Article

Phylogenetic Evidence for the Lissorchiid Concept of the Genus *Anarhichotrema* Shimazu, 1973 (Trematoda, Digenea)

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Abstract: *Anarhichotrema* Shimazu, 1973 is a monotypic digenean genus, with the type- and only species, *Anarhichotrema ochotense* Shimazu, 1973, known to infect North Pacific fishes. This genus was originally described as a member of the Lissorchiidae (Monorchioidea) and later moved to the Zoogonidae (Microphalloidea). Its exact phylogenetic position has remained unresolved due to the lack of molecular data. In this study, we isolated specimens of *A. ochotense* from the Bering wolffish, *Anarhichas orientalis* Pallas, 1814 caught in the Sea of Okhotsk, described them morphologically and performed a molecular phylogenetic analysis of their nuclear 18S and 28S rDNA regions. The specimens examined in our study generally corresponded to previous morphological descriptions of *A. ochotense* but were noticeably smaller, possibly due to the crowding effect. The phylogenetic analysis placed *Anarhichotrema* within the Lissorchiidae as a sister taxon to the group comprising freshwater lissorchiids. Thus, we restore *Anarhichotrema* to the Lissorchiidae, as originally assigned.

Keywords: Sea of Okhotsk; *Anarhichas orientalis*; Lissorchiidae; Monorchioidea

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1. Introduction

Anarhichotrema Shimazu, 1973 is a monotypic digenean genus erected by Shimazu [1] for *A. ochotense* Shimazu, 1973 described from the Bering wolffish *Anarhichas orientalis* Pallas, 1814 (Anarhichadidae) caught in the Sea of Okhotsk. Twelve years later Machida [2] described the genus *Neolissorhis* Machida, 1985 with the type and only species *Neolissorhis genge* Machida, 1985 from the intestine of Zoarcid fish *Lycodes* sp. in the Sea of Japan (East Sea). Machida [2] was apparently unaware of the publication by Shimazu [1] since he did not mention *A. ochotense* in his work. However, the two species had extremely similar morphology. Thereafter, Bray [3] reasonably synonymised *Neolissorhis* with *Anarhichotrema* and *N. genge* with *A. ochotense*. Later Kuramochi [4] found *A. ochotense* in a Zoarcid fish *Petroschmidtia toyamensis* Katayama, 1941 (= *Lycodes toyamensis* (Katayama, 1941)) in the Sea of Japan (East Sea).

Since *Anarhichotrema* is similar to *Lissorhis* Magath, 1917, the type-genus of the Lissorchiidae Magath, 1917, in general morphology, it was originally described as a member of the Lissorchiidae [1], and so was *Neolissorhis* [2]. However, Bray [3,5] moved *Anarhichotrema* into the Zoogonidae based on the understanding that lissorchiids did not typically parasitise marine fish. According to the current zoogonid concept, *Anarhichotrema* is affiliated to the Lecithostaphylinae Odhner, 1911 *sensu lato* [6]. However, the lack of molecular genetic data for this genus significantly complicates evaluation of its phylogenetic position.

In this paper, we redescribe the morphology of *A. ochotense* based on specimens from the type-host and type-locality and test its phylogenetic position using 28S rRNA and concatenated 18S+28S rRNA gene sequences.

2. Materials and Methods

2.1. Sample Collection and Morphological Observation

In total, 375 specimens of *A. ochotense* were found in a single individual of *A. orientalis* (total length 88.2 cm) caught off the western shore of Iturup Island (Sea of Okhotsk, 44°44'59" N 147°8'2" E). For the morphological study 15 specimens were fixed in 70% ethanol without being pressed with a cover glass and stained with acetocarmine. Five of them were dehydrated in 96% ethanol, cleared in dimethyl phthalate, and mounted in Canada balsam in whole view. The terminal part of the reproductive system and the ovarian complex were extracted from the bodies of the remaining ten specimens using needles. These isolated organs were also mounted in Canada balsam with preliminary dehydration and clearing, same as the whole worms. Three specimens for phylogenetic analysis were fixed in 96% ethanol. Paragenophores were deposited in the Museum of Helminthological Collections, Centre for Parasitology, A.N. Severtsov Institute of Ecology and Evolution RAS Moscow, Russia (IPEE RAS).

