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Phylogenetic relationships of Hemiuridae (Digenea: Hemiuroidea) with new morphometric and molecular data of *Aphanurus mugilis* Tang, 1981 (Aphanurinae) from mullet fish of Vietnam

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A B S T R A C T

Adult *Aphanurus mugilis* Tang, 1981 worms were detected in the intestine of *Moolgarda engeli* in the shallow waters off Cat Ba Island, Vietnam. Tang (1981) first described this species in *Mugil cephalus* off China. The worms in Vietnamese mullet were identical to Chinese specimens in a number of morphometric characteristics, with the exception of body and ovary size. In the present study, morphological characteristics, and the first molecular data for *A. mugilis* are provided. Additionally, molecular phylogenetic analysis of the family Hemiuridae was performed. The results of our molecular phylogenetic study indicate that the presence or absence of an ecsoma was not associated with molecular data for hemiurid subfamilies differentiation. The basal position of Bunocotylinae on the molecular-based phylogenetic tree indicated a primordial nature of ecsoma of hemiurid trematodes. Considerable molecular differentiation of Bunocotylinae from other hemiurids indicated the possibility of the recognition of the family Bunocotylidae Dollfus, 1950. Assuming that *Machidatrema chilostoma* is considered within the Bunocotylinae, the paraphyly of the Lecithasterinae was supported.

1. Introduction

Trematodes of the genus *Aphanurus* are intestinal parasites of different teleosts in seas and brackish waters. This genus includes 16 nominal species, although the species composition varies within the literature [1]. Of these, two species, *A. microrchis* Chauhan, 1945 and *A. mugilis* Tang, 1981, have been detected in the intestines of mullet *Mugil parsia* from India and *M. cephalus* from China [2–5]. In the present study, the species *A. mugilis* was identified in the intestine of *Moolgarda engeli* from the shallow waters off Cat Ba Island, Vietnam. The morphological characteristics and first molecular data for this species are given. The phylogenetic relationships of *A. mugilis*, a representative of the subfamily Aphanurinae, were analyzed within the Hemiuroidea. Some families within this superfamily are composed of morphologically dissimilar species, and are polyphyletic according to the molecular data. For example, obvious molecular polyphyly is typical for Hemiuridae and Lecithasteridae [6]. Currently, there are sufficient molecular data for these two families, including two additional species of Lecithasteridae, *Lecithaster mugilis* Yamaguti, 1970 and a new species *L. sudzuhensis* [7], for the representative phylogenetic analysis that was performed in our study. Additionally, we have performed the analysis to determine the consistency of molecular differentiation in Hemiuridae subfamilies, and the morphological characteristics used for species identification for hemiurid trematodes.

2. Materials and methods

2.1. Trematode collection and preparation

Adult worms of the species *A. mugilis* were detected in the intestine of *Moolgarda engeli* in the shallow waters of Cat Ba Island, Vietnam. Seventy specimens of mullet fish were prepared. Of these, eleven specimens were infected with one to nine trematode specimens per fish. Following removal from the intestine, the worms from each fish species were rinsed in distilled water, killed in hot distilled water and preserved in 70% ethanol. Following fixation, flukes were transferred to 96% ethanol and whole mounts were stained with alum carmine, dehydrated in an ethanol series, cleared in xylene and mounted in Canada balsam.

2.2. DNA extraction, amplification and sequencing

Adult specimens of *Aphanurus mugilis*, collected from *Moolgarda*

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Table 1
List of taxa, incorporated into molecular analysis (n - number of specimens).

