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RESEARCH PAPER

First Molecular Data for Trematode *Bianium tonkinensis* Nguyen, Nguyen, Ha, Ermolenko, 2017 (Digenea: Lepocreadiidae) from Vietnam and the Problem of Paraphyly of the Genus *Bianium*

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Abstract

Adult worms of the genus *Bianium* (Lepocreadiidae) were collected from the intestines of fish *Lagocephalus lunaris* from the coastal waters of Cat Ba Island, Vietnam. Species identification and validation of these trematodes based on morphological and molecular datasets with the analysis of phylogenetic relationships within the Lepocreadiidae were carried out. The morphological characteristics of the worms from our study agree with the diagnosis of the genus *Bianium* and most similar morphologically to *B. tonkinensis* from Vietnam based on comparative analysis of our material with type material, published earlier. Based on molecular data (ITS2 and 28S rDNA), trematode from our material is close to several lepocreadiid species, namely *B. arabicum,* and *Diploproctodaeum monstrorum* from Australia by p-distance values at the interspecific level. Results of phylogenetic analysis show that the genus *Bianium* is paraphyletic, confirming results of earlier studies. Trematodes from our study belong to the species *Bianium tonkinensis*. The taxonomical status of *B. arabicum* from New Caledonia is still questionable. We support the statement that the Australian trematode, misidentified earlier as *B. plicitum*, is unknown species, which presumably belong to the genus *Bianium*.

Introduction

The large family Lepocreadiidae Odhner, 1905 (Platyhelminthes: Digenea) contains intestine parasites of marine teleosts, including deep-sea species that inhabit tropical to subtropical waters (Bray, 2005; Bray et al., 2009). The genus *Bianium* Stunkard, 1930, is one of the problematic taxa within this family. A number of species within this genus increase each time after recurrent taxonomical revisions (Yamaguti, 1971; Bray & Cribb, 1998; Bray, 2005; Bray & Cribb, 2012). At the present time, nine species of the genus *Bianium* are recognised, namely *B. arabicum* Sey, 1996, *B. hemistoma* (Ozaki, 1928) Yamaguti, 1934, *B. indicum* (Gupta, 1967) Bray, Cribb & Baker, 1996, *B. isostoma* Liu, 1997, *B. lianyungangense* Shen, 1990, *B. plicitum* (Linton, 1928) Stunkard, 1931, *B. purii* Gupta, 1968, *B. rewa* Bray, Cribb & Baker, 1996, and *B. tonkinensis* Nguyen, Nguyen, Ha & Ermolenko, 2017. (Stunkard, 1931; Yamaguti, 1934; Gupta, 1968; Shen, 1990; Bray et al., 1996; Sey, 1996; Liu, 1997; Nguyen et al., 2017). Dronen et al. (2016) established the new lepocreadiid genus *Pelopscreadium* Dronen, Blend, Khalifa, Mohamadain & Karer, 2016 with the type species earlier

known as *Bianium spongiosum* Bray, Cribb, 1998. Following that, a new species of the genus *Bianium*, *B. tonkinensis* collected from the lunartial puffer, *Lagocephalus lunaris* (Bloch & Schneider, 1801) from the Gulf of Tonkin, Vietnam, was described morphologically (Nguyen et al., 2017). The first molecular data on *Bianium* species was generated by Bray et al. (2009) for species *B. spongiosum*. Later, this species was transferred to the new genus *Pelopscreadium* based on a combination of the three main morphological features, which distinguishes members of this genus from all the other *Bianium* species assigned to the genus at the time (Dronen et al., 2016). Based on molecular data, Bray et al. (2018a) carried out the study of *Bianium* species, including *Bianium arabicum* Sey, 1996, from non-type location and host. Additionally, in this study, membership of trematode specimens that thought to be members of *Bianium plicitum* (Linton, 1928) Stunkard, 1931 wasn't confirmed. Later, Curran et al. (2021) provided new molecular data on the specimens of *Bianium cf. plicitum* from the Gulf of Mexico. Results of phylogenetic analysis in the studies of Bray et al. (2018a) and Curran et al. (2021) show that the genus *Bianium* is paraphyletic.

During parasitological studies of the Vietnamese fish *Lagocephalus lunaris* from the coastal water of Cat Ba Island, we found intestinal trematodes, which were similar to the Lepocreadiidae morphological diagnosis (Bray, 2005). Thus, the main goal of the present study was species identification and validation of these trematodes based on morphological and molecular datasets with the analysis of phylogenetic relationships within the Lepocreadiidae.

Material and Methods

Collection of Trematodes and Morphological Analysis

Adult worms, *Bianium* (Lepocreadiidae), were collected from the intestines of fish *Lagocephalus lunaris* from the coastal waters of the Cat Ba Island, Vietnam. Three and eight trematodes were detected in two of six dissected fish specimens. All detected worms were firstly identified under a microscope. The worms were separately rinsed in 3% saline, killed in hot distilled water (95°C), and preserved in 70% ethanol. After fixation, they were replaced with 96% ethanol. Whole-mounts were made by staining specimens with alum carmine, dehydrating the worms in a graded ethanol series, and clearing in clove oil. The clove oil treatment was followed by mounting the specimens in Canada balsam under a coverslip on a glass slide. Alongside this, additional studies of slides of type specimens of *Bianium tonkinensis* (Holotype: VNMN 2013; paratypes: VNMN 2014-2020) from the museum of the Institute of Ecology and Biological Resources, VAST, were performed. All measurements are given in micrometers.

