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Morphometric and molecular data of the two digenean species *Lasiotocus lizae* Liu, 2002 (Monorchiidae) and *Paucivitellosus vietnamensis* sp. n. (Bivesiculidae) from mullet fish in Tonkin Bay, Vietnam

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Abstract

Adults of *Lasiotocus lizae* Liu, 2002 (Monorchiidae) were found in the mullet *Liza longimanus* (Günther) from Tonkin Bay, near Cat Ba Island, Vietnam. In this region, flukes belonging to the genus *Paucivitellosus* (Bivesiculudae) were found in *Liza subviridis* (Valenciennes), *Liza engeli* (Bleeker) and *Valamugil seheli* (Forskåi). Results of investigations showed that morphological features of *L. lizae* (like *L. glebulentus* Overstreet, 1971) do not meet the criteria of the genus *Lasiotocus*. In addition, *L. lizae* is highly differentiated from other species of *Lasiotocus* from which molecular data were obtained, including *L. arrhichostoma* Searle, Cutmore et Cribb, 2014 and *L. typicum* (Nicoll, 1912). Phylogenetic analyses revealed that *L. lizae* differs considerably from other species of the genus *Lasiotocus* presented in the GenBank database. We have identified a new species of the genus *Paucivitellosus* – *P. vietnamensis* sp. n. – from *L. subviridis*, which differs from *P. fragilis* Coil, Reid et Kuntz, 1965 by metrical and molecular (18S rRNA) data, and from *P. hanumanthai* Mani, 1989 by metric features. Our results also show considerable molecular differentiation between *P. vietnamensis* sp. n. and *Paucivitellosus* spp. recovered from *L. engeli* and *V. seheli* in Vietnam.

Introduction

Liu (2002) first reported information about adult worms of *Lasiotocus lizae* from the intestines of *Liza carinata* (Valenciennes) from the Taiwan Strait. Type species of the genus *Paucivitellosus*, *P. fragilis* Coil, Reid & Kuntz, 1965 (Bivesiculidae), have been found in *Chelon troscheli* Bleeker (now *C. macrolepis*) from the same area (Coil *et al*., 1965). Peng *et al*. (2004) presented morphometrics and illustrations for *P. fragilis* from *L. carinata* of the Taiwan Strait. Pearson (1968) studied the life cycle and morphology of various developmental stages of this species. Mani (1989) included a new species, *P. hanumanthai*, in the genus *Paucivitellosus* from the Indian *Mugil cephalus*, and provided data on the life cycle and morphology of various developmental stages of the described trematode species. Subsequently, Cribb *et al*. (1994) analysed numerous specimens of the genus *Paucivitellosus* from various species of Mugilidae and Blenniidae, and concluded that *P. hanumanthai* is a synonym of *P. fragilis*.

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We have identified adults of *L. lizae* and *Paucivitellosus* spp. in mullet fish from the coastal waters of Cat Ba Island, Tonkin Bay, Vietnam and present the morphometric and molecular data for these species.

Materials and methods

Collection of trematodes

Adult worms, including 49 specimens of *L. lizae* from the intestines of *Liza longimanus* and a number of specimens (*n*) of *Paucivitellosus* spp. from *Liza subviridis* ($n = 14$), *Liza engeli* ($n = 2$) and *Valamugil seheli* ($n = 2$) were collected in the coastal waters near Cat Ba Island Tonkin Bay, Vietnam. Trematode specimens were rinsed in saline and then identified under a light microscope. For DNA analysis, worms were killed in hot distilled water and preserved in 70% ethanol. Following fixation, flukes were transferred to 96% ethanol and whole mounts were stained in alum carmine, dehydrated in an ethanol series, cleared in xylene and mounted in Canada balsam.

We were unable to perform detailed morphological analysis of *Paucivitellosus* spp. from *L. engeli* and *V. seheli* due to the small number of specimens collected. These worms were analysed with molecular methods only.

All measurements are given in millimetres (mm).

DNA extraction, amplification and sequencing

Five adult *L. lizae* specimens from *L. longimanus* and six trematode specimens of the genus *Paucivitellosus* from *L. subviridis* ($n = 2$), *L. engeli* ($n = 2$) and *V. seheli* ($n = 2$) were used for molecular phylogenetic analysis. Total DNA was extracted from whole worms using the 'hot shot' technique (Truett, 2006).

Nuclear 28S rDNA was amplified by polymerase chain reaction (PCR) using the following primers: forward DIG12 (5′-AAG CAT ATC ACT AAG CGG-3′), forward LSU5 (5′-TAG GTC GAC CCG CTG AAY TAA AGC-3′) and reverse 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach *et al.*, 2003). Nuclear 18S rDNA was amplified using the primers 18S-E and 18S-F (Littlewood & Olson, 2001). Initial PCR reactions were carried out using Q5 High Fidelity polymerase (New England Biolabs, Hitchin, UK). Amplification was performed in a GeneAmp 9700 thermo cycler (Applied Biosystems, Waltham, Massachusetts, USA) with annealing temperatures of 55°C for the partial 28S rRNA gene, 58°C for the complete 18S rRNA gene and 54°C for the complete internal transcribed spacer (ITS)1–5.8S–ITS2 rDNA fragment. Negative and positive controls were used. The 28S rDNA PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems), as recommended by the manufacturer, with the internal sequencing primers described in Tkach *et al.* (2003). Amplified 18S rDNA fragments were sequenced with the primers for amplification and additional internal primers, as described in Littlewood & Olson (2001). The PCR products were analysed using an ABI 3130 genetic analyser (Applied Biosystems) at the Institute of Biology and Soil Science. The sequences were submitted to the European Nucleotide Archive (ENA) (see table 1).