Species	n	Author	GenBank Accession number	
			18S	28S
Hemiuridae				
Aphanurus sp., Vietnam	3	Present study	LT607804-LT607806	LT607807-LT607809
Bunocotyle progenetica	1	Pankov et al., 2006	DQ354369	DQ354365
Dinurus longisinus	1	Cribb et al., 2001, Olson et al., 2003	AJ287501	AY222202
Lecithochirium caesionis	1	Cribb et al., 2001	AJ287528	–
Lecithocladium. excisum	1	Cribb et al., 2001, Olson et al., 2003	AJ287529	AY222203
Lecithochirium microstomum	1	Calhoun et al., 2013	–	KC985235
Merlucciotrema praeclarum	1	Cribb et al., 2001, Olson et al., 2003	AJ287535	AY222204
Opisthadena dimidia		Cribb et al., 2001, Olson et al., 2003	AJ287549	AY222198
Pleurusdigitatus	1	Cribb et al., 2001, Olson et al., 2003	AJ287562	AY222201
Robinia aurata	1	Pankov et al., 2006	DQ354371	DQ354367
Hemiurus appendiculatum	2	Bao et al., 2015	–	KR349118- KR349121
Saturnius gibsoni	1	Marzoug et al., 2014	–	KJ010542
Saturnius sp.	1	Pankov et al., 2006	–	DQ354366
Lecithasteridae				
Aponurus sp.	2	Pankov et al., 2006, Carreras-Aubets et al., 2011	DQ354372	DQ354668, HQ713441
Lecithaster gibbosus	1	Cribb et al., 2001, Olson et al., 2003	AJ287527	AY222199
L. mugilis	1	Beprozvannykh et al., 2016	LN865007	LN865016
L. sudzuhensis	1	Beprozvannykh et al., 2016	LN865013	LN865022
Lecithophyllum botryophorum	1	Olson et al., 2003	AY222107	AY222205
Machidatrema chilostoma	1	Olson et al., 2003	AY222106	AY222197
Accacoeliidae				
Accacoelium contortum	1	Cribb et al., 2001, Olson etr al., 2003	AJ287472	AY222190
Azygioidae				
Otodistomum cestoides	1	Cribb et al., 2001, Olson etr al., 2003	AJ287553	AY222187
Bivesiculidae				
Bivesicula claviformis	1	Cribb et al., 2001/Olson et al., 2003	AJ287485	AY222182
B. fusiformis	1	Olson et al., 2003	AY222100	AY222183
B. unexpecta	1	Olson et al., 2003	AY222099	AY222181
Derogenidae				
Derogenesvaricus	1	Cribb et al., 2001, Olson et al., 2003	AJ287511	AY222189
Hemiperamanterie	1	Olson et al., 2003	AY222105	AY222196
Didymozoidae				
Didymozoon scombri	1	Cribb et al., 2001, Olson et al., 2003	AJ287500	AY222195
Didymozoid. sp. 2-PO-200	1	Olson et al., 2003	AY222102	AY222192
D. sp. 3-PO-200	1	Olson et al., 2003	AY222104	AY222194
Hirundeliidae				
Hirudinella ahi	1	Calhoun et al., 2013	–	KC985238
Hirudinella ventricosa	1	Calhoun et al., 2013	–	KC985232
Sclerodistomidae				
Prosogonotrema bilabiatum	1	Olson et al., 2003	–	AY222191
Derogenidae				
Derogenes varicus	1	Olson et al., 2003	–	AY222189
G. muraenolepisi	2	Sokolov et al., 2016	–	LN865025- LN865026
Thometrema lotzi	1	Calhoun et al., 2013	–	KC985236
Transversotrematidae				
Crusziella formosa	1	Cribb et al., 2001/Olson et al., 2003	AJ287491	AY222185
Prototransversotrema steeri	1	Olson et al., 2003	AY222101	AY222184
Transversotrema haasi	1	Cribb et al., 2001/Olson et al., 2003	AJ287583	AY222186

engeli from Cat Ba Island were used for molecular analysis (Table 1). Total DNA was extracted from flukes, which were fixed in 96% ethanol, using the "hot shot" technique [8].

18S rDNA was amplified with the following primers: 18S-E (5′ CCG AAT TCG TCG ACA ACC TGG TTG ATC CTG CCA GT 3′), 18S-F (5′ CCA GCT TGA TCC TTC TGC AGG TTC ACC TAC 3′), as previously described [9]. The initial PCR reaction was performed in a total volume of 20 μl containing 0.25 mM of each primer pair, approximately 10 ng of total DNA in water, 10 × Dream *Taq* buffer, 1.25 mM dNTPs and 1 unit of Dream *Taq* polymerase (Thermo Scientific, USA). Amplification of a 2000 bp fragment of the 18S rRNA gene was performed in a GeneAmp 9700 (Applied Biosystems, USA) with 5 min of denaturation at 96 °C, 35 cycles of 1 min at 96 °C, 20 s at 58 °C and 5 min at 72 °C, followed by 10 min of extension at 72 °C. Negative and positive controls using both primers were included.

28S rDNA was amplified with the primers DIGl2 (5′-AAG CAT ATC ACT AAG CGG -3′) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′), as previously described [10]. The master mix for the initial PCR reaction for 28S rDNA was identical to that described above for 18S rDNA. Amplification of a 1200 bp fragment of 28S rDNA was performed in a GeneAmp 9700 (Applied Biosystems, USA) with 3 min of denaturation at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C, followed by 7 min of extension at 72 °C. Negative and positive controls using both primers were included.

PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA), as recommended by the manufacturer, with the internal sequencing primers described in [9] for 18S rDNA and in [10] for 28S rDNA. PCR products were analyzed using an ABI 3130 genetic analyzer at the

Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of Russian Academy of Science. Sequences were submitted to the European Nucleotide Archive (ENA) with the accession numbers described in Table 1.