DNA Extraction, Amplification and Sequencing

One specimen of all detected worms was fixed in 96% ethanol and used for the molecular analysis. Total DNA was extracted from flukes using a hot shot technique (Truett, 2006). The polymerase chain reaction (PCR) amplification volume amounted to 25 µl containing 12.5 µl GoTaq® Green Master Mix, 1.25 µl of 10 pm/µl each primer, 5 µl DNA template, and 5 µl sterile deionized water.

28S ribosomal DNA (rDNA) was amplified with the primers 28SA (5'-TCG ATT CGA GCG TGA WTA CCC GC-3') (Matejusova & Cunningham, 2004) and 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') (Tkach et al., 2003) with an annealing temperature of 55 °C. Products were sequenced using the internal primers 300F (5'-CAA GTA CCG TGA GGG AAA GTT G-3'), ECD2 (5'-CTT GGT CCG TGT TTC AAG ACG GG-3'), 1200R (5'-GGG CAT CAC AGA CCT G-3') and 900F (5'-CCG TCT TGA AAC ACG GAC CAA G-3') from Tkach et al. (2003).

The ribosomal ITS2 fragment was amplified with the primers BD1 (5'-GTC GTA ACA AGG TTT CCG TA-3') and BD2 (5'-TAT GCT TAA ATT CAG CGG GT-3') (Luton et al., 1992) with an annealing temperature of 54 °C with following sequencing with internal primer 3S (5'-GGT ACC GGT GGA TCA CGT GGC TAG TG-3') and the PCR primer BD2 (Luton et al., 1992).

Negative and positive controls of PCR using both primer pairs were included. Polymerase chain reaction (PCR) products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following the manufacturer's recommendations with the internal sequencing primers described by Tkach et al. (2003) for 28S rDNA. PCR product sequences were analysed using an ABI 3500 genetic analyzer at the Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS. The sequences were submitted to the GenBank database (NCBI) (Table 1).

Alignments and Phylogenetic Analyses

Ribosomal DNA sequences were assembled with SeqScapev.2.6 software provided by Applied Biosystems. Alignment, estimations of the number of variable sites and p-distance calculation were performed using MEGA 7.1 software (Kumar et al., 2016). The values of genetic p-distances were calculated for both the 28S rDNA and the ITS2 rDNA fragments.

Phylogenetic analyses of the Lepocreadioidea was performed using the Maximum Likelihood (ML) and Bayesian Inferrence (BI) algorithm based on the 28S rDNA sequence dataset 1334 bp in length. The best-fit model of sequence evolution for the phylogenetic analysis was estimated based on Akaike's information criterion AIC (Akaike, 1974) using jModeltest version 2.1.5 software (Darriba et al., 2012). The model of best fit was GTR + I + G (Posada, 2003) for both ML and BI algorithms. The ML and Bayesian phylogenetic analyses

Table 1. List of taxa, incorporated into phylogenetic analysis

Trematode species	Host	Location	NCBI accession numbers		Reference
			28S rDNA	ITS2 rDNA	
Lepocreadioidea					
Lepocreadiidae					
Bianium tonkinensis	***Lagocephalus lunaris***	**Vietnam, Tonkin Bay**	**PP941785**	**PP941784**	**This study**
Bianium arabicum	*Lagocephalus lunaris*	New Caledonia	MH157076	MH157054	Bray et al., 2018a
Bianium sp.	*Torquigener pleurogramma*	Australia, Moreton Bay	MH157066	MH157055	Bray et al. 2018a
Bianium cf. plicitum	*Sphoeroides testudineus*	USA, Florida	MZ345681-82	MZ345681	Curran et al., 2021
Diploproctodaeum monstrosum	*Arothron stellatus*	Australia: Lizard Island, Great Barrier Reef	FJ788473	—	Bray et al., 2009
Diploproctodaeum cf. monstrosum	*Arothron hispidus*	Australia, Moreton Bay	MH157069	MH157059	Bray et al., 2018a
Diploproctodaeum momoaafata	*Ostracion cubicus*	Australia: Heron Island, Great Barrier Reef	FJ788474	—	Bray et al., 2009
Diplocreadium tsontso	*Balistoides conspicillum*	Australia: Heron Island, Great Barrier Reef	FJ788472	—	Bray et al., 2009
Echeneidocoelium indicum	*Echeneis naucrates*	Australia: Swain Reefs, Great Barrier Reef	FJ788475	—	Bray et al., 2009
Hypocreadium toombo	*Pseudobalistes fuscus*	New Caledonia	FJ788480	—	Bray et al., 2009
Hypocreadium cf. *patellare*	*Balistoides viridescens*	Australia: Lizard Island, Great Barrier Reef	FJ788478	—	Bray et al., 2009
Lepocreadium oyabitcha	*Abudefduf sordidus*	Australia	OM777006	OM777008	Duong et al., 2022
Lepotrema amblyglyphidodonis	*Amphiprion akindynos*	Australia: Heron Island, Queensland	MH730017	MH730002	Bray et al., 2018b
Lobatocreadium exiguum	*Pseudobalistes fuscus*	New Caledonia	FJ788484	—	Bray et al., 2009
Mobahincia teirae	*Mobahincia teirae*	Australia: Moreton Bay	MH157068	—	Bray et al., 2018a
Multitestis magnacetabulum	*Platax teira*	Australia: Heron Island, Great Barrier Reef	FJ788485	—	Bray et al., 2009
Neohypocreadium dorsoporum	*Chaetodon flavirostris*	Australia: Heron Island, Great Barrier Reef	FJ788487	—	Bray et al., 2009
Neomultitestis aspidogastriformis	*Platax teira*	Australia: Heron Island, Great Barrier Reef	FJ788489	—	Bray et al., 2009
Pelopscreadium spongiosum	*Ostracion cubicus*	Australia: Lizard Island, Great Barrier Reef	FJ788469	—	Bray et al., 2009
Aephnidiogenidae					
Austroholorchis sprenti	*Sillago ciliata*	Australia: Moreton Bay	MH157075	MH157065	Bray et al., 2009
Gorgocephalidae					
Gorgocephalus yaaji	*Kyphosus cinerascens*	South Africa: Sodawana Bay	MW353896	—	Huston et al., 2021