2.2. DNA Extraction, Amplification and Sequencing, and Phylogenetic Analysis

In order to obtain 18S and 28S rRNA gene sequences, several specimens of *A. ochotense* were separately dehydrated in a dry block heater for 1.5 h at 37 °C, digested with a mixture of 48 µL 0.1% Chelex-100 and 2 µL Proteinase K (concentration 10 mg/mL), and incubated for 2.5 h at 55 °C and 35 min at 95 °C. The partial sequence of 18S rRNA gene was amplified with WormA (5'-GCGAATGGCTCATTAAATCAG-3') and WormB (5'-ACGGAACCTTGTTACGACT-3') primers [7] using the following PCR parameters: initial denaturation at 95 °C (3 min); 35 cycles of 20 s at 95 °C; 20 s at 50.1 °C; 180 s at 72 °C; 5 min at 72 °C for final extension. The D1–D3 domains of 28S rRNA gene were amplified using the dig12 (5'-AAGCATATCACTAAGCGG-3') and L0 (5'-GCTATCCTGAGRGAACTTCG-3') [8]. The thermal cycle parameters were as follows: initial denaturation at 95 °C (3 min); 35 cycles of 20 s at 95 °C; 20 s at 53.4 °C; 85 s at 72 °C; 5 min at 72 °C for final extension. All amplicons were sequenced directly with the same pair of primers at the Research Park of St. Petersburg State University (Centre for Molecular and Cell Technologies). The newly obtained sequences from both forward and reverse primers were assembled using Chromas Pro 1.7.4. These sequences were assembled and trimmed, and the resultant sequence lengths were: 1798 bp for single 18S rRNA gene, 1264–1280 bp for 28S rRNA gene. For comparative purposes and phylogeny construction, 18S and 28S rRNA gene sequences of monorchiod, microphalloid and apocreadiid trematodes from the GenBank database were also used (Table 1).

Table 1. Trematode species involved in phylogenetic analyses.

Taxa	18S rRNA Gene, Accession Number	Reference	28S rRNA Gene, Accession Number	Reference
Monorchioidea				
Lissorchiidae				
<i>Anarhichotrema ochotense</i>	OM108704.1	Present Study	OM108706.1– OM108708.1	Present Study
<i>Asaccotrema vietnamiense</i>	-	-	MK863409.1	[9]
<i>Asymphyiodora progenetica</i>	-	-	MT103403.1	[10]
<i>Asymphyiodora</i> sp.	-	-	MT153917.1	[10]
<i>Lissorchis</i> cf. <i>gullaris</i>	-	-	MT928353.1	[11]
<i>Lissorchis kritskyi</i>	AY222136.1	[12]	EF032689.1	[13]
<i>Lissorchis</i> cf. <i>nelsoni</i>	-	-	MT928354.1	[11]
<i>Palaeorchis incognitus</i>	-	-	MT103408.1	[10]
<i>Posthoviteilinum psiloterminae</i>	-	-	MT928351.1	[11]

Table 1. Cont.

Taxa	18S rRNA Gene, Accession Number	Reference	28S rRNA Gene, Accession Number	Reference
Monorchioidea				
Monorchiidae				
<i>Ancylocoelium typicum</i>	AJ287474.1	[14]	AY222254.1	[12]
<i>Cableia pudica</i>	AJ287486.1	[14]	AY222251.1	[12]
<i>Diplomonorchis leiostomi</i>	AY222137.1	[12]	AY222252.1	[12]
<i>Genolopa ampullacea</i>	-	-	MN984474.1	[15]
<i>Helicometroides longicollis</i>	-	-	KJ658287.1	[16]
<i>Infundiburictus arrhichostoma</i>	-	-	KJ658289.1	[16]
<i>Lasiotocus mulli</i>	MT669013.1	[17]	MT669011.1	[17]
<i>Madhavia fellaminuta</i>	-	-	MG920219.1	[18]
<i>Monorchis monorchis</i>	-	-	AF184257.1	[19]
<i>Parachrisomon delicatus</i>	-	-	MG920218.1	[18]
<i>Pseudohurleytrema yolandae</i>	-	-	MT649300.1	[20]
<i>Proctotrema addisoni</i>	-	-	KJ658291.1	[16]
<i>Provitellus turrum</i>	AJ287566.1	[14]	AY222253.1	[12]
<i>Provitellus infrequens</i>	-	-	MK501985.1	[21]
<i>Retroporomonorchis pansho</i>	-	-	MT672340.1	[22]
Deropristidae				
<i>Skrjabinopsolus nudidorsalis</i>	MN700960.1	[23]	MN700996.1	[23]
Microphalloidea				
Faustulidae sensu lato				
<i>Bacciger lesteri</i>	-	-	AY222269.1	[12]
<i>Trigonocryptus conus</i>	-	-	AY222270.1	[12]
Zoogonidae				
<i>Deretrema nahaense</i>	-	-	AY222273.1	[12]
<i>Diptherostomum</i> sp.	-	-	AY222272.1	[12]
<i>Lepidophyllum steenstrupi</i>	-	-	AY157175.1	[24]
<i>Plectognathotrema kamegaii</i>	-	-	KM505035.1	[25]
<i>Proctophantastes gillissi</i>	-	-	KU163452.1	[26]
<i>Steganoderma</i> cf. <i>eamiqtrema</i>	-	-	MW264135.1	[6]
<i>Zoogonoides viviparus</i>	-	-	AY222271.1	[12]
Apocreadioidea				
Apocreadiidae				
<i>Homalometron octopapillatum</i>	-	-	JQ389865.1	[27]