2.3. Alignments and the phylogenetic analysis

Ribosomal DNA sequences were assembled with SeqScape v.2.6 software. Alignments and an estimation of the number of variable sites and p-distance calculation were performed using MEGA 6.0 [11]. The distribution analysis of p-distance values performed with Statistica 10 software [12]. Phylogenetic analyses of the nucleotide sequences were performed using Bayesian algorithms (BI) with MrBayes v. 3.1.2 software [13]. The best nucleotide substitution model was estimated with jModeltest v. 2.1.5 software [14] Bayesian Information Criterion (BIC) [13]. The best nucleotide substitution model for combined 18S rRNA + 28S rRNA gene sequence data was the general time reversible with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + G) [15]. Bayesian analysis was performed using 10,000,000 generations, with two independent runs. Summary parameters and the phylogenetic tree were calculated with a burnin of 1,500,000 generations. The significance of the phylogenetic relationships was estimated using posterior probabilities [13]. The phylogenetic relationships of the species of Hemiuridae were inferred from our data, along with the nucleotide sequences of the 18S rDNA and 28S rDNA of other trematode specimens obtained from the NCBI GenBank database [24–26] (Table 1).

3. Results

3.1. Description

Aphanurus mugilus Tang, 1981

Host: *Moolgarda* (*Valamugil*) *engeli*.
Locality: coastal water off Cat Ba Island, Halong Bay, northern Vietnam (20°84′ N, 106°59′E).
Site: Intestine.
Intensity of infection: One to nine specimens.
Description (based on seven specimens) (Fig. 1; Table 2):

Body elongated, tapered anteriorly, wide at ventral sucker level. Vestige of ecsoma absent. Body surface annular plications along entire length of body. Forebody short. Oral sucker subglobular; aperture subterminal. Ventral sucker large, muscular, rounded; aperture oriented antero-ventrally. Prepharynx absent. Pharynx transverse-oval. Oesophagusis extremely short or apparently absent. Caeca wide, and reaches posterior third of body. Testes transverse-oval, symmetrical or slightly diagonal, pre-equatorial, contiguous or separated. Seminal vesicle large, globular at testes level and partially overlapping them, and the ovary and vitellarium. Pars prostatica tubular, long, with wide lumen, enveloped by several layers of large gland cells. Pars prostatica joins base of sinus sac at about level of anterior edge of ventral sucker. Sinus sac tubular. Hermaphroditic duct long, tubular, straight, extends throughout length of sinus sac; distal half eversible, forming temporary sinus organ lined with small tubercles. Genital pore median at level of posterior edge of oral sucker. Ovary transversely oval, post-testicular, close to left testis. Seminal receptacle uterine. Vitellarium single, large, compact, four-lobed masses, contiguous with ovary. Uterine coils reach well posterior to vitellarium, pass forward medio-dorsally toward gonads, seminal vesicle and ventral sucker, and enters base of sinus sac. Eggs operculate. Excretory vesicle Y-shaped; lateral arms wide, and unite dorsally to oral sucker. Excretory pore terminal.

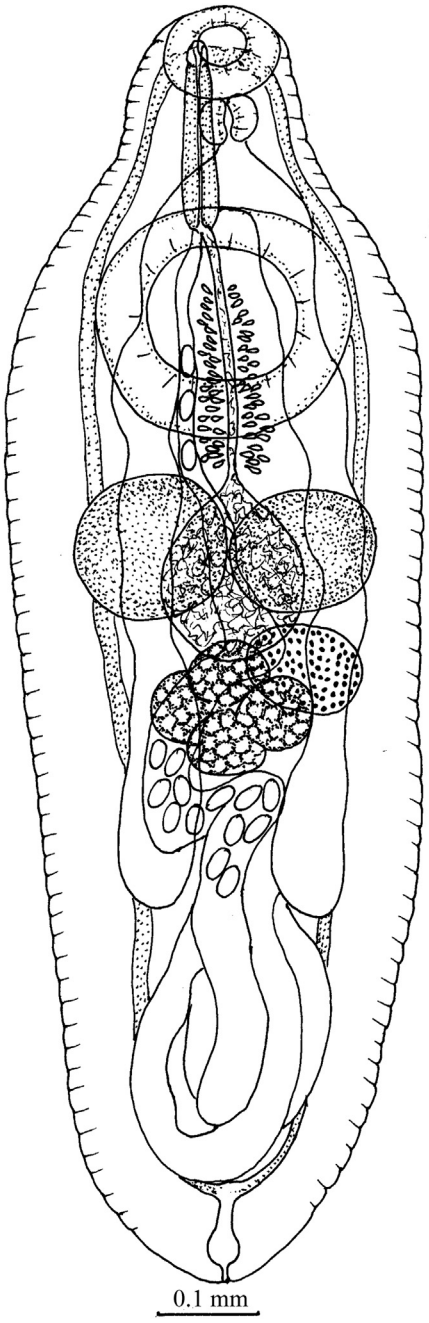

Fig. 1. *Aphanurus mugilis* Tang, 1981, adult worm.