were performed using the MEGA 7.1 and MrBayes v.3.2.6 software (Ronquist et al., 2012), respectively. Bayesian analysis was used with the following set of parameters of the likelihood model: nst = 6, rates = invgamma. The main priors, including frequencies of nucleotide substitutions, nucleotide composition, gamma shape and amount of invariant sites were fixed. The Markov Chain Monte Carlo (MCMC) algorithm was performed with the following parameters: ngen=10000000 via two independent runs (nruns=2), four simultaneous Markov chains (nchains=4) with every 100th tree saved (samplefreq=100) and the standard deviation of split frequencies at 0.0099. Summary parameters and the phylogenetic tree were calculated with a burn-in of 25% of generations. Nodal supports were estimated as bootstrap values generated with 1000 replications (Felsenstein, 1985) in the ML and as posterior probabilities in the BI analyses (Huelsenbeck et al., 2001). Accession numbers, authority, and supporting information about rDNA sequences from GenBank used for the phylogenetic analyses are provided in Table 1.

Results

Description

Bianium tonkinensis Nguyen, Nguyen, Ha & Ermolenko, 2017

Host: Lagocephalus lunaris (Bloch & Schneider)

Site: Intestine

Locality: Gulf of Tonkin, Cat Ba Island shell waters,Vietnam, 20°43'59.3"N 107°00'29.9"E.

Intensity of infection: 3 and 8 worms.

Materials deposited. Materials № 243-250 Tr are deposited in the parasitological collection of the Zoological Museum (deposited 20 November 2023, Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia; e-mail: petrova@biosoil.ru).

Adult worm (material examined: 8 specimens) (Figure 1, Table 2)

Body elongate with slightly tapered anterior end. Tegument with spines. Pre-oral lobe with median V-shaped deflection. Large internal patches of large cells forming sponge-like pads laterally in forebody between anterior edge of oral sucker and about level of genital pore or anterior end of ventral sucker. Along lateral sides of body longitudinal folds present, bending ventrally and reach middle of post-testicular field. Sponge-like pads and longitudinal folds forming incomplete of scoop. Oral sucker subterminal, round. Ventral sucker round, slightly smaller them oral sucker, in middle of anterior half of body. Prepharynx absent. Pharynx round or transversely oval, with papillae along anterior ventral rim. Oesophagus short, bifurcating anteriorly to ventral sucker at level midway between pharynx and ventral sucker. Caeca thick, opening outside through separate ani one on each side of excretory pore. Testes two, tandem or slightly oblique, longitudinally oval, in middle of posterior half body. External seminal vesicle saccular, reaching the level of ovary. Cirrus-sac claviform, does not reach ovary slightly. Internal seminal vesicle oval. Pars prostatica oval, vesicular, lined with anuclear cell-like bodies. Ejaculatory duct long, muscular is eversible. Genital atrium distinct. Genital pore just post-bifurcal, sinistrally

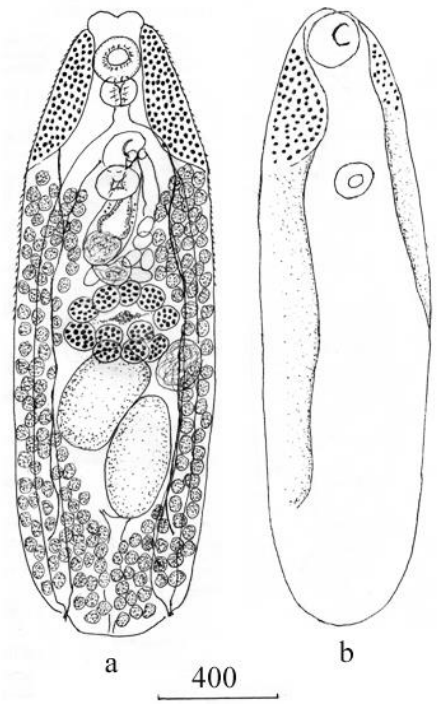

Figure 1. *Bianium tonkinensis* Nguyen, Nguyen, HA, Ermolenko, 2017 from the present study: a. Ventral view, b. Location sponge-like pads and longitudinal folds (scale-bar: µm).