The sequences involved in phylogenetic analyses were aligned using MUSCLE algorithm as implemented in R studio ‘msa’ package [28,29]. Sequences for general alignments were downloaded from GenBank database with custom script based on ‘ape’ package in R studio [30,31]. The alignments were then checked manually in SeaView software [32]. The final length of alignment of 28S rRNA gene sequences was 1270 bp. To concatenate sequences of 18S and 28S rRNA genes “catfasta2phym.pl” script [33] was used with “-f” parameter. Final length of the concatenated alignment was 3050 bp.

The evolutionary model for maximum likelihood (ML) and Bayesian Inference (BI) analysis was chosen with MrModeltest v. 2.4 [34]. The model of best fit was GTR + G + I for both the concatenated and separated alignments. Maximum likelihood analysis was performed through the CIPRES portal [35] with non-parametric bootstrap with 1000 pseudoreplicates. Bayesian Inference analysis was performed using MrBayes 3.2.7a. The analysis was carried out using computational resources provided by Resource Center “Computer Center of SPbU” (<http://cc.spbu.ru>, project No. 110-21448, accessed on 30 March 2021). Trees were run for 35,000,000 generations, by which time they ceased converging (final

average standard deviation of the split frequencies was less than 0.01). The quality of the chains was estimated using built-in MrBayes tools. Based on the estimates, the first 15,000 generations were discarded for burn-in. The phylogenetic trees were rooted based on the findings of Olson et al. [12] and Sokolov et al. [23]: the Apocreadiidae for the trees generated on 28S rRNA gene data, the Deropristidae for 18S + 28S rRNA gene sequences-based trees. Estimates of evolutionary divergence (p-distances) were made with MEGA X software [36].

3. Results

3.1. Phylogenetic Relationships

We obtained partial sequences of the 28S rRNA gene for three specimens and partial sequences of the 18S rRNA gene for one specimen. The 28S rRNA gene sequences were almost identical to each other after trimming and alignment. Only one gap was observed in position 1266 of general alignment in sequence OM108706. The BLAST hit results showed that the 28S rRNA sequences of *A. ochotense* were more similar to those of lissorchiid and monorchiid digeneans.

Maximum likelihood and Bayesian Inference analyses based on 28S rRNA gene data placed *A. ochotense* within the Monorchioidea, sister to the well-supported ‘freshwater lissorchiids’ clade; the position of *A. ochotense* was poorly-supported (Figure 1). The ‘freshwater lissorchiids’ clade included the paraphyletic assemblage of the lissorchiine digeneans and the monophyletic Asymphyllodorinae. However, the Asymphyllodorinae clade was not well-supported in any of the analyses. In turn, the *A. ochotense* + ‘freshwater lissorchiids’ clade resolved sister to the Monorchioidea; this larger clade was well-supported. The degree of divergence between the 28S rRNA gene sequences of *A. ochotense* and the basal species in the ‘freshwater lissorchiids’ clade (*Palaeorchis incognitus* Szidat, 1943) did not exceed that between the basal monorchioids (*Cableia pudica* Bray, Cribb & Barker, 1996, *Helicometroides longicollis* Yamaguti, 1934), being 10% and 17%, respectively. In the trees based on 18S+28S rRNA gene data, *A. ochotense* formed a well-supported clade with *Lissorchis kritskyi* Barnhart & Powell, 1979 (Figure 2).

3.2. Systematics

Monorchioidea Odhner, 1911

Lissorchiidae Magath, 1917

Anarhichotrema Shimazu, 1973

Anarhichotrema ochotense Shimazu, 1973 Figure 3

Taxonomic Summary

Host: *Anarhichas orientalis* Pallas, 1814 (Anarhichadidae).