3.2. Molecular data

A total of 1912 and 1257 alignable characters were available for analysis of the 18S rRNA gene and 28S rRNA gene datasets, respectively. Of these, 1236 constant and 544 parsimony-informative sites were detected for the 18S rRNA gene and 486 and 620 for 28S rRNA gene, respectively. No variable and parsimony-informative sites for either 18S or 28S rRNA genes of *Aphanurus mugilis* were present.

Phylogenetic tree topology, based on combined complete 18S rRNA gene and partial 28S rRNA gene sequence data, showed two main large clades within a monophyletic Hemiuroidea (Fig. 2). The first clade contained two families, Hemiuridae and Lecithasteridae, and the second clade included species of Didymozoidae, Accacoelidae, Derogenidae, Sclerodistomidae and Syncoeliidae. The species *Hemipera manteri* (Derogenidae) formed a basal branch within the Hemiuroidea.

Table 2
Sizes (mm) of adult *Aphanurus* from mullets.

Features	*Aphanurus mugilus*			*A. microrchis* Chauhan, 1945
	Present study		Tang, 1981	
	Range	Mean		
Body	1.093–2.23 × 0.354–0.616	1.482 × 0.471	2.69–2.96 × 0.532–0.741	0.58 × 0.144
Oral sucker	0.085–0.127 × 0.100–0.131	0.100 × 0.118	0.095–0.114 × 0.114–0.152	0.025 × 0.039
Pharynx	0.050–0.065 × 0.054–0.069	0.058 × 0.062	0.062–0.076 × 0.066–0.086	0.013 × 0.019
Ventral sucker	0.181–0.296 × 0.223–0.343	0.248 × 0.274	0.152–0.237 × 0.182–0.266	0.063 × 0.050
Forebody	0.200–0.323	0.259		
Sinus-sak	0.173–0.258 × 0.042–0.050	0.207 × 0.045		0.013 × 0.041
Pars prostatica	0.177–0.231 × 0.069–0.085	0.202 × 0.078		
Testes				
Left testes	0.085–0.250 × 0.089–0.219	0.165 × 0.159	0.095–0.244 × 0.171–0.246	0.025
Right testes	0.092–0.212 × 0.096–0.193	0.147 × 0.153	0.115–0.266 × 0.123–0.231	
Seminal vesicle	0.116–0.193 × 0.108–0.154	0.167 × 0.128		
Ovary	0.054–0.135 × 0.073–0.169	0.096 × 0.110	0.133–0.182 × 0.190–0.257	0.039 × 0.044
Vitellarium	0.104–0.185 × 0.116–0.231	0.142 × 0.174	0.228–0.368 × 0.209–0.337	0.037 × 0.050
Post-testicular field length	0.485–0.832	0.637		
Post-caecal field length	0.154–0.470	0.295		
Bw/Bl[a]	1:2.54-3.62	3.14		
Fo/Bl	1:4.30–6.31	5.72		
Eggs	0.027–0.039 × 0.015–0.019	–	0.022–0.035 × 0.013–0.017	0.018 × 0.009

[a] Bw/Bl – body width as a percentage of body length, Fo/Bl – length of the forebody as a percentage of body length.

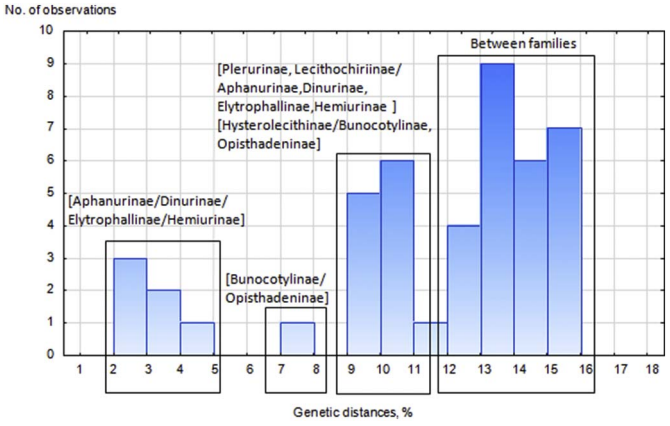

Fig. 2. Histogram of genetic p-distance values distribution within Hemiuroidea by 28S rRNA gene partial sequences.