Table 2. Measurements (µm) of adult worms of *Bianium* species

	Bianium tonkinensis **n = 8**	*Bianium spongiosum* ex *Ostracion cubicus*, Australia (Bray, Cribb, 1998)	*Bianium tonkinensis* ex *Lagocephalus lunaris*, Vietnam (Nguyen et al., 2017)	*Bianium arabicum* ex *Lagocephalus sceleratus*, New Caledonia (Bray et al., 2010)	*Bianium arabicum* ex *Lagocephalus lunaris*, Kuwait Bay (Sey, 1996)
Body length	1001-2174	1352-1412	976-1656	889-1225	650-1025
Body width	293-693	438-489	320-488	312-416	300-380
Bl/Bw (%)*	29.4-34.4	32-35	26-37	29-38	-
Forebody length	254-508	467-487	240-436	280-358	-
Fo/Bl (%)*	24.3-29.7	35	19-28	29-34	-
Pre-oral lobe	39-92	19-32	-	45-74	-
Oral sucker length	92-158	134-148	84-120	106-131	70-104
Oral sucker width	96-158	187-193	96-132	107-133	65-113
Ventral sucker length	85-139	129-135	64-128	82-108	67-90
Ventral sucker width	85-139	136-142	60-112	89-105	65-90
Suckers length ratio	1:0.86-0.93	1:0.73-0.74	1:0.54-1.12	1:0.74-0.94	-
Suckers width ratio	1:0.86-0.96	-	-	1:0.71-0.83	-
Pharynx length	62-119	131-135	56-100	89-126	65-95
Pharynx width	62-131	129	48-104	91-135	65-78
Oesophagus	15-27	64-71	12-80	12-22	-
Ovary length	96-262	122-142	84-196	79-158	80-100
Ovary width	162-308	122-135	144-212	115-182	45-60
Metraterm length	150-204	-	-	-	-
Testis anterior length	127-347	138-155	140-240	86-163	85-104
Testis anterior width	135-250	129-148	112-200	96-143	88-130
Testis posterior length	146-423	155-180	140-268	101-194	104-130
Testis posterior width	127-204	116-155	44-188	76-131	85-130
Cirrus sac length	173-404	276-277	144-284	195-294	120-128
Cirrus sac width	77-135	88-90	66-108	46-121	40-50
Ovary lobes number	9-11	10-16	8-11	-	7-10
Post-testicular field length	135-416	236-245	124-400	162-253	-
Pt/Bl (%)*	12.7-19.1	17-18	13-25	16-23	-
Eggs length	58-62	60-63	48-68	58-65	59-65
Eggs width	39-42	40-45	32-54	27-39	34-36

*Bl – body length, Bw – body width, Pt - Post-testicular field length

submedian. Ovary immediately pre-testicular or slightly overlap anterior testis, middling, consists from separate subglobular follicles. Seminal receptacle oval, sinistral to ovary. Uterus between ovary and ventral sucker. Eggs few. Metraterm muscular sinistrally to cirrus-sac. Vitellarium from numerous follicles from level ventral sucker to posterior end of the body, present laterally, confluent or not in post-testicular region. Excretory vesicle I-shaped, pore terminal.

Molecular Differentiation and Phylogenetic Analysis

Results of phylogenetic analysis based on the 28S rRNA gene partial sequences show that the trematode from our material was within polytomic clade, denoted as "BDDLP" on the ML tree (Figure 2) and the Bayesian tree (Figure 3), which contain trematode species from five genera (species names provided according to respective GenBank submissions). Within this clade there were three lineages: the first one represents with two specimens of *Bianium cf. plicitum* ex *Sphoeroides testudineus* (USA, Florida), the second lineage appears as two species, *Pelopscreadium spongiosum* and *Diploproctodaeum momoaafata.* The third lineage was presented by the polytomic terminal clade, which includes the trematode from our material and representatives of four lepocreadiid genera. Within this terminal clade, the trematode from our study and *Bianium arabicum* (New Caledonia) formed separate lineages, whereas other species, *Bianium* sp. ex *Torquigener pleurogramma* (Australia), *Diploproctodaeum monstrorum, Lobatocreadium exiguum* and *Diplocreadium tonso* formed internal well resolved clade. Genetic distances between these three lineages ranged from 1.0±0.23% (lineages 1/3) to 2.07±0.31% (lineages 2/3). Based on genetic distance values, trematode from our material close to several species, namely *Diploproctodaeum cf. monstrorum*, d = 0.62±0.23%, *Bianium* sp. (MH157066), d = 0.68±0.23%, *Bianium arabicum*, d=0.84±0.26% and *Diploproctodaeum monstrorum*, 0.84±0.26%. Alongside this, two *Diploproctodaeum* specimens differ from each other by 0.4±0.17% (Table 3).

Unfortunately, morphological data for the *Diploproctodaeum cf. monstrosum* unavailable. In the case of unambiguous confirmation of validity of *Diploproctodaeum cf. monstrosum,* the considerable morphological differences in form of the body, scoop structure, length and width ratios between this species as well as *Bianium plicitum* from original description and *B. tonkinensis* can be declared (Table 2). These differences are significant for recognition of these trematodes as different species. Consequently, the p-distance value 0.62 ± 0.23% – 0.68 ± 0.23% based on 28S rRNA gene sequence data between *B. tonkinensis* and these species can be considered as interspecific for trematodes from the clade.

Based on ITS2 sequence data, the minimal p-distance values were between *Bianium tonkinensis* and *Diploproctodaeum cf. monstrorum*, d=2.25±0.7%. The last specimen was closer to Australian *B. arabicum* (d=1.56±0.59%) and *Bianium* sp. (d=1.78±0.6%), whereas p-distance values between these two species and *B. tonkinensis* ranged from 2.47±0.74% to 2.7±0.75% (Table 4).