Alignments and phylogenetic analysis

The ribosomal DNA sequences were assembled with SeqScape (v. 2.6) software (Applied Biosystems) and aligned using the MEGA (v. 6.0) alignment explorer (Tamura *et al.*, 2013) with default options. Estimation of a number of variable and parsimony-informative sites was performed using MEGA 6.0. Phylogenetic analysis of the nucleotide sequences was performed using Bayesian algorithms with MrBayes (v. 3.1.2) software (Huelsenbeck *et al.*, 2001). Phylogenetic algorithms were used with the TPM3uf + I model (Darriba *et al.*, 2012) for 28S data of trematodes of *Paucivitellosus*; general time reversible model, including gamma-distributions (GTR + G) (Tavare, 1986) for 28S data of *L. lizae*; K80 + G model (Kimura, 1980) for 18S data of trematodes of *Paucivitellosus*; and TPM3 + G model (Darriba *et al.*, 2012) for 18S data of *L. lizae*. These models showed the best fit to the data using Bayesian information criterion (BIC) in jModeltest (v. 3.07) software (Darriba *et al.*, 2012). Bayesian analysis was performed using 10,000,000 generations and with four independent runs. The first 1,000,000–2,500,000 generations were burned, depending on the DNA fragment and species investigated. These values were established with the Tracer software (Rambaut & Drummond, 2009). The significance of the phylogenetic relationship was estimated by posterior probabilities (Huelsenbeck *et al.*, 2001).

The phylogenetic relationships of *L. lizae* and *Paucivitellosus* species were inferred from our data and the 18S rDNA and 28S rDNA nucleotide sequences of other trematode specimens obtained from GenBank (National Center for Biotechnology Information (NCBI)) (Cribb *et al.*, 2001; Tkach *et al.*, 2001; Olson *et al.*, 2003; Bray *et al.*, 2005; Besprozvannykh *et al.*, 2012) (table 1).

Results

Lasiotocus lizae *Liu, 2002*

Taxonomic summary

Host. Liza longimanus – four fish examined, one infected with 49 worms.

Locality. Coastal waters off Cat Ba Island, Tonkin Bay, northern Vietnam (20°84′N, 106°59′E).

Site. Intestine.

Deposited. Slides (numbers 68–72Tr.) were placed in the collection of the Zoological Museum (Institute of Biology and Soil Sciences, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia); E-mail: petrova@ibss.dvo.ru. Deposited 25 September 2015.

Description

Based on seven specimens (fig. 1a, b; table 2). Body oval, spined from anterior to posterior end. Oral sucker subterminal, prepharynx short or absent, oesophagus present, oesophageal bifurcation before ventral sucker or on level of its anterior margin. Caeca terminated at level of middle of testis. Ventral sucker on border of anterior and middle third of body, larger than oral one. The ratio of sucker's length was 1:1.12–1.57 and of sucker's width

Table 1. List of taxa incorporated in the molecular analysis of different trematode families, with the number of DNA sequences given in parentheses.

Species	Author (18S/28S)	Accession number in the European Nucleotide Archive	
		18S	28S
Monorchiidae			
Lasiotocus lizae	This study	LN864994–LN864996	LN831720– LN831724
L. arrhichostoma	Olson *et al.*, 2003	–	AY222254
Monorchis monorchis	Tkach *et al.*, 2001	–	AF184257
Proctotrema addisoni	Searle *et al.*, 2014	–	KJ658291
Provitellus turrum	Cribb *et al.*, 2001/Olson *et al.*, 2003	AJ287566	AY222253
Pleorchis polyorchis	Bray *et al.*, 2005	DQ248202	–
Lasiotocus typicum	Cribb *et al.*, 2001/Olson *et al.*, 2003	AJ287474	AY222254
Diplomonorchis leiostomi	Olson *et al.*, 2003	AY222137	AY222252
Helicometroides longicollis	Searle *et al.*, 2014	–	KJ658287
Lissorchiidae			
Asymphylodora perccotti		–	FR822715–FR822716
Lissorchis kritskyi	Olson *et al.*, 2003/Curran *et al.*, 2006; Olson *et al.*, 2003	AY222136	EF032689, AY222250
Bivesiculidae			
Paucivitellosus vietnamensis sp. n. ($n = 2$)	This study	LN865001–LN865002	LN831715– LN831716
Paucivitellosus sp. 1 ($n = 2$)	This study	LN864997– LN864998	LN865003–LN865004
Paucivitellosus sp. 2 ($n = 2$)	This study	LN864999– LN865000	LN865005–LN865006
Paucivitellosus fragilis	Cribb *et al.*, 2001	AJ287557	–
Bivesicula claviformis	Cribb *et al.*, 2001/Olson *et al.*, 2003	AJ287485	AY222182
Bivesicula fusiformis	Olson *et al.*, 2003	AY222100	AY222183
Bivesicula unexpecta	Olson *et al.*, 2003	AY222099	AY222181
Crusziella formosa	Cribb *et al.*, 2001/Olson *et al.*, 2003	AJ287491	AY222185
Prototransversotrema steeri	Olson *et al.*, 2003	AY222101	AY222184
Transversotrema haasi	Cribb *et al.*, 2001/Olson *et al.*, 2003	AJ287583	AY222186