There were five lineages within the first clade; the first (H1) included representatives of five Hemiuridae subfamilies: Aphanurinae (*Aphanurus mugilis*), Dinurinae (*Dinurus longisinus*), Elytrophallinae (*Lecithocladium excisum*), Plerurinae (*Plerurus digitatus* and *Merlucciotrema praeclarum*) and Lecithochiriinae (*Lecithochirium caesionis*). Dinurinae and Elytrophallinae are closely related and Aphanurinae appears as a sister group for these two subfamilies. Two species of Plerurinae were separated from each other and Lecithochiriinae had an intermediate position relative to *Plerurus digitatus* and *Merlucciotrema praeclarum*. The last species was basal for the H1 lineage. Three species of the genus *Lecithaster* (Lecithasteridae, Lecithasterinae) formed the second lineage (L1), which was closely related to H1. The species of Lecithasterinae, *Aponurus* sp. and *Lecithophyllym botryophorum*, were closely related to each other and formed a lineage (L2). Two species from different families, *Opisthadena dimidia* (Hemiuridae) and *Machidatrema chilostoma* (Lecithasteridae) clustered together and formed a fourth lineage (H2/L3) with poor statistical support. The last one (lineage H3) was basal within the first clade and was represented by hemiurid trematodes of the subfamily Bunocotylinae (*Bunocotyle progenetica* and *Robinia aurata*). The same tree topology was obtained by Bayesian analysis based on 28S rRNA partial sequences with the highest number of species. *Hemiurus appendiculatum*, a member of the type-genus of Hemiuridae, was closely related with the Aphanurinae/Dinurinae/Elytrophallinae group within the L1 lineage, and an additional species of Bunocotylinae, *Saturnius gibsoni*, was closely related with *B. progenetica* within lineage H3.

Genetic p-distance values calculated by pairwise comparative analysis of different Hemiuridae and Lecithasteridae subfamilies by 28S rRNA gene sequence data are shown in Table 3, and the distribution of these values is presented in a histogram (Fig. 3). There were four ranges of values distinguished from each other. The first range (2–5%) was obtained by pairwise comparison of the four subfamilies Aphanurinae, Dinurinae, Elytrophallinae and Hemiurinae. The second range was

Table 3
Genetic p-distance values (%, below diagonal) and std. error (above diagonal) calculated by pairwise comparative analysis of different Hemiuridae and Lecithasteridae subfamilies by 28S rRNA gene sequence.

		1	2	3	4	5	6	7	8	9	10
1	Aphanurinae		0.57	0.65	0.64	0.86	1.11	1.24	1.45	1.08	1.37
2	Dinurinae	2.99		0.69	0.71	0.88	1.13	1.21	1.41	1.1	1.43
3	Elytrophallinae	2.99	3.32		0.72	0.95	1.12	1.27	1.49	1.18	1.44
4	Hemiurinae	2.82	3.82	4.32		0.86	1.12	1.3	1.38	1.11	1.47
5	Plerurinae	9.55	10.3	10.1	9.97		0.93	1.01	1.08	0.87	1.12
6	Lecithocheriinae	9.63	11	11	10.5	10.9		1.22	1.27	1.06	1.34
7	Bunocotylinae	12.7	13.7	13.8	13.1	11.9	13.7		0.97	0.89	1.03
8	Opisthodeninae	14.6	15.9	15.8	14.8	12	14.8	7.77		1.02	1.15
9	Lecithastetinae	14.5	15.6	15.4	15.3	13.6	15.3	12.9	13.5		1.03
10	Hysterolecithinae	14	15	15	15.4	12.4	14	9.68	9.14	13.5	