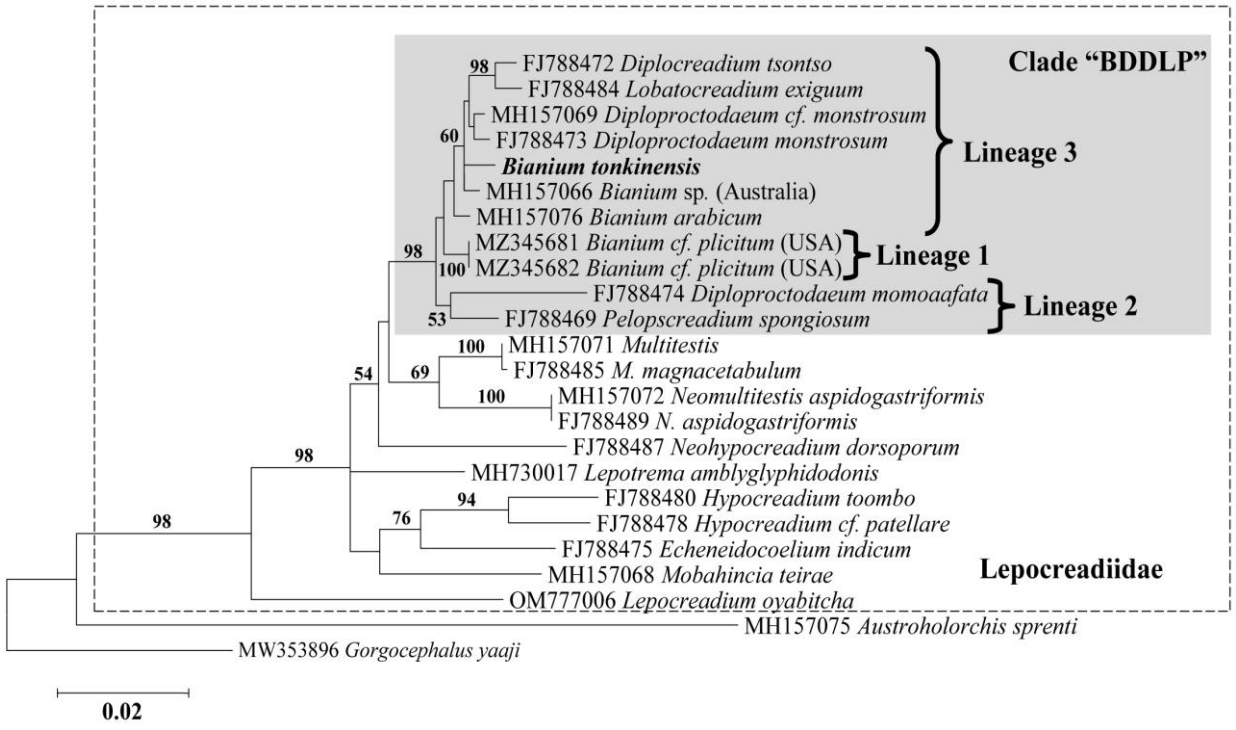

Figure 2. Phylogenetic relationships reconstruction of the Lepocreadiidae, generated with the maximum likelihood (ML) algorithm based on partial 28S rRNA gene sequence dataset. Nodal numbers – bootstrap values that indicate statistical support of phylogenetic relationships; only significant values (50 – 100) are showed.