was 1:0.96–1.26. Testis single, round or oval, in posterior half of body on median line. Two seminal ducts, which merge before cirrus-sac, moving away from testis. Anterior part of cirrus-sac before ventral sucker. Distal part of cirrus-sac with sac-shaped seminal vesicle.

Posterior part of cirrus-sac right from median line, for most part covered by ventral sucker, never crosses the posterior margin. Pars prostatica tubular, surrounded by small number of prostatic cells. Cirrus spined. Genital pore lateral or sublateral, left from ventral sucker, on level of oesophageal bifurcation. Genital atrium non-armed. Ovary large, three-lobed, to the right of median line of body, a short distance before or adjacent to the testis. Anterior margin of ovary on a level with anterior border-middle of ventral sucker. Seminal receptacle uterine. Mehlis' gland and Laurer's canal present. Uterine loops from posterior end of body to anterior margin of ventral sucker. Metraterm thin-walled, opening in bipartite terminal organ. Anterior part of terminal organ spined. Vitellarium from two compact masses, each from four large undivided follicles. Vitelline fields lateral in middle part of body, from posterior margin of ovary to anterior margin of testis. Eggs light yellow, operculated. Excretory bladder I-shaped, consists of one large or one large and 1–3 small concretions.

Molecular data

A 1345-bp fragment of the 18S rRNA gene was successfully sequenced and aligned for *L. lizae*. There were no variable sites between 18S sequences of *L. lizae*.

A 1068-bp fragment of the 28S rRNA gene sequence of *L. lizae* from Vietnam, obtained in our study, was aligned with 28S rDNA sequences of trematodes of the family Monorchidae from the GenBank database. There were three variable sites within *L. lizae* from Vietnam, which provide 0.28% of intraspecific genetic differentiation.

Remarks

The morphology and morphometrics of the present trematode species are similar to those of *L. glebulentus* Overstreet, 1971 from *M. cephalus* of the estuarine waters of the Gulf of Mexico (Overstreet, 1971) and *L. lizae* Liu, 2002 from *L. carinata* of Taiwan Strait, China (Liu, 2002). The main differences relate to the cirrus-sac, terminal organ, ovary and excretory bladder (table 2). The cirrus-sac lies between the anterior margin of the testis and the oesophageal bifurcation, and its distal part reaches the level of the vitellarium, vs. cirrus-sac is placed between the anterior margin of the ventral sucker and the pharynx, and its distal part is partially covered by the ventral sucker. The proximal part of terminal organ is spined or non-spined vs. the proximal part of terminal organ is non-spined. The ovary is non-lobed vs. the ovary is three-lobed. The excretory bladder contains 5–13 concretions vs. an excretory bladder with 1–4 concretions. Based on these features, the worms from our study are most similar to *L. lizae*, differing insignificantly from the latter only by the position of the cirrus-sac. We expect that adult worms collected from mullet fish from Tonkin Bay belong to *L. lizae*.