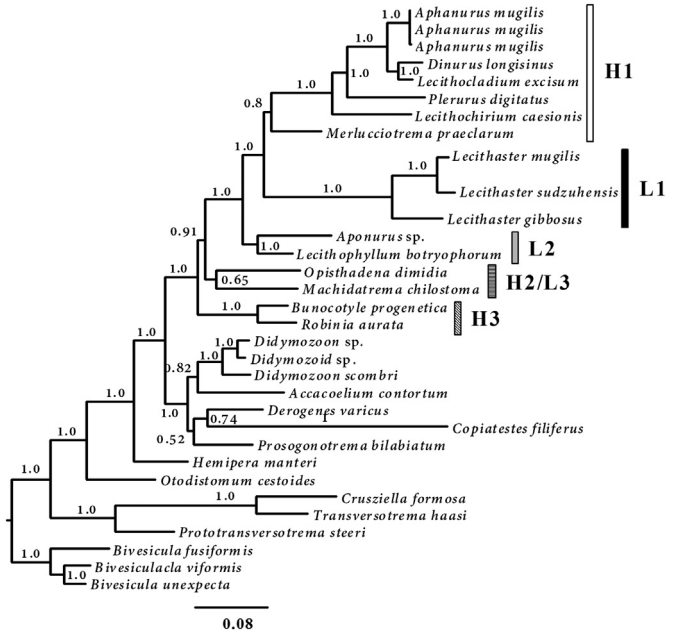

Fig. 3. Phylogenetic relationships of the superfamily Hemiuroidea obtained with Bayesian algorithm based on concatenated nucleotide sequence data set of complete 18S rRNA gene and partial 28S rRNA gene. Nodal numbers - posterior probabilities that indicate statistical support of phylogenetic relationships.

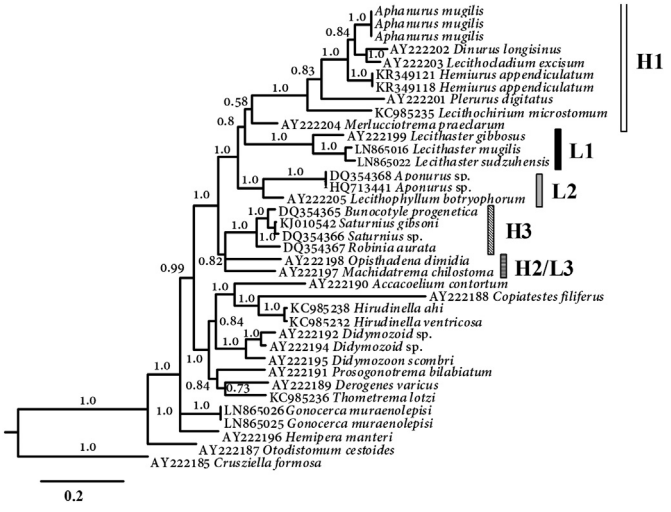

Fig. 4. Phylogenetic relationships of the superfamily Hemiuroidea obtained with Bayesian algorithm based on concatenated nucleotide sequence data set of partial 28S rRNA gene. Nodal numbers - posterior probabilities that indicate statistical support of phylogenetic relationships.

represented by a single value (7.77%) and was obtained for Bunocotylinae/Opisthadeninae. The next range (9–11%) was obtained by comparative analysis of Plerurinae and Lecithochiriinae with four other subfamilies (Aphanurinae, Dinurinae, Elytrophallinae and Hemiurinae). Genetic p-distance values were obtained for the pairs Hysterolecithinae (Lecithasteridae)/Opisthadeninae (Hemiuridae), and Hysterolecithinae (Lecithasteridae)/Bunicotylinae (Hemiuridae) were within the third range. The highest range (12–16%) represents the interfamiliar differentiation level. Nevertheless, the same values were obtained by pairwise comparisons of Bunocotylinae and Opisthadeninae with other hemiurid trematodes as for the Lecithasterinae/Hysterolecithinae pair.

4. Discussion

4.1. Morphological analysis of Aphanurus Mugilis

Trematode specimens from Vietnamese *Moolgarda (Valamugil) engeli* were identical to *Aphanurus mugilis* from *M. cephalus* in Fujian China (Tang, 1981) [2] according to most morphometric characters, with the exception of a slightly smaller body and ovary size in *A. mugilis* (Table 2). These differences are conditioned by age-specific parameters and the wide range in body size and ovary size variation. In other respects, these worms are identical to each other. Our main finding is that these trematodes belong to the species *A. mugilis*, based on the results of morphometric analysis, definitive host specificity and geographical localization.

4.2. Taxonomical interpretation of molecular-based phylogenetic relationships

The genus *Aphanurus* (Aphanurinae) was the one of the earliest taxa included in the family Hemiuridae on the basis of a number of metric and morphological characters [2,16]. The present study provides taxonomical and phylogenetic analyses of Aphanurinae based on the first molecular data for *Aphanurus mugilis*. This species was in the same clade as most hemiurid subfamilies (lineage H1) and was closely related to Dinurinae, Elytrophallinae and Hemiurinae (d = 2.8 ± 0.57%–3.0 ± 0.64% by 28S rRNA gene sequence data). These results appear to support previous findings on the systematic position of Aphanurinae within Hemiuridae. However, genetic p-distances between Aphanurinae, Dinurinae, Elytrophallinae and Hemiurinae were considerably lower in comparison with values calculated between each of these four subfamilies and Plerurinae and Lecithochiriinae (Fig. 2). The last two subfamilies were also distinctly distant from each other (d = 10.9 ± 0.93%). It is notable that two species of Plerurinae (*P. digitatus* and *M. praeclarum*) differ from each other at the same level (d = 10 ± 0.9%). These results indicate that lineage H1 represents the true Hemiuridae family. On the other hand, our data show that the taxonomical status of Aphanurinae, Dinurinae, Elytrophallinae and Hemiurinae should be reconsidered in light of the molecular differentiation found in the present study, with the inclusion of these trematodes into the same subfamily, Hemiurinae.