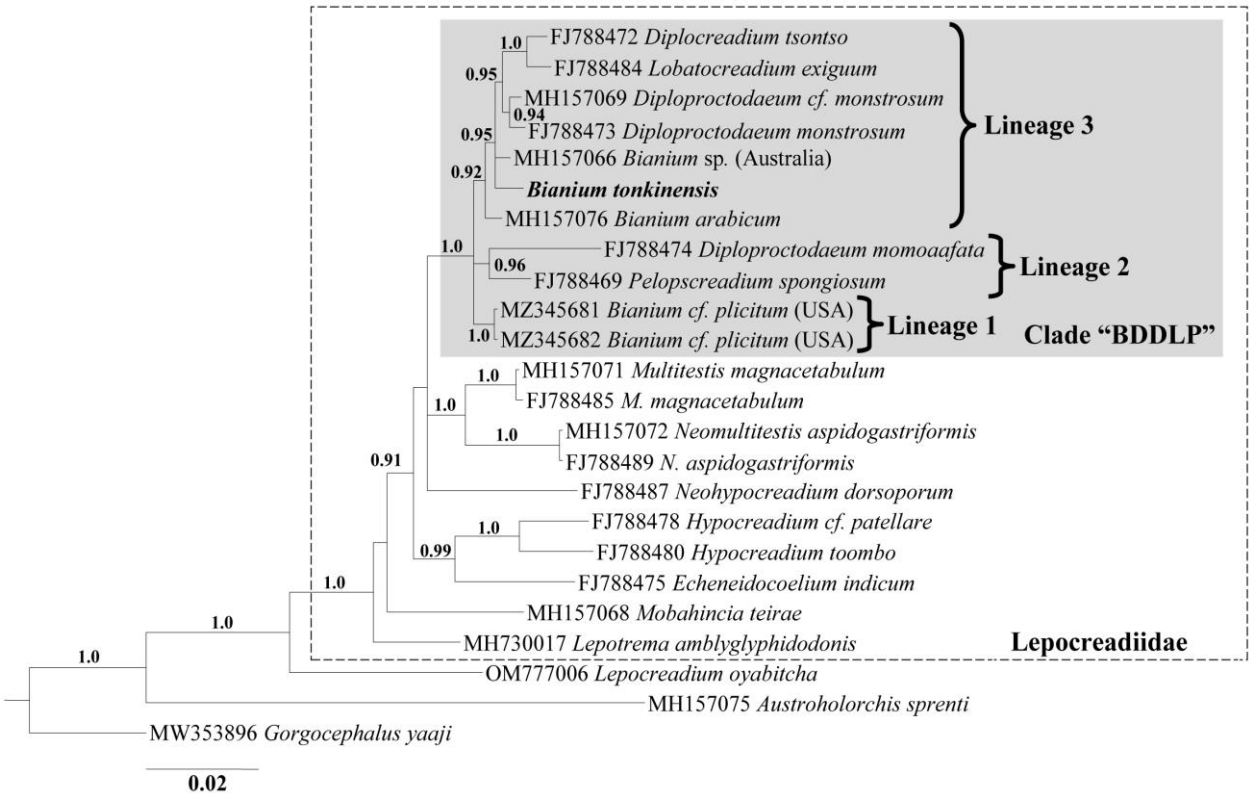

Figure 3. Phylogenetic relationships reconstruction of the Lepocreadiidae, generated with the Bayesian inference (BI) algorithm based on partial 28S rRNA gene sequence dataset. Nodal numbers – a posterior probabilities that indicate statistical support of phylogenetic relationships; only significant values (0.9 – 1.0) are showed.

Table 3. P-distance values (d, %) between nucleotide sequences of trematodes from the Clade I (Figure 2) based on 28S rRNA gene sequence dataset. Values between *Bianium tonkinensis* and other species from the clade are bolded.

		1	2	3	4	5	6	7	8	9	10	11
1	***Bianium tonkinensis***		**0.232**	**0.296**	**0.316**	**0.259**	**0.218**	**0.261**	**0.442**	**0.321**	**0.325**	**0.355**
2	MH157066 *Bianium* sp.	**0.683**		0.247	0.264	0.217	0.185	0.21	0.409	0.273	0.281	0.309
3	MZ345681 *Bianium cf. plicitum*	**1.068**	0.838		0	0.228	0.253	0.258	0.411	0.338	0.313	0.325
4	MZ345682 *Bianium cf. plicitum*	**1.139**	0.894	0		0.243	0.266	0.276	0.439	0.362	0.335	0.337
5	MH157076 *Bianium arabicum*	**0.837**	0.609	0.683	0.728		0.222	0.25	0.407	0.3	0.325	0.327
6	MH157069 *Diploproctodaeum cf. monstrosum*	**0.623**	0.543	0.936	0.985	0.7		0.17	0.41	0.268	0.266	0.327
7	FJ788473 *D. monstrosum*	**0.839**	0.607	0.835	0.891	0.76	0.389		0.412	0.277	0.263	0.326
8	FJ788474 *Diploproctodaeum momoaafata*	**2.659**	2.498	2.493	2.579	2.173	2.48	2.336		0.466	0.463	0.456
9	FJ788472 *Diplocreadium tsontso*	**1.223**	0.99	1.379	1.471	1.146	0.936	0.992	2.906		0.223	0.379
10	FJ788484 *Lobatocreadium exiguum*	**1.302**	1.068	1.301	1.388	1.224	1.016	0.914	2.822	0.682		0.367
11	FJ788469 *Pelopscreadium spongiosum*	**1.616**	1.224	1.379	1.387	1.381	1.494	1.379	2.498	1.772	1.692	

The d values are below diagonal, the standard error values - above diagonal.

Table 4. P-distance values (d, %) between nucleotide sequences of trematodes from the Clade I (Figure 2) based on ITS2 rDNA sequence dataset. Values between new species *Bianium tonkinensis* and other species from the clade are bolded. The d values are below diagonal, the standard error values - above diagonal.

		1	2	3	4	5	6
1	***Bianium tonkinensis***		**0.698**	**0.739**	**0.748**	**0.748**	**0.921**
2	MH157059 *Diploproctodaeum cf. monstrosum*	**2.255**		0.591	0.605	0.605	0.864
3	MH157054 *Bianium arabicum*	**2.472**	1.560		0.601	0.601	0.816
4	MH157056 *Bianium* sp.	**2.703**	1.782	1.569		0.000	0.814
5	MH157055 *Bianium* sp.	**2.703**	1.782	1.569	0.000		0.814
6	MZ345681 *Bianium cf. plicitum*	**3.689**	3.220	2.975	2.968	2.968	

The ML and BI phylogenetic trees based on concatenated ribosomal ITS2 and 28S rDNA sequence dataset showed the same topology (Figure 4). On these trees, *B. tonkinensis* closely related with *Diploproctodaeum cf. monstrorum,* keeping paraphyly of the genus *Bianium*.