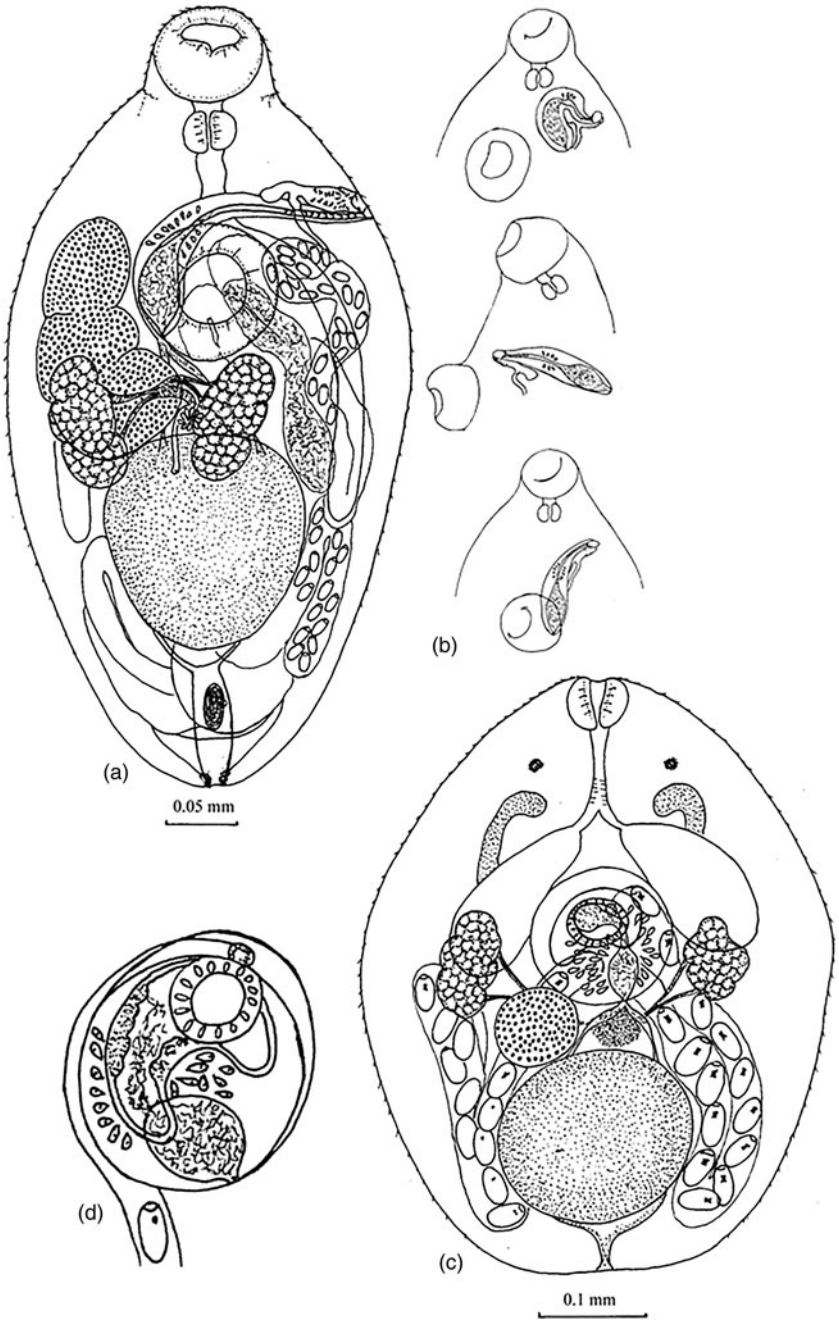

Fig. 1. (a, b) *Lasiotocus lizae* Liu, 2002: (a) adult worm; (b) variants of location of the cirrus-sac. (c, d) *Paucivitellosus vietnamensis* sp. n.: (c) adult worm; (d) cirrus-sac.

The type species of *Lasiotocus*, *L. mulli* (Stossich, 1883), like the most numerous species of this genus, has a median or submedian genital pore before the ventral sucker. For the species *L. odhneri* (Srivastava, 1939), *L. minutus* (Manter, 1931), *L. elongates* (Manter, 1931) and *L. mugilis* Overstreet, 1969, the submedian genital pore is placed in the borders of projection of the ventral sucker. For the trematode specimens studied in the present work, the genital pore is sublaterally or laterally located. At the same time, the genital pore lies left from the ventral sucker in the species *L. glebulentus* and *L. lizae*. Unlike *L. mulli*, which has a vitellarium consisting of a group of separate follicles, these two species have vitellaria consisting of two compact masses of undivided follicles. Similar vitellarial structures are inherent for the type species of *Pseudoproctotrema*, *P. parupenei* Yamaguti, 1924. Ovaries

Table 2. A comparison of the morphometrics (in mm) of adult *Lasiotocus lizae* with *L. glebulentus*.

Features	*L. lizae* (present study)		*L. lizae* (Liu, 2002)		*L. glebulentus* (Overstreet, 1971)
	Range	Mean	Range	Mean	
Body	0.554–0.612 × 0.280–0.327	0.594 × 0.294	0.528–0.732 × 0.248–0.304	0.610 × 0.275	0.458–1.124 × 0.201–0.310
Anterior end	0.127–0.219	0.165	0.036–0.056*	0.047*	0119–0.275
Oral sucker	0.058–0.089 × 0.077–0.104	0.073 × 0.088	0.056–0.074 × 0.070–0.094	0.064 × 0.078	0.035–0.079 × 0.058–0.102
Prepharynx length	0–0.0077	–	–	–	0.019–0.056
Pharynx	0.031–0.046 × 0.042–0.050	0.038 × 0.047	0.022–0.038 × 0.032–0.040	0.030 × 0.036	0.026–0.040 × 0.030–0.051
Oesophagus length	0.023–0.062	0.044	0.016–0.036	0.024	0.023–0.063
Ventral sucker	0.085–0.116	0.101 × 0.105	0.074–0.084 × 0.082–0.094	0.079 × 0.088	0.060–0.102 × 0.067–0.107
Sucker ratio	1:1.10–1.51	1:1.28	1:1.09–1.25	1:1.18	1:1.0–1.3
Ovary	0.108–0.162 × 0.054–0.100	0.134 × 0.076	0.108–0.158 × 0.048–0.080	0.134 × 0.063	0.044–0.170 × 0.028–0.109
Metraterm	0.077–0.100 × 0.020–0.035	0.086 × 0.030	–	–	–
Testis	0.146–0.158 × 0.104–0.154	0.153 × 0.126	0.110–0.210 × 0.092–0.148	0.167 × 0.115	0.123–0.233 × 0.047–0.119
Cirrus-sac	0.135–0.250 × 0.039–0.058	0.214 × 0.050	0.100–0.166 × 0.034–0.048	0.135 × 0.040	0.180–0.393 × 0.037–0.068
Terminal organ	0.077–0.100 × 0.020–0.035	0.086 × 0.030	0.038–0.052 × 0.020–0.032	0.045 × 0.024	0.088–0.135 × 0.037–0.072
Vitelline fields	0.062–0.123 × 0.046–0.073	0.097 × 0.061	0.048–0.108 × 0.032–0.070	0.080 × 0.050	–
From posterior end of body to testis	0.085–0.212	0.156	0.106–0.244	0.173	–
From posterior end of body to vitellarium	0.189–0.270	0.211	–	–	–
Eggs	0.018–0.020 × 0.009–0.011		0.018–0.022 × 0.008–0.010	0.019 × 0.009	0.016–0.026 × 0.009–0.012[a] 0.021–0.030 × 0.009–0.013[b]