Site in host: Intestine.

Locality: The Sea of Okhotsk (44°44′59″ N, 147°8′2″ E).

Deposited material: Five whole-mounted gravid specimens, IPEE RAS 14313, 14314.

Representative DNA sequences: Partial 28S rRNA gene sequences of *A. ochotense* are deposited under GenBank numbers OM108706.1–OM108708.1. Partial 18S rRNA gene sequence is deposited under GenBank number OM108704.1.

Description (based on five paragenophores–whole-mounted gravid specimens; five slides with isolated ovarian complex and five slides with isolated terminal genitalia; measurements in Table 2).

Body fusiform, maximum width near mid-level of body. Tegument spined. Forebody with numerous gland-cells in parenchyma. Oral sucker round, mouth subterminal. Ventral sucker rounded, unspecialised. Prepharynx short, muscular. Pharynx muscular, elongate-oval. Oesophagus contracted to some extent in all specimens. Intestinal bifurcation dorsal to ventral sucker. Caeca blind, terminate near posterior extremity of body.

Figure 1. Phylogenetic relationships of *Anarhichotrema ochotense* based on dataset of 28S rRNA gene sequences. The bootstrap (≥50) and posterior probability (≥0.90) values are given near the nodes for ML and BI analyses, respectively.

Figure 2. Phylogenetic relationships of *Anarhichotrema ochotense* based on concatenated dataset of 18S+28S rRNA gene sequences. The bootstrap (≥ 50) and posterior probability (≥ 0.90) values are given near the nodes for ML and BI analyses, respectively.

Figure 3. *Anarhichotrema ochotense* from intestine of *Anarhichas orientalis*, the Sea of Okhotsk: (a) whole mount, ventral view; (b), terminal genitalia, ventral view; (c), ovarian complex, dorsal view. Abbreviations: c, invaginated cirrus; ej, ejaculatory duct; f, fertilization chamber; ga, common genital atrium; isv, internal seminal vesicle; lc, Laurer's canal; md, middle part of oviduct; mt, metraterm; oc, ovicapt; oo, oötype with Mehlis' gland cells; pc, prostatic cells; pp, pars prostatica; src, canalicular seminal receptacle; sru, uterine seminal receptacle; v, vitelline reservoir. Scale bars: (a) = 500 μm , (b,c) = 50 μm .

Testes two, irregularly indented (in four specimens) or entire (in one specimen), tandem, slightly overlapping, in posterior half of body. Cirrus sac rectilinear to comma-shaped, passes beyond posterior margin of ventral sucker, terminating in a common genital atrium. Vas deferens absent; vasa efferentia directly joining seminal vesicle. Internal seminal vesicle unipartite, tubular, sinuous. Pars prostatica vesicular; lined with anuclear cell-like bodies; field of prostatic cells extensive. Ejaculatory duct rectilinear, invaginated cirrus

unarmed. Common genital atrium small, distinct. Genital pore sinistro- (in two specimens) or dextro-marginal (in three specimens), located near midlevel of ventral sucker.

Table 2. Measurements (μm) of *Anarhichotrema ochotense* from present and previous studies: n—number of specimens measured, *—estimated from the published illustration.

Morphometric Characters		Present Study; n = 5	Shimazu [1], (1973); n = 5	Machida [2], as <i>Neolissorchis genge</i> ; n = 5
Body	Length (L)	875–986	1750–2750	1660–2140
	Width (W)	438–506	900–1380	870–950
Forebody	L, % of body L	25.7–30.4	24.1 *	22.7 *
Post-testicular region	L, % of body L	16.7–23.6	13.9 *	10 *
Oral sucker	L	147–167	180–280	168–194
	W	147–160	220–320	204–230
Ventral sucker	L	167–173	260–380	280–306
	W	179–199	340–480	311–332
Sucker ratio	W	1:1.17–1.30	1:1.32–1.54	1:1.40–1.60
Prepharynx	L	32–44	52 *	51–77
Pharynx	L	115–128	170–180	148–158
	W	83–96	170–220	117–138
Anterior testis	L	122–128	230–340	204–255
	W	103–173	260–420	230–342
Posterior testis	L	128–147	260–410	230–332
	W	96–147	280–340	316–408
Cirrus sac	L	205–237	510–550	229–538
	W	64–80	100–140	95–108
Ovary	L	90–115	170–340	178–316
	W	154–180	350–550	255–362
Eggs	L, without filament	25	24–30	27–35
	W	12	14–16	15–18

