20–25
First lateral spine	52–73 × 8–12 (n = 18)	–	–
Other lateral spines	73–105 × 8–19 (n = 17)	–	–
Dorsal spines	72–94 × 8–14 (n = 32)	64–84 × 12–14	–

[a] As *Euparyphium anhingae*; [b] One spine distinctly larger (see also Dietz, 1910)

Fig. 2 Scanning electron micrographs of *Euparyphium capitaneum* Dietz, 1909 ex *Anhinga anhinga*. A, Entire specimen, ventral view; B, C, Head collar; D, Forebody, lateral view (note everted cirrus); E, Head collar, lateral view; F, G, Region of ventral sucker [note large papillae (arrows) and small sensory receptors (arrowheads)]. *Scale-bars*: A, 1000 µm; B, C, E, 100 µm; D, 200 µm; F, G, 50 µm
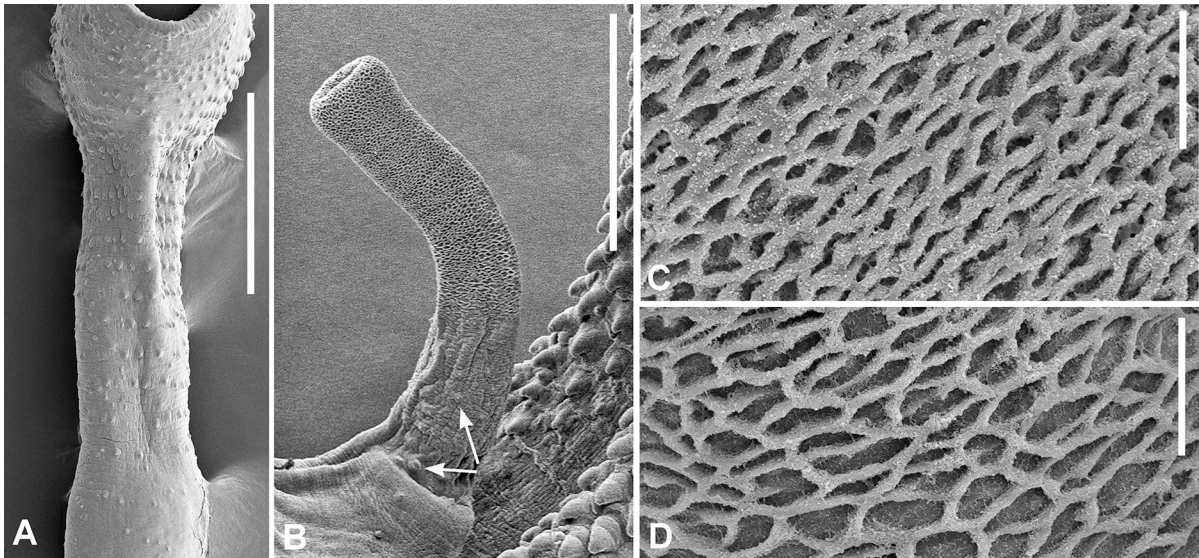
Fig. 3 Scanning electron micrographs of *Euparyphium capitaneum* Dietz, 1909. A, Anterior hindbody, ventral view, showing spination; B, cirrus, lateral view – note the basal sensory papillae (indicated by arrows); D, E, Detail of spongiform cirrus surface. *Scalebars*: A, 300 µm; B, 100 µm; C, D, 5 µm

The BI analysis resulted in a tree with overall well-supported topologies. Disregarding weakly-supported branches/clades, the tree contained six strongly (100%) supported clades which are numbered 1–6 in Fig. 4. The largest clade (Clade 1) included representatives of the genera *Echinostoma* Rudolphi, 1809, *Echinoparyphium* Dietz, 1909, *Ishthmiophora*, *Petasiger* Dietz, 1909, *Drepanocephalus* Dietz, 1909 and *Euparyphium*, all belonging to the subfamily Echinostomatinae Looss, 1899 of the family Echinostomatidae. Within this large clade, *Echinostoma* + *Echinoparyphium* clustered in a rather weakly-supported clade whereas the members of the remaining genera formed a 100% supported grouping. Interrelationships among genera within the latter group are not well defined.