* Probably, Liu (2002) made a mistake. Length of anterior end of the body, described by him, is not identical to the percentage of body length. According to Liu, the anterior end of the body is 28–33% (mean 31%) of the body length, which corresponds to a mean length of 0.189.
[a]Mounted specimens; [b]living ones.

Table 3. Genetic differentiation (%) of species of Monorchioidea by 18S rRNA gene sequences.

		1	2	3	4	5	6
1	*Lasiotocus lizae*						
2	*L. typicum*	8.05					
3	*Diplomonorchis leiostomi*	4.89	6.77				
4	*Provetellus turrum*	5.49	7.22	4.59			
5	*Pleorchis polyorchis*	6.09	8.57	5.56	6.54		
6	*Lissorchis kritskyi*	6.99	9.02	6.09	6.99	3.83	

of *L. lizae* are three-lobed and longitudinally extended, similar to the ovaries in the type species of *Paraproctotrema*, *P. fusiforme* Yamaguti, 1934. The presence of significant morphological differences between *L. glebulentus* and *L. lizae* and the type species of *Lasiotocus*, *L. mulli*, and the similarity of these species with other species of this genus by a number of diagnostic criteria suggest that *L. glebulentus* and *L. lizae* may belong to the genus of Monorchiinae Odhner, 1911. Accordingly, the molecular data demonstrate that *L. lizae* is highly differentiated from other Monorchiidae (tables 3 and 4). Genetic differentiation by 18S rDNA analyses of *L. lizae* with members of Monorchiidae and Lissorchiidae ranged from 4.9% (*Diplomonorchis leiostomi*) to 8% (*Lasiotocus typicum*). The Bayesian algorithm of phylogenetic reconstructions based on 18S rRNA gene sequences showed unresolved topology for Monorchiidae, and put *L. lizae* and *Provitelus turrum* (Monorchiidae) into the same cluster with high statistical support (fig. 2a). Analysis of genetic distances, calculated for 28S rDNA sequence data, showed that *L. lizae* has the same level of genetic differentiation (9.42–9.59%) as *Monorchis monorchis*, *P. turrum* and *Lasiotocus arrhichostoma* (table 4). However, Bayesian analysis of the phylogenetic reconstruction showed that *L. lizae* was closely related to *M. monorchis* (fig. 2b), and *L. arrhichostoma* appears as a sister species to the cluster *L. lizae*/*M. monorchis*.

In spite of the non-compliance of morphological characteristics of *L. glebulentus* and *L. lizae* to diagnostic criteria for *Lasiotocus*, we consider the data inappropriate to establish a new genus or include these two species into any existing genera of Monorchiinae. Our data support the point of Searle *et al*. (2014) about the necessity for revision of the genus *Lasiotocus* and the subfamily Monorchiinae, using molecular data for all type species.

Table 4. Genetic differentiation (%) of species of Monorchioidea by 28S rRNA gene sequences.

		1	2	3	4	5	6	7	8	9	10
1	*Lasiotocus lizae*										
2	*L. arrhichostoma*	9.59									
3	*Monorchis monorchis*	9.42	10.5								
4	*Proctotrema addisoni*	11.2	9.32	11.6							
5	*Provitellus turrum*	9.49	8.13	9.81	7.53						
6	*Lasiotocus typicum*	12.8	12.6	14.8	14.1	11.4					
7	*Diplomonorchis leiostomi*	10.3	9.02	10.6	9.12	7.63	11.7				
8	*Helicometroides longicollis*	11	10.2	13.1	11.4	9.91	14	10.6			
9	*Asymphylodora perccotti*	15	13.1	15.6	13	12	15.5	12.6	10.7		
10	*Lissorchis kritskyi*	13.1	12.3	14.1	11.7	10.1	14.2	11	8.82	9.02	

Paucivitellosus vietnamensis *sp. n.*

Taxonomic summary

Type host. *Liza subviridis*.

Site. Intestine.

Intensity of infection. Ten fish examined, one infected with 14 worms.

Type locality. Coastal waters of Cat Ba Island, Tonkin Bay, Vietnam (20°84′N, 106°59′E).