Bayesian phylogenetic analysis revealed polyphyly for the family Lecithasteridae (Fig. 4). The genus *Lecithaster* was located separately from *Aponurus* and *Lecithophyllum* despite it generally being recognized in the same subfamily, Lecithasterinae. There is considerable molecular differentiation between these three genera; p-distance values ranged from 8.5% (*Lecithophyllum/Aponurus*) to 17.5% (*Lecithaster/Aponurus*). The genus *Lecithaster* was considered as a member of Hemiuridae (subfamily Lecithasterinae) for a long time, until Skrjabin and Gushanskaya (1954) erected the family Lecithasteridae with six subfamilies and 13 genera, including *Aponurus*, *Lecithophyllum* and *Lecithaster* [2]. The paraphyly of Lecithasterinae has been previously shown using the V4 region of 18S rRNA gene sequence data [17]. Our results support this point, supporting the assumption that *Machidatrema chilostoma* is within Bunocotylinae by Leon-Regagnon et al. (1998) [18], which is contrary to the concept of Bray and Cribb (2000) that *Machidatrema* shares a lineage with hysterolecithine lecithasterids [19].

The subfamily Bunocotylinae in our study formed a basal branch for the Hemiuridae + Lecithasteridae clade that confirmed results of a molecular phylogenetic study of Hemiuridae performed by Pankov et al. (2006) [6]. However, these authors followed the concept of Gibson (2002) who recognized the subfamily Bunocotylinae with two genera in a recent revision of Hemiuridae [16]. Our results of genetic p-distances and phylogenetic analyses indicate considerable molecular differentiation between bunocotylines and other hemiurids (Figs. 2–4). Despite most studies following the concept of Bunocotylinae [6,18,20,21,22], we do not exclude the possibility of recognition of the family Bunocotylidae Dollfus, 1950.

4.3. Molecular analysis of Hemiuridae with major diagnostic morphological characteristics

Blair et al. (1998) first used molecular and morphological matrices for phylogenetic reconstructions of Hemiuroidea. The main conclusion of the study of Blair et al. (1998) was that molecular and morphological matrices for a large group of digeneans are not incongruent leading to the belief that both kinds of data are of value in inferring relationships among the Digenea [17]. We have made progress in resolving a high level of disagreement between molecular and morphological data by comparative analysis of molecular differentiation that was reflected in Bayesian phylogenetic tree topology and major diagnostic morphological characteristics used for species identification for Hemiuridae [16]. According to Gibson and Bray (1979) [23], the major diagnostic feature of the family Hemiuridae is the ecsoma, a protrusible, posterior region of the body that has been entirely lost or is vestigial in four subfamilies (according to Gibson, 2002): Aphanurinae, Bunocotylinae, Opisthadeninae and Pulmoverninae. Gibson (2002) also noted that the ecsoma is a functionally considerable structure that enables the worms to exist in the acid regions of the stomach [16]. Our results indicate that trematodes of the subfamily Aphanurinae which lack an ecsoma are clustered with Dinurinae, and that Elytrophallinae are characterized by well-developed protrusible posterior parts of the body, i.e. ecsoma (Fig. 5). These three subfamilies are genetically close to each other by p-distance values − 3.9% (Fig. 2). Moreover, species of Plerurinae and Lecithochiriinae, which are also characterized by the presence of an ecsoma, are included in the H1 lineage with Aphanurinae, Dinurinae and Elytrophallinae by molecular data. Trematodes of Bunocotylinae with a vestige of an ecsoma formed a basal branch (lineage H3) for the Hemiuridae + Lecithasteridae cluster. The species *Opisthadena dimidia* (Opisthadeninae), also characterized by an absence of an ecsoma, is phylogenetically distant from Aphanurinae (d = 16.9% by 28S rRNA gene partial sequence data). Thus, the presence of an ecsoma, a major diagnostic feature, is not congruent with molecular differentiation or phylogenetic relationships of Hemiuridae (Fig. 5). Moreover, the basal position of Bunocotylinae possibly indicates a primordial nature of the ecsoma of hemiurid trematodes.