Clade 2 (Fig. 4) included representatives of four fasciolid genera; see below for comments on their interrelationships. Clade 3 (Fig. 4) comprised four species representing at least two genera of the Philophthalmidae Looss, 1899. The internal topology of this clade was fully resolved with 100% support of its two sub-clades. The cyclocoelid *C. mutabile* formed its own independent branch (Clade 4 in Fig. 4). One of the two remaining 100% supported clades (Fig. 4) included two species of *Echinochasmus* Dietz, 1909 (Clade 5) whereas Clade 6 consisted

of representatives of two genera of the Psilostomidae Looss, 1900.

Discussion

In this study we provide the first adequately detailed description of *E. capitaneum* based on well-fixed, high quality specimens observed on total mounts under light microscope as well as under scanning electron microscope. This allowed the observation of previously unreported details of the species morphology. The SEM study allowed description of the pattern of the tegumental spination, particularly the posterior extent of the spination and the presence of the spine-free area on the ventral surface of the forebody. Among other morphological features observed under SEM were the sensory papillae on the ventral sucker and the basal sensory papillae on the cirrus (Figs. 2F, 3B). The morphological observations of the surface ultrastructure of the cirrus confirmed the lack of spines; this further corroborates the value of this morphological character as a distinguishing feature between the two closely related genera, *Isthmiophora* and *Euparyphium*.

The content of the genus *Euparyphium*, including its type-species, has been called into question by

Table 3 Digenean species used in the phylogenetic analysis, their hosts, geographical origin and GenBank accession numbers