Type deposited. Holotype (no. 60-Tr.) and paratypes (nos 61–64-Tr.) were placed in the collection of the Zoological

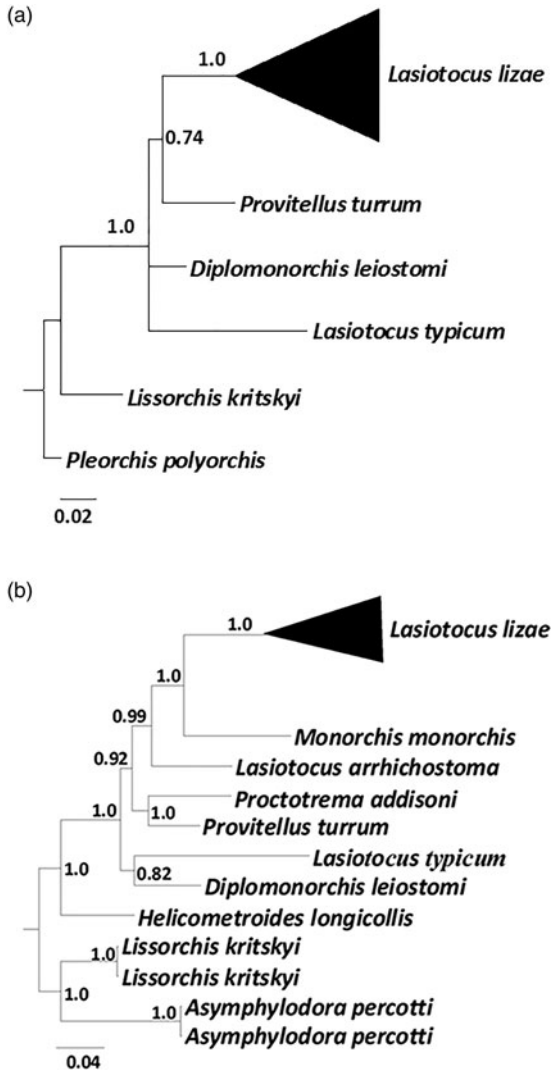

Fig. 2. Bayesian phylogenetic tree of species of Monorchioidea based on: (a) partial 18S rRNA gene sequences; (b) partial 28S rRNA gene sequences.

Museum (Institute of Biology and Soil Sciences, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia); E-mail: petrova@ibss.dvo.ru. Deposited 25 September 2015.

Etymology: The specific name refers to Vietnam, the type locality.

Description

Based on six specimens (fig. 1c and d; table 5). Body trapezoidal, spined. Two eyespots in first third of body, on level of oesophagus. Oral sucker absent, pharynx opened in short oesophagus, oesophageal bifurcation before cirrus-sac. Caeca sac-shaped, short, terminated level of posterior margin of cirrus-sac or anterior margin of testis. Ventral sucker absent, testis single, round or cross-

Table 5. A comparison of the morphometrics (in mm) of adult *Paucivitellosus vietnamensis* sp. n. with *P. fragilis*.

Features	*Paucivitellosus vietnamensis* sp. n. (present study)			*P. fragilis*						*P. hanumanthai* (Mani, 1989)	
				(Coil *et al.*, 1965)		(Pearson, 1968)		(Peng *et al.*, 2004)			
	Holotype	Range	Mean	Range		Range	Mean	Range	Mean	Range	Mean
Body length	0.554	0.508–0.647	0.598	0.390–0.540		0.390–0.450	0.420	0.560–0.820	0.726	0.480–0.592	0.512
Body width	0.447	0.385–0.523	0.452	0.150–0.360		0.230–0.290	0.270	0.534–0.712	0.620	0.320–0.384	0.338
Ratio length/ width of body	1.24	1:1.06–1.68	1.32	–		–	–	–	–	–	–
Pharynx length	0.046	0.046–0.054	0.051	0.036–0.050		0.033–0.041	0.038	0.052–0.070	0.061	0.040–0.052	0.046
Pharynx width	0.058	0.058–0.069	0.062	0.042–0.054		0.050–0.058	0.054	0.058–0.084	0.070	0.060–0.064	0.058
Oesophagus length	0.065	0.065–0.119	0.096	0.020–0.070		0.035–0.054	0.045	0.072–0.160	0.105	–	–
Cirrus-sac length	0.135	0.11–0.177	0.139	–		0.076–0.093	0.084	0.100–0.156	0.135	0.080–0.108	0.095
Cirrus-sac width	0.119	0.077–0.135	0.118	–		0.058–0.076	0.065	0.100–0.166	0.140	0.100–0.112	0.092
Testis length	0.154	0.135–0.166	0.153	0.080–0.160		0.052–0.100	0.077	0.122–0.242	0.194	0.120–0.188	0.140
Testis width	0.185	0.135–0.193	0.163	0.072–0.120		0.045–0.063	0.054	0.062–0.138	0.097	0.096–0.120	0.103
Ovary length	0.073	0.058–0.080	0.068	0.030–0.064		0.034–0.061	0.044	0.054–0.088	0.070	0.052–0.076	0.063
Ovary width	0.077	0.062–0.089	0.075	0.032–0.064		0.032–0.047	0.038	0.038–0.054	0.047	0.036	0.036
Eggs, length	–	0.054–0.065	–	0.046–0.052		0.043–0.052	0.047	0.056–0.078	0.063	0.056–0.068	0.061
Eggs, width	–	0.027–0.035	–	0.024–0.028		0.022–0.026	0.025	0.030–0.038	0.032	0.024–0.032	0.027

oval, in posterior half of body. Two seminal ducts, which merge before cirrus-sac, moving away from testis. Cirrus-sac large, round with oval seminal vesicle. Duct of seminal vesicle thin-walled, often with sperm, opens in C-shaped pars prostatica in middle part. Pars prostatica opens in genital atrium by thick-walled pore. Prostatic cells numerous. Ovary round, to the right of median line between cirrus-sac and testis. Uterine loops lie from posterior margin on both sides of testis to cirrus-sac. Genital pore opens in genital atrium on median line of body. Vitellarium two- to four-lobed, in two lateral fields, placed on both sides of median line from cirrus-sac to ovary. Eggs oval, operculated, with miracidiae. Miracidium with eyespot. V-shaped excretory bladder, branches reach level of oesophagus.