Other morphological features of hemiurids (e.g. structure of vitellarium, seminal vesicle, ovary) are distributed randomly on the phylogenetic tree. Surprisingly, molecular differentiation of Hemiuridae subfamilies was associated with texture of the body surface (Fig. 5). According to Gibson (2002), the body surface of trematodes of the Aphanurinae, Dinurinae and Elytrophallinae subfamilies is mainly plicated, in contrast to species from the other subfamilies which possess smooth body surfaces [16]. This feature is associated with genetic differentiation and phylogenetic tree topology, where trematodes with plicated body surfaces are closely related to each other (Figs. 3, 4), which indicates a significance of this character for generic diagnosis.

Additionally, we have analyzed the association of molecular differentiation and host-specificity of hemiurid and lecithasterid trematodes. It is known that definitive hosts for trematodes of Hemiuridae are mainly marine teleosts [16]. Nevertheless, trematodes of Bunocotylinae were described chiefly from freshwater or euryhaline fish species [6, 16, 18, 20–22]. In the light of this host-specificity, a basal position of Bunocotylinae on the Bayesian phylogenetic tree can be interpreted to imply different pathways for definitive host adoption of ancestral trematodes of Hemiuridae and Lecithasteridae.

5. Conclusions

The results of our study have revealed several taxonomical questions that should be resolved. Firstly, the main characteristic used to identify subfamilies of Hemiuridae is the presence or absence of ecsoma, which is not congruent with the molecular differentiation of these taxa. The significance of this characteristic for use in identifying subfamilies should be reconsidered. Secondly, according to Gibson (2002), the ecsoma is a vestige in four subfamilies of Hemiuridae, including Bunocotylinae. We propose that the basal position of Bunocotylinae on the molecular-based phylogenetic tree indicates a primordial nature of the ecsoma of hemiurid trematodes. Thirdly, our results of genetic p-distances and phylogenetic analyses indicate considerable molecular differentiation of Bunocotylinae and other hemiurids; thus, we cannot exclude the possibility of recognition of the family Bunocotylidae Dollfus, 1950. Finally, our results support the paraphyly of Lecithasterinae with the assumption that *Machidatrema chlostoma* is considered a bunocotyline.

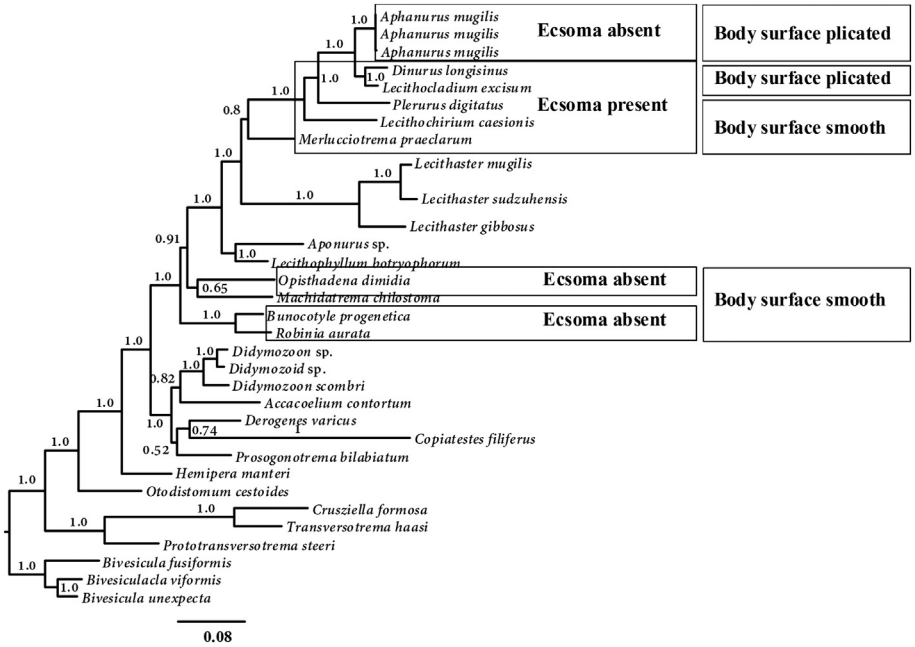

Fig. 5. Phylogenetic relationships of the superfamily Hemiuroidea obtained with Bayesian algorithm based on concatenated nucleotide sequence data set of complete 18S rRNA gene and partial 28S rRNA gene associated with morphological characters. Nodal numbers - posterior probabilities that indicate statistical support of phylogenetic relationships.

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