| Family/Species	Host	Country	GenBank No.	Reference
Family Echinostomatidae				
Drepanocephalus spathans Dietz, 1909	*Planorbella trivolvis* (Say)	USA	JN993270	Griffin et al. (2012)
Echinochasmus sp.	*Lithoglyphus naticoides* (C. Pfeiffer)	Lithuania	JQ088098	Staneviciute et al. (unpublished)
Echinochasmus japonicus Tanabe, 1926	–	Vietnam	JQ890579	Besprozvannykh et al. (unpublished)
Echinoparyphium cinctum (Rudolphi, 1803)	*Anas platyrhynchos* L.	Ukraine	AF184260	Tkach et al. (2001)
Echinoparyphium rubrum (Cort, 1914)	*Helisoma trivolvis* (Say)	USA	JX262943	Tkach et al. (2012)
Echinostoma revolutum (Frölich, 1802)	*Mesocricetus auratus* Waterhouse	UK	AY222246	Olson et al. (2003)
Echinostoma paraensei Lie & Basch, 1967	"hamster"	USA	EU025867	Lotfy et al. (2008)
Echinostomatidae gen. sp.	*Physa acuta* (Draparnaud)	USA	GU270100	Hicks et al. (unpublished)
Euparyphium capitaneum Dietz, 1909	*Anhinga anhinga* (L.)	USA	KP009616	Present study
Isthmiophora melis (Schrank, 1788)	*Nyctereutes procyonoides* (Gray)	Ukraine	AF151941	Tkach et al. (2000)
Isthmiophora hortensis (Asada, 1926)	*Procyon lotor* (L.)	Japan	AB189982	Sato & Suzuki (2006)
Petasiger islandicus Kostadinova & Skirnisson, 2007	*Gyraulus* cf. *laevis*	Iceland	JQ425592	Georgieva et al. (2012)
Family Fasciolidae				
Fasciola gigantica Cobbold, 1855	–	Thailand	HM004190	Thaenkham et al. (unpublished)
Fasciola hepatica Linnaeus, 1758	*Bubalus bubalis* (L.)	Egypt	EU025874	Lotfy et al. (2008)
Fascioloides jacksoni (Cobbold, 1869)	*Elephas maximus* L.	USA	EU025871	Lotfy et al. (2008)
Fascioloides magna (Bassi, 1875)	"domestic pig"	USA	EU025872	Lotfy et al. (2008)