Discussion

Species Validation Based on Morphological Data

The worms ex *Lagocephalus lunaris* caught in the Gulf of Tonkin, Vietnam, agree with the diagnosis of the genus *Bianium* by morphological characteristics. Particularly, the trematodes from our study are most similar to *Bianium spongiosum* Bray, Cribb, 1998, from Australia; *Bianium arabicum* from Kuwait and New Caledonia; and *Bianium tonkinensis* from Vietnam; all of these species were detected in teleost fish species of the order Tetraodontiformes (Sey, 1996; Bray, Cribb, 1998; Bray et al., 2010; Nguyen et al., 2017). The key morphological characters for the differentiation of these species are the presence or absence of an incomplete scoop, sponge-like pads with cells, and ani. *Bianium spongiosum* and *B. tonkinensis* possess sponge-like pads with cells, as well as trematodes from our material. However, by other parameters, these two species differ from newly found trematodes: *B. spongiosum* reported no scoop as such, and *B. tonkinensis* denoted a scoop incomplete with lateral flaps in the forebody, and individuals of these two species have no ani (Bray, Cribb, 1998; Nguyen et al., 2017). As for the worm *Bianium arabicum* from Kuwait*,* Sey (1996) found an incomplete scoop with lateral flaps ending at the posterior extremity, ani, and numerous parenchymal gland cells in the anterior part of the body, extending to the ventral sucker. At the same time, for *B. arabicum* specimens from New Caledonia, the only incomplete scoop with an indefinite length was denoted (Bray et al., 2010). Despite the similarity between *B. arabicum* from Kuwait and *B. arabicum* from New Caledonia, Kuwaiti specimens, unlike New Caledonian specimens, possess flaps ending at the posterior extremity, sponge-like pads and ani, as well as smaller testes, ovary, and cirrus sacs (Table 2). These features and the geographical distance of these representatives argue for the validity of *B. arabicum* sensu Sey (1996) from Kuwait. The taxonomical status of *B. arabicum* from New Caledonia is still questionable, as is that of *B. dayawanense* Shen & Tong, 1990, from China, which differs from New Caledonian *B. arabicum* only by large sizes (Shen, Tong, 1990).

Alongside the above-mentioned arguments, for all considered *Bianium* species, there are different testes arrangements: tandem for *B. spongiosum* and *B. arabicum* from Kuwait; tandem or oblique for *B. tonkinensis,* and oblique for *B. arabicum* from New Caledonia.

The results of the morphological analysis of the worms in our study show that in some specimens, an incomplete scoop and folds reaching the middle of the post testicular field, sponge-like pads, and anuses were identified; in some individuals, these structures are poorly visible or not visible. The testes are arranged tandemly in small specimens or obliquely in large ones. Based on the obtained morphological data, the trematodes from our study differ from *B. spongiosum*, *B. arabicum* from New Caledonia, and *B. tonkinensis* and are similar to *B. arabicum* from Kuwait by the following characteristics: incomplete scoops, length folds, sponge-like pads, and ani. However, the trematodes we detected and *B. arabicum* from Kuwait differ from each other by metric parameters, including the sizes of suckers, ovary, testes, and cirrus sac (Table 2). Because trematodes from our material and *B. tonkinensis* were detected in *Lagocephalus lunaris* from the Gulf of Tonkin, Vietnam, we carried out additional morphological studies of slides of type specimens of *B. tonkinensis* from the study of Nguyen et al. (2017). Results of these studies show that type specimens of *B. tonkinensis* possess folds that reach the middle posterior testis and ani that weren't revealed in the original description of this species (Nguyen et al., 2017). Accepting these results and the metric identity of

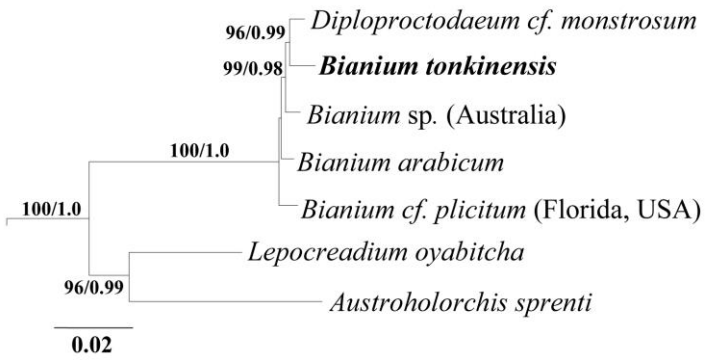

Figure 4. Fragment of the phylogenetic tree of Lepocreadiidae, based on concatenated ITS2 + 28S rDNA sequence dataset. Nodal numbers – bootstrap values / a posterior probabilities that indicate statistical support of phylogenetic relationships for the Maximum Likelihood / the Bayesian algorithms; only significant values (50 – 100 / 0.9 – 1.0) are showed.

trematodes from our material and from Nguyen et al. (2017) ex *Lagocephalus lunaris* from the Gulf of Tonkin, Vietnam (Table 2), we conclude that new trematodes belong to *Bianium tonkinensis*.

In our opinion, the generic membership of *Bianium spongiosum* should be discussed. Dronen et al. (2016) established the new genus *Pelopscreadium* within Lepocreadiidae based on morphological characteristics, and two species were recognised as members of this genus, including specimens denoted as *Bianium spongiosum* in the study of Bray & Cribb (1998); the last was recognised as a type species for the new genus. *Bianium spongiosum* was transferred to the *Pelopscreadium* on the basis of absence of scoop and presence sponge-like pads in the specimens from Bray & Cribb (1998) that are representative for specimens of the new genus. However, sponge-like pads are present in *B. arabicum* from Kuwait; Sey (1996) described this character as "numerous parenchymal gland cells found in anterior part of body, extending to ventral sucker" and presented it at the figure. Dronen et al. (2016) do not consider the data provided by Sey (1996) for the differentiation of *Pelopscreadium* and *Bianium.* Moreover, the presence or absence of a scoop highly depends on the quality of the slides of the worms, and sometimes it can be missed. Bray et al. (2010) assigned for their specimens of *B. arabicum* "…folds of the body (or scoop-sides), the full extent is not always visible on both sides of the worm." Based on all these data, we believe that the inclusion of *B. spongiosum* in the genus *Pelopscreadium* is unreasonable.