Ovary multilobate, median, overlapping anterior edge of anterior testis. Ovicapt distinct. Proximal part of oviduct forms thick-walled fertilization chamber terminating with sphincter. Middle part of oviduct long, linked to canalicular seminal receptacle and Laurer's canal, and vitelline reservoir. Distal part of oviduct forms oötype. Canalicular seminal receptacle slender, without sperm. Laurer's canal bulbous proximally, opens medially, anterior to ovary. Oötype with compact Mehlis' gland, antero-ventral to ovary. Uterus tortuous, reaching into post-testicular region, proximal portion functions as uterine seminal receptacle. Metraterm short, separated from uterus by constriction, terminating with sphincter, unarmed, ventral to cirrus sac. Eggs numerous, operculate, with long anopercular filament. Vitellarium follicular; follicles form two ventrolateral fields and median dorsal group. Ventrolateral fields extend from level of posterior edge or posterior third of ventral sucker to posterior extremity of body, confluent in post-testicular region. Follicles of dorsal group in two submedian longitudinal rows; rows asymmetrical in most specimens, anterior-most follicle at midlevel of anterior testis to midpoint of ventral sucker-ovary distance, posterior-most follicle at level of posterior edge of posterior testis or anterior third or middle of post-testicular region or near posterior extremity of body. Vitelline reservoir median, ventral to ovary.

Excretory vesicle I-shaped, extends to middle or anterior edge of posterior testis, posterior end surrounded by small muscular sphincter; excretory pore terminal.

4. Discussion

The specimens of *A. ochotense* described in our study are similar to those from Shimazu [1] in most of the morphological features related to the shape and location of the organs. Only three features appear to be different, based on the drawing in Shimazu [1]: the relative position of the testes (overlapping vs. separate), the position of the anterior border of the ventrolateral fields of the vitelline follicles (at the level of or slightly anteriorly to the posterior edge of the ventral sucker vs. distinctly posterior to the ventral sucker), and the arrangement of the cirrus sac (overlapped by the ventral sucker vs. lying outside the projection of the sucker). However, Machida [2] demonstrated that these features can be variable.

At the same time, the trematode specimens in our study were noticeably smaller than those studied by T. Shimazu and M. Machida. This difference concerned all morphometric characteristics except egg length (Table 2). The small size of the parasites could be associated with the crowding effect: *A. ochotense* infection intensity was 375 individuals in our study as compared with six individuals in Shimazu [1] and five individuals in Machida [2]. The so-called crowding effect is a result of competition for host's finite resources by parasites. Typically, this effect increases with increasing numbers of parasites within a host and manifests in reduced body size and thus fitness [37–39].

Phylogenetic analyses conducted in this study indicate that *Anarhichotrema* is a member of the Lissorchiidae, thus, we restore the genus to this family, where it was originally assigned [1]. The family Lissorchiidae is currently divided into two subfamilies, the Lissorchiinae Magath, 1917 and the Asymphylogorinae Szidat, 1943 [5]. The division is based on the number of testes: two in the former and one in the latter subfamily [5]. Phylogenetic data support the unification of the lissorchiine and the asymphylogorine digeneans into the same family but do not support the monophyly of the Lissorchiinae ([9–11,40], Present Study). Notably, the position of two lissorchiine genera, *Lissorchis* and *Palaeorchis* Szidat, 1943, is unstable in the phylograms of different authors [10,11], Present Study. *Anarhichotrema*, in contrast to the other lissorchiid genera, has a marine life history and the filamented eggs compare with [5,9,11]. Taking into account the ecological and morphological features of *Anarhichotrema* as well as the phylogenetic data (the basal position of *Anarhichotrema* and the paraphyly of the Lissorchiinae), we conjecture that a separate subfamily may have to be erected for this genus in the future and that all freshwater lissorchiids should probably be considered within one subfamily (Lissorchiinae). However, for now, we conditionally consider *Anarhichotrema* to the Lissorchiinae.

The results of our phylogenetic analysis allow us to hypothesise about the marine ancestor of the Lissorchiidae. This is evidenced by both the basal position of a taxon with a marine life history (*Anarhichotrema*) in the clade of lissorchiid trematodes and by the marine ecology of the main number of members of the Monorchidae, that is a sister to this clade. However, verification of this hypothesis requires a phylogenetic assessment of the few known freshwater genera of monorchids: *Heckmannia* Bilqees, Hadi, Khan & Perveen, 2012 and *Sphericomonorchis* Thatcher, 1996.

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