Fasciolopsis buski (Lankester, 1857)	"domestic pig"	Vietnam	EU025870	Lotfy et al. (2008)
Parafasciolopsis fasciolaemorpha Ejsmont, 1932	*Bison bonasus bonasus* (L.)	Poland	EU025869	Lotfy et al. (2008)
Family Philophthalmidae				
Cloacitrema narrabeenensis Howell & Bearup, 1967	*Batillaria australis* (Quoy & Gaimard)	Australia	AY222248	Olson et al. (2003)
Philophthalmidae gen. sp.	*Batillaria australis* (Quoy & Gaimard)	Australia	AY222247	Olson et al. (2003)
Philophthalmus sp.	*Tachuris rubrigastra* (Vieillot)	Peru	JQ627832	Literák et al. (2013)
Philophthalmus gralli Mathis & Leger, 1910	*Rhea americana* (L.)	USA	JQ246435	Church et al. (2013)
Family Psilostomidae				
Psilochasmus oxyurus (Creplin, 1825)	*Anas platyrhynchos* L.	Ukraine	AF151940	Tkach et al. (2000)
Sphaeridiotrema monorchis Lin & Chen, 1983	–	Vietnam	JQ890547	Besprozvannykh et al. (unpublished)
Sphaeridiotrema pseudoglobulus McLaughlin, Scott & Huffman, 1993	"duck"	Canada	GQ890330	Bergmame et al. (2011)
Family Cyclocoeliidae				
Cyclocoelum mutabile (Zeder, 1800)	*Calidris canutus* (L.)	UK	AY222249	Olson et al. (2003)
Family Notocotylidae (outgroup)				
Notocotylus attenuatus (Rudolphi, 1809)	*Aythya ferina* (L.)	Ukraine	AF184259	Tkach et al. (2001)
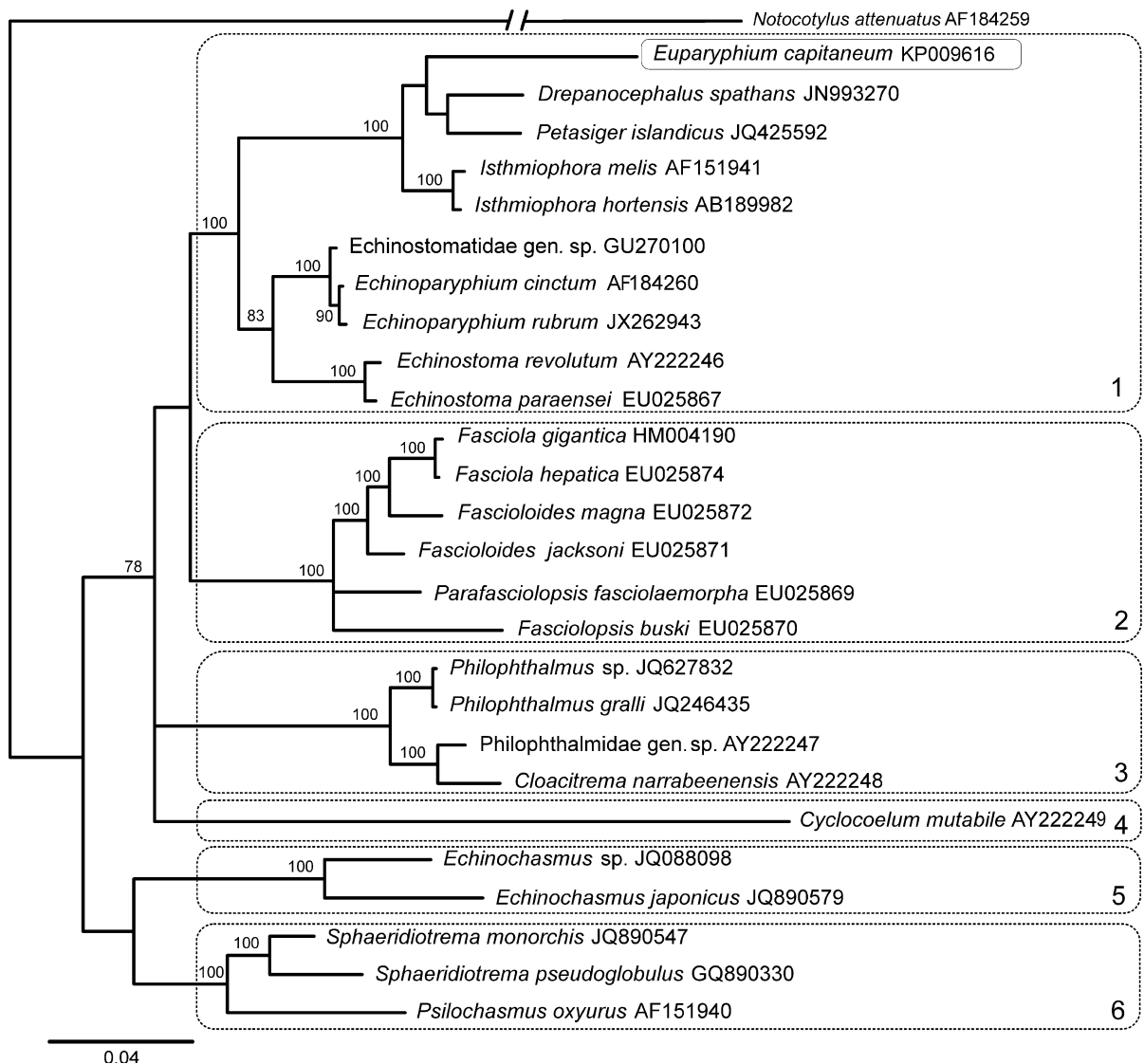
Fig. 4 Phylogenetic relationships among 25 taxa of the Echinostomatoidea resulting from Bayesian analysis (3,000,000 generations) based on the nuclear ribosomal sequences of the 28S rRNA gene. Only bootstrap values > 70 are shown. Dotted rectangles indicate six strongly supported clades. Branch length scale bar indicates number of substitutions per site