Molecular Based Species Delimitation and the Paraphyly of the Genus *Bianium*

The 28S rDNA sequence differentiation of all available *Bianium* species ranged from 0.61 ± 0.26% between *B. tonkinensis* and Australian *B. arabicum* to 1.14±0.32% between *B. tonkinensis* and Australian trematode *Bianium* sp. (Table 3). Specimens denoted as *Bianium cf. plicitum* from the Atlantic Ocean (USA, Florida) from the study of Curran et al. (2021) and *Bianium* sp. from Australia provided in Bray et al. (2018a) differ from each other by 0.84±0.25% – 0.89±0.26%. Bray et al. (2018a) proposed in their study that Australian "*B. plicitum*" from their study is not conspecific to *B. plicitum* from the Atlantic Ocean. Later, Curran et al. (2021) confirmed this proposition based on molecular data on *B. cf. plicitum* from the Atlantic Ocean (USA, Florida), denoting that "Australian specimens are likely undescribed species." The availability of new molecular data on *B. tonkinensis* from the present study and accepting the results of several earlier studies that indicate considerably high phylogenetic closeness of trematode species from the Indo-West Pacific, including Australia, Vietnam, and the Russian Far East (Atopkin et al., 2015; Besprozvannykh et al., 2015; Cribb et al., 2016; Atopkin et al., 2021; Pérez-Ponce de León, 2024) provided a little chance to find out that *B. tonkinensis* and Australian *Bianium* sp. are conspecific. In the 28S rDNA sequences of these two trematodes and *B. cf. plicitum* from Florida (USA), we found eight parsimony informative sites containing fixed substitutions (Table 5). By these sites, *B. tonkinensis* and Australian *Bianium* sp. were identical. However, we also found nine substitutions between the 28S rDNA sequences of these two trematodes (Table 6). Unfortunately, we cannot say whether these substitutions are fixed or not because there is only one sequence for each species available. However, we tend to believe these nine substitutions to be interspecific characters. Additionally, *B. tonkensis* from our study and Australian *Bianium* sp. ex *Torquigener pleurogramma* (Australia) from the study of Bray et al. (2018a) differ by most of the metric parameters. Hafeezullah (1970) provides morphological data for "*Bianium plicitum*" ex *Lagocephalus lunaris,* the same host species as *B. tonkinensis.* Metric parameters of this "*B. plicitum,*" taken from an illustration from Hafeezullah's (1970) paper and provided by Bray et al. (2018a), also considerably differ from those of *B.*

Table 5. Parsimony-informative sites between 28S rDNA (1349 bp) sequences of different *Bianium* species.

Species	Site Nos.							
	221	222	223	431	436	599	766	813
Bianium tonkinensis (Vietnam)	G	C	G	T	G	G	G	T
Bianium sp. (MH157066, Australia)	G	C	G	T	G	G	G	T
Bianium cf. plicitum (MZ345681, Florida, USA)	A	A	T	C	A	A	A	C
Bianium cf. plicitum (MZ345682, Florida, USA)	A	A	T	C	A	A	A	C

Table 6. Variable sites in 28S rDNA sequences (1349 bp) of *B. tonkinensis* and Australian *Bianium* sp.

Species	Site Nos.								
	133	186	233	241	300	689	709	720	1056
Bianium tonkinensis (Vietnam)	G	A	C	G	T	T	C	C	G
Bianium sp. (MH157066, Australia)	A	T	T	A	C	G	T	T	A

tonkinensis. Based on these results, we support the viewpoint of Curran et al. (2021) regarding the taxonomical status of Australian trematodes as an undescribed species, presumably member of the genus *Bianium*.

In terms of molecular phylogenetic data, the genus *Bianium* can be interpreted as a paraphyletic group on the ML tree (Figure 2) and a polyphyletic on the Bayesian tree (Figure 3). All members of this genus involved in the analysis have different close ancestral nodes. Different species of the genus *Bianium* are presented as separate lineages or dispersed within the large polytomic clade and closely related to representatives of other lepocreadiid genera. Unfortunately, the molecular data available at present are insufficient for taxonomical interpretation of the trematodes that gathered within this clade. This clade does not result in a dichotomy, hampering adequate taxonomical interpretation at a specific level. Nevertheless, based on the results of phylogenetic analysis based on a partial 28S rRNA gene sequence dataset, it does not exclude that all trematode species, representing three lineages within a large polytomic clade, can be members of the same genus. However, discrete morphological data for specimens included in molecular analysis hampers our ability to state to what genus these trematodes can be attributed. In our opinion, a more representative species sample is necessary for morphological and phylogenetic analyses to resolve this problem.

Ethical Statement

Sample collection and all experimental procedures were approved by Committee of Bioethics of Federal Scientific Center of Biodiversity FEB RAS

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Author Contribution

First author carried out molecular analyses on the samples, analysed the molecular phylogenetic data and drafted the manuscript. Second author collected the trematodes and write some parts of the paper. Third author collected and dissected fish specimens, provided material for comparative morphological analysis and drafted the manuscript. Fourh author collected and dissected fish specimens, carried out morphological identification of fish specimens. Fifth author carried out morphological analysis of trematodes, prepared a parts of Results and Discussion sections concerned morphological data.

Conflict of Interest

The authors declare no conflict of interest and compliance with all relevant ethical standards. All original molecular data are verified and can be approved with protocols and raw data.

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