Molecular data

Two sequences of the 18S rRNA gene fragment (268 bp) were successfully sequenced and aligned for *P. vietnamensis* sp. n. from *L. subviridis* from Vietnam. These two sequences were identical to each other.

Two 1081-bp sequences of the 28S rRNA gene fragment were obtained for *P. vietnamensis* sp. n. from *L. subviridis* from Vietnam. These sequences were also identical to each other.

Trematodes identified as *Paucivitellosus* sp. 1 and *Paucivitellosus* sp. 2 from *L. engeli* and *V. seheli*, respectively, have no intraspecific rRNA gene sequence variations.

Remarks

According to WoRMS Editorial Board (2014), this genus includes two species: *P. fragilis* Coil, Reid & Kuntz, 1965 and *P. hanumanthai* (Mani, 1989). Adult *P. vietnamensis* sp. n. from *L. subviridis* meets all morphological criteria (Cribb, 2002) of the genus *Paucivitellosus*. On the other hand, *P. vietnamensis* sp. n. differs from *P. fragilis* and *P. hanumanthai* by a number of metric features (Coil *et al*., 1965; Pearson, 1968; Mani, 1989). In particular, *P. vietnamensis* sp. n. is characterized by larger body size, oesophagus, cirrus-sac and eggs in comparison with *P. fragilis* (table 5). The newly described species is closer to *P. hanumanthai* by most metric characteristics, with the exception of sizes of body width, testis, ovary and cirrus-sac length (table 5).

Cribb *et al*. (1994) reported that *Paucivitellosus* includes the single type-species, *P. fragilis*. On the basis of analysis of numerous specimens of *Paucivitellosus* from various Australian, Indian and Chinese Mugilidae and Blenniidae, Cribb *et al*. (1994) have come to the conclusion that *P. hanumanthai* is a synonym of *P. fragilis*. They reported that high variation in length (from 0.272 to 0.812 mm) and width (from 0.216 to 0.525 mm) of the body is typical for *P. fragilis*. However, cercariae of *P. hanumanthai* have sizes of 0.272–0.304 × 0.196–0.256 mm (Mani, 1989). On the contrary, sizes of cercariae of *P. fragilis* are of size 0.150–0.170 × 0.110–0.130 mm (Pearson, 1968). According to Mani (1989), it is unlikely that adult worms from naturally infected hosts (Cribb *et al*., 1994) have smaller body sizes than cercariae. Mani (1989) also established that specimens of mature *P. hanumanthai* at 10 days of age have a minimum body length and width of 0.480 and 0.320 mm, respectively. Usually, small worms that can be attributed to specimens of the genus *Paucivitellosus* increase in size insignificantly after puberty. Based on the data of Crib *et al*. (1994), we can note that specimens of *P. fragilis* almost tripled their body lengths after puberty. The largest specimens, identified as *P. fragilis*, were detected in *L. carinata* of the Taiwan Strait (Peng *et al*., 2004) (table 5). These specimens are characterized by higher values of morphometric parameters in comparison with specimens of *P. fragilis* and *P. hanumanthai* reported by Coil *et al*. (1965), Pearson (1968) and Mani (1989), respectively. Given that specimens of various species can be barely discernible morphologically, metric criteria, together with molecular data, can determine the main features for species differentiation.

The results of genetic investigation showed that specimens of *P. vietnamensis* sp. n. from *L. subviridis*, and *Paucivitellosus* sp. 1 and *Paucivitellosus* sp. 2 from *L. engeli* and *V. seheli*, are highly differentiated from specimens of *P. fragilis* from the Australian *Crenimugil crenilabis* (Cribb *et al*., 2001). Specimens of *Paucivitellosus* spp. were genetically distant from Australian *P. fragilis* by 4.17–4.55%, by analysis of 18S rRNA gene sequences (table 6). Our study also showed that genetic differentiation of *P. vietnamensis*

Table 6. Genetic differentiation (%) of species of Bivesiculidae by 18S rRNA gene sequences.

		1	2	3	4	5	6	7
1	*Paucivitellosus vietnamensis* sp. n.							
2	*Paucivitellosus* sp. 1	0.38						
3	*Paucivitellosus* sp. 2	0.00	0.38					
4	*P. fragilis* (*Crenimugil crenilabis*)	4.17	4.55	4.17				
5	*Bivesicula claviformis*	8.33	8.71	8.33	4.17			
6	*Bivesicula fusiformis*	7.95	8.33	7.95	3.79	2.27		
7	*Bivesicula unexpecta*	7.58	7.95	7.58	3.41	1.89	0.38	

Table 7. Genetic differentiation (%) of species of Bivesiculidae by 28S rRNA gene sequences.