several authors (Mendheim, 1943; Skrjabin & Bash-kirova, 1956; Yamaguti, 1958) and more recently by Kostadinova & Gibson (2002). The number of the species within this genus has changed several times, mostly due to the convoluted taxonomic history of the genera *Isthmiophora* and *Echinocirrus*. The main reason for the lack of systematic and nomenclatural stability among these digeneans has been the relative paucity of morphological variation among these genera and the lack of phylogenetic data. Based on

the examination of the type-species of *Euparyphium* and *Isthmiophora* and newly-collected material, plus a critical evaluation of the previously published data, Kostadinova & Gibson (2002) re-established the validity of *Isthmiophora* with *Isthmiophora melis* as the type-species. Our molecular phylogenetic analysis has confirmed the systematic conclusions by Kostadi-nova & Gibson (2002) regarding the separation of *Euparyphium* and *Isthmiophora* and the utility of the morphological characters proposed by these authors

for generic differentiation. In the phylogenetic tree (Fig. 4) *Isthmiophora* and *Euparyphium* appear as distinct genus-level clades within the cluster comprising the Echinostomatinae. Inclusion of the type-species of both genera in our analysis gives credibility to our results and provides the basis for future detailed systematic revision of the content of these genera.

Of the remaining species of *Euparyphium* only a short (618 bp) sequence of 28S rRNA gene of *Euparyphium albuferensis* Esteban, Toledo, Sanchez & Munoz-Antoli, 1997 was available on GenBank (AY219697) and compatible with our dataset, albeit too short to be included in the phylogenetic analysis. However, we found high degree of sequence divergence (8.5%) in the overlapping, generally conserved 28S region of *E. capitaneum* and *E. albuferensis* suggesting that these species are not very closely related and most likely not congeneric. Moreover, a BLAST search using the sequence of *E. albuferensis* has demonstrated that this species is much closer to members of the genera *Hypoderaeum* Dietz, 1909 and *Echinoparyphium* (1% divergence) than to *E. capitaneum*, the type-species of *Euparyphium*. Therefore, either *E. albuferensis* does not belong to *Euparyphium* or the specimen that was used as a source for the sequence has been misidentified. The clustering of this species with *Echinoparyphium* and *Hypoderaeum* in the tree based on ITS2 sequences of nuclear ribosomal DNA published by Heneberg (2013) supports the above considerations. It is worth noting that in the original description of *E. albuferensis* Esteban et al. (1997) indicated that this species may belong to either *Euparyphium* or *Echinoparyphium* and distinguished the new species from all members of the *Echinoparyphium* 'recurvatum' species complex. Some features in the description by Esteban et al. (1997), i.e. the size of the dorsal spines (dorsal oral spines being longer than aboral), the postequatorial location of the testes and the more anterior extent of the vitelline fields, also agree better with the diagnosis of *Echinoparyphium* (see Kostadinova, 2005). Therefore, although *E. albuferensis* may appear valid (and the specimen sequenced identified correctly), additional morphological and molecular phylogenetic analyses are required before a definitive conclusion for its generic affiliation is reached. Further, the morphological and molecular similarity of *E. albuferense* with *Echinoparyphium* spp. and the novel data for the type-species of *Euparyphium* stress the rather mechanistic

nature of the diagnosis of the latter genus by Kostadinova (2005). The molecular and morphological results of our study indicate that *Euparyphium* requires further revision. Sequencing of the Asian species currently allocated to *Euparyphium*, i.e. *E. guerreroi* and *E. murinum*, would help resolve this issue; we do not exclude the possibility of *Euparyphium* being monotypic.

Although in the most recent systematic treatment of the Cyclocoelidae Stossich, 1902 by Kanev et al. (2002) this family was included into its own superfamily, the subsequent molecular phylogenetic analyses by Olson et al. (2003) firmly placed it within a well-supported clade with other echinostomatoideans. A more detailed study including broader representation of cyclocoelids is necessary to evaluate its interrelationships with other families within the superfamily.

Among the other taxa of the Echinostomatoidea included in the present phylogenetic analysis the position of *Fascioloides jacksoni* (Cobbold, 1869) deserves a comment. This species is positioned among the Fasciolidae Railliet, 1895 (Clade 2) as the basal taxon to *Fascioloides magna* (Bassi, 1875) and two species of *Fasciola* Linnaeus, 1758 in a 100% supported clade. The position of *F. jacksoni* in this clade and the high level of support of all branches in it do not unequivocally support the conclusion of Heneberg (2013) that phylogenetic data suggest inclusion of *F. jacksoni* in *Fascioloides* Ward, 1917. Our result based on 28S sequences is identical to that obtained by Heneberg (2013) who used the same gene as well as ITS1 region of the nuclear ribosomal DNA. On the other hand, the ribosomal ITS2 region and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) gene in Heneberg's study favoured the inclusion of *F. jacksoni* in *Fascioloides*. Considering the above inconsistencies in the outcome of phylogenetic analyses, the systematic position of *F. jacksoni* as well as morphological and biological characters used to delineate genera in the Fasciolidae deserve further consideration.

The only somewhat unexpected result in our phylogenetic analysis was the separation of the clade of *Echinochasmus* spp. (subfamily Echinochasminae Odhner, 1910, Clade 5) from the remaining representatives of the family Echinostomatidae (subfamily Echinostomatinae, Clade 1) in its own family-level group. This result indicates that the systematic

position of *Echinochasmus* may deserve a detailed analysis and possibly, re-consideration. It should be mentioned, however, that the sequence for one of these species, *Echinochasmus* sp. (JQ088098), was based on a cercarial isolate and that the sequence of *Echinochasmus japonicus* Tanabe, 1926 (JQ890579) is not otherwise published and does not have associated host data. Therefore, at this time we prefer to consider this result with some caution until more *Echinochasmus* spp. (and other members of the subfamily) are available for phylogenetic analysis.

Our results are in complete agreement with those obtained by Heneberg (2013) who used essentially the same set of taxa with the exception of *E. capitaneum* and a different outgroup. The pattern was also largely preserved in the tree based on ITS1 sequences presented by Heneberg (2013). At the same time, the ITS2 tree in Heneberg (2013) shows some striking differences in the position of several taxa. These include the position of *Isthmiophora* as a sister group of the Cathemasiidae Fuhrmann, 1928 and Psilostomidae Looss, 1900 and the nested position of *Sphaeridiotrema* Odhner, 1913, basal to several echinostomatine genera. Considering these obvious inconsistencies and low support of many topologies in the ITS2 tree in the study of Heneberg (2013) this DNA region seems to be unsuitable for higher level phylogenetics and classification within the Echinostomatoidea.

References

Bergmame, L., Hyffman, J., Cole, R., Dayanandan, S., Tkach, V., & McLaughlin, J. D. (2011). *Sphaeridiotrema globulus* and *Sphaeridiotrema pseudoglobulus* (Digenea): species differentiation based on mtDNA (Barcode) and partial LSU-rDNA sequences. *Journal of Parasitology, 97*, 1132–1136.

Church, M. L., Barrett, P. M., Swenson, J., Kinsella, J. M., & Tkach, V. V. (2013). Outbreak of *Philophthalmus gralli* in four greater rheas (*Rhea americana*). *Veterinary Ophthalmology, 16*, 65–72.

Dietz, E. (1909). Die Echinostomiden der Vögel. *Zoologischer Anzeiger, 34*, 180–192.

Dietz, E. (1910). Die Echinostomiden der Vögel. *Zoologische Jahrbücher, 12*, 256–512.

Esteban, J. G., Toledo, R., Sánchez, L., & Muñoz-Antolí, C. (1997). Life-cycle of *Euparyphium albuferensis* n. sp. (Trematoda: Echinostomatidae) from rats in Spain. *Systematic Parasitology, 38*, 211–219.

Georgieva, S., Kostadinova, A., & Skírnisson, K. (2012). The life-cycle of *Petasiger islandicus* Kostadinova & Skirnisson, 2007 (Digenea: Echinostomatidae) elucidated with the aid of molecular data. *Systematic Parasitology, 82*, 177–183.

Griffin, M. J., Khoo, L. H., Quiniou, S. M., O'Hear, M. M., Pote, L. M., Greenway, T. E., Wise, D. J. (2012). Genetic sequence data identifies the cercaria of *Drepanocephalus spathans* (Digenea: Echinostomatidae), a parasite of the double-crested cormorant (*Phalacrocorax auritus*), with notes on its pathology in juvenile channel catfish (*Ictalurus punctatus*). *Journal of Parasitology, 98*, 967–972.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor v. 5.0.9. *Nucleic Acids Symposium, 41*, 95–98.

Heneberg, P. (2013). Phylogenetic data suggest the reclassification of *Fasciola jacksoni* (Digenea: Fasciolidae) as *Fascioloides jacksoni* comb. nov. *Parasitology Research, 112*, 1679–1689.

Huelsenbeck, J. P., & Ronquist, F. (2001). MrBayes: Bayesian inference of phylogeny. *Bioinformatics, 17*, 754–755.

Kanev, I., Radev, V., & Fried, B. (2002). Family Cyclocoelidae Stossich, 1902. In: Gibson, D. I., Jones, A. & Bray, R. A. (Eds) *Keys to the Trematoda, Volume 1*. Wallingford: CAB International, pp. 131–145.

Kostadinova, A. (2005). Family Echinostomatidae Looss, 1899. In: Jones, A., Bray, R. A. & D. I. Gibson, D. I. (Eds) *Keys to the Trematoda, Volume 2*. Wallingford: CAB International and The Natural History Museum, pp. 9–64.