		1	2	3	4	5	6
1	*Paucivitellosus vietnamensis* sp. n.						
2	*Paucivitellosus* sp. 1	1.58					
3	*Paucivitellosus* sp. 2	0.62	0.95				
4	*Bivesicula claviformis*	11.24	11.81	10.86			
5	*Bivesicula fusiformis*	14.68	15.39	14.44	10.98		
6	*Bivesicula unexpecta*	7.42	8.00	7.04	7.04	11.22	

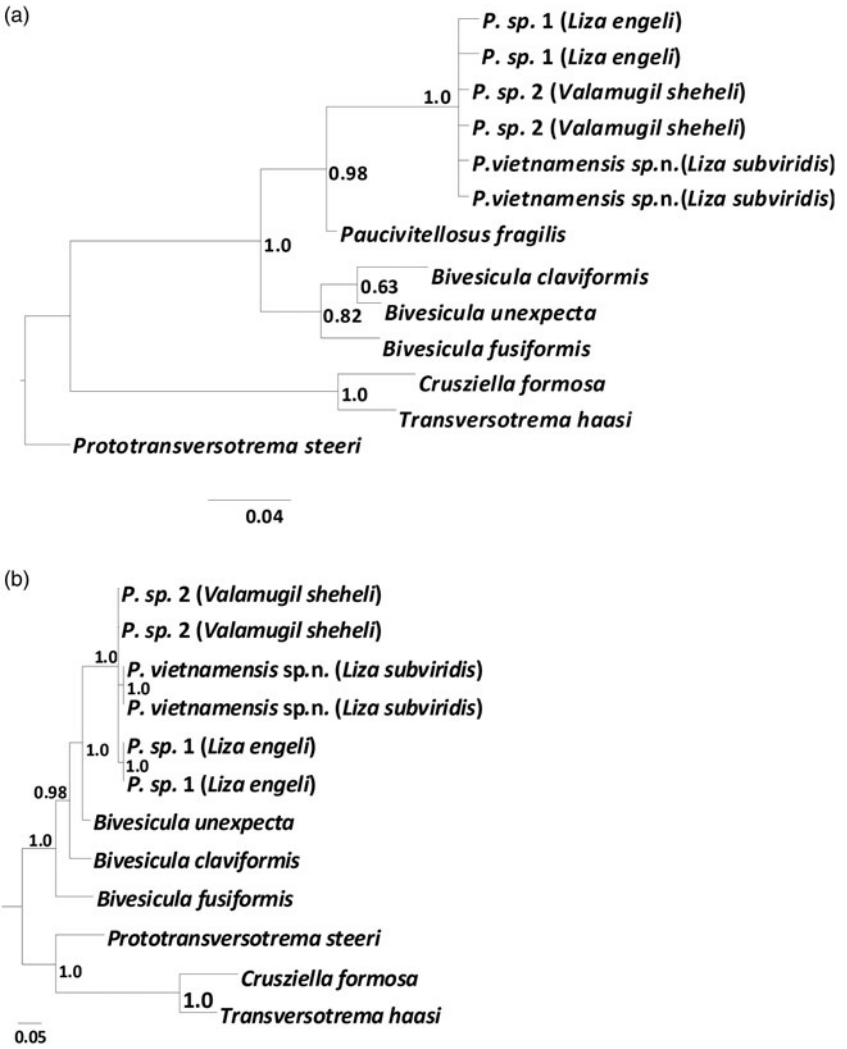

Fig. 3. Bayesian phylogenetic tree of Bivesiculidae based on: (a) partial 18S rRNA gene sequences; (b) partial 28S rRNA gene sequences.

sp. n. from *Paucivitellosus* sp. 1 from *L. engeli* was 0.38% by 18S rRNA gene sequences, and 1.58% by 28S rRNA gene sequences (tables 6 and 7). Nucleotide sequences of 18S rRNA of *P. vietnamensis* sp. n. and *Paucivitellosus* sp. 2 were identical. These worms differ from each other by a 28S rRNA gene fragment (0.62%). Moreover, specimens of *Paucivitellosus* from *L. engeli* and *V. seheli* show a slight genetic differentiation by analysis of the 268-bp 18S rRNA gene fragment (0.38%) and 1081-bp 28S rRNA gene fragment (0.95%) (tables 6 and 7). Bayesian phylogenetic analysis also showed high differentiation between trematode specimens of the genus *Paucivitellosus* from Vietnam and *P. fragilis* from *C. crenilabis* (fig. 3a) and indicated genetic heterogeneity of specimens of this genus from different mugilid species from Vietnam (fig. 3b). These observations do not preclude *P. fragilis* sensu Cribb *et al.* (1994) from representing a set of cryptic species.

On the basis of the observed genetic differences between *Paucivitellosus* specimens from different Vietnamese species of mullet fish and the absence of morphological data of trematodes from *L. engeli* and *V. seheli*, we recognize a new trematode species, *P. vietnamensis* sp. n., from *L. subviridis*.

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Conflict of interest

None.

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