Kostadinova, A., & Gibson, D. I. (2002). *Isthmiophora* Lühe, 1909 and *Euparyphium* Dietz, 1909 (Digenea: Echinostomatidae) re-defined, with comments on their nominal species. *Systematic Parasitology, 52*, 205–217.

Literák, I., Heneberg, P., Sitko, J., Wetzel, E. J., Cardenas Callirgos, J. M., Čapek, M., Valle Basto, D., & Papoušek, I. (2013). Eye trematode infection in small passerines in Peru caused by *Philophthalmus lucipetus*, an agent with a zoonotic potential spread by an invasive freshwater snail. *Parasitology International, 62*, 390–396.

Lotfy, W. M., Brant, S. V., DeJong, R. J., Le, T. H., Demiaszkiewicz, A., Rajapakse, R. P., Perera, V. B., Laursen, J. R., & Loker, E. S. (2008). Evolutionary origins, diversification, and biogeography of liver flukes (Digenea, Fasciolidae). *American Journal of Tropical Medicine and Hygiene, 79*, 248–255.

Mendheim, H. (1943). Beiträge zur Systematik und Biologie der Familie Echinostomatidae (Trematoda). *Archiv für Naturgeschichte, 12*, 175–302.

Nylander, J. A. A. (2004). MrModelTest 2.3. Program Distributed by the Author. Uppsala University; Uppsala, Switzerland.

Olson, P. D., Cribb, T. H., Tkach, V. V., Bray, R. A., & Littlewood, D. T. J. (2003). Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal for Parasitology, 33*, 733–755.

Pérez Vigueras, I. (1944). Trematodes de la super-familia Echinostomatidae, con descripción de siete especies nuevas de Cuba. *Revista de la Universidad de la Habana, 57*, 221–234.

Premvati, G. (1968). Echinostome trematodes from Florida birds. *Proceedings of the Helminthological Society of Washington, 35*, 197–200.

Rambaut, A. (2012). FigTree v. 1.4. Molecular evolution, phylogenetics and epidemiology. Edinburgh, UK: University of Edinburgh, Institute of Evolutionary Biology, http://tree.bio.ed.ac.uk/software/figtree/.

Sato, H., & Suzuki, K. (2006). Gastrointestinal helminthes of feral raccoons (Procyon lotor) in Wakayama Prefecture, Japan. *Journal of Veterinary Medical Science, 68*, 311–318.

Skrjabin, K. I., & Bashkirova, E. Y. (1956). Family Echinostomatidae. *Osnovy Trematodologii, 12*, 53–930 (In Russian).

Tkach, V. V., Littlewood, D. T. J., Olson, P. D., Kinsella, J. M., & Świderski, Z. (2003). Molecular phylogenetic analysis of the Microphalloidea Ward, 1901. *Systematic Parasitology, 56*, 1–15.

Tkach, V. V., & Pawlowski, J. (1999). A new method of DNA extraction from the ethanol-fixed parasitic worms. *Acta Parasitologica, 44*, 147–148.

Tkach, V., Pawlowski, J., & Mariaux, J. (2000). Phylogenetic analysis of the suborder Plagiorchiata (Platyhelminthes, Digenea) based on partial lsrDNA sequences. *International Journal for Parasitology, 30*, 83–93.

Tkach, V., Pawlowski, J., Mariaux, J., & Swiderski, Z. (2001). Molecular phylogeny of the suborder Plagiorchiata and its position in the system of Digenea. In: Littlewood, D. T. J. & Bray, R. A. (Eds) *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, pp. 186–193.

Tkach, V. V., Schroeder, J. A., Greiman, S. E., & Vaughan, J. A. (2012). New genetic lineages, host associations and circulation pathways of *Neorickettsia* endosymbionts of digeneans. *Acta Parasitologica, 57*, 285–292.

Yamaguti, S. (1958) *Systema helminthum. Vol. 1. The digenetic trematodes of vertebrates*. New York: Interscience Publishers, 1,575 pp.