

DESCRIPTION AND PHYLOGENETIC AFFINITIES OF A NEW SPECIES OF *NEOPSILO TREMA* (DIGenea: PSILOSTOMIDAE) FROM LESSER SCAUP, *AYTHYA AFFINIS* (ANSERIFORMES: ANATIDAE)

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KEY WORDS

ABSTRACT

Psilostomidae
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Neopsilotrema itasca n. sp.
Molecular phylogeny
28S
NDI

Neopsilotrema is a small genus of psilostomid digeneans parasitic in the intestine of birds in the Palearctic and Nearctic. At present, the genus includes 4 species: *Neopsilotrema lisitsynae* from the Palearctic and *Neopsilotrema affine*, *Neopsilotrema lakotae*, and *Neopsilotrema marilae* from the Nearctic. Herein, we describe a new species, *Neopsilotrema itasca* n. sp., from lesser scaup *Aythya affinis* collected in Minnesota. The species can be distinguished from congeners on the basis of the ventral sucker:oral sucker width ratio, body width:length ratio, and cirrus sac size, along with other characters. We generated new 28S ribosomal deoxyribonucleic acid (DNA) and NADH dehydrogenase (NDI) mitochondrial DNA sequence data of a variety of psilostomids from the Palearctic and Nearctic along with sequences of the ribosomal internal transcribed spacer (ITS) region (ITS1 + 5.8S + ITS2) from 3 *Neopsilotrema* species. The molecular phylogenetic affinities of a variety of psilostomid taxa were studied using 28S sequence data. The 28S sequences of psilostomids demonstrated 1–7.9% intergeneric divergence, whereas the sequences of NDI had 17.7–34.1% intergeneric divergence. The interspecific divergence among members of *Neopsilotrema* was somewhat lower (0.2–0.5% in 28S; 0.3–0.4% in ITS; 12–15.7% in NDI). Our comparison of DNA sequences along with morphologic study suggests Holarctic distribution of *N. lisitsynae*.

The Psilostomidae Looss, 1900 is a small family of echinostomatoidean digeneans that parasitize the intestines of their avian and mammalian definitive hosts. The majority of psilostomids have echinostome-like morphology but lack a head collar with spines (Kostadinova, 2005; Tkach et al., 2016; Kudlai et al., 2017). Some members of the family are known to have significant ecological impacts; for instance, *Sphaeridiotrema pseudoglobulus* McLaughlin, Scott, and Huffman, 1993 has been associated with mass mortality events of aquatic birds in the midwestern United States (Hoeve and Scott, 1988; Herrmann and Sorensen, 2009). Tkach et al. (2016) revised the system of the Echinostomatoidea Looss, 1899 on the basis of partial 28S ribosomal deoxyribonucleic acid (rDNA) sequences, which included re-evaluation of the constituent genera of the Psilostomidae; subsequently Kudlai et

al. (2016, 2017) further amended the content of the Psilostomidae and described 4 new genera primarily on the basis of specimens collected from aquatic birds in the Nearctic (*Byrdtrema* Kudlai, Kostadinova, Pulis and Tkach, 2017, *Longisaccus* Kudlai, Kostadinova, Pulis and Tkach, 2017, *Macracetabulum* Kudlai, Kostadinova, Pulis and Tkach, 2017, and *Neopsilotrema* Kudlai, Pulis, Kostadinova and Tkach, 2016).

At present, *Neopsilotrema* contains 4 species (Kudlai et al., 2016). Three *Neopsilotrema* species are known from the Nearctic: *Neopsilotrema affine* Kudlai, Pulis, Kostadinova and Tkach, 2016, *Neopsilotrema lakotae* Kudlai, Pulis, Kostadinova and Tkach, 2016, and *Neopsilotrema marilae* (Price, 1942); 1 species, *Neopsilotrema lisitsynae* Kudlai, Pulis, Kostadinova and Tkach, 2016, is known from the Palearctic. No member of the genus is currently known to be distributed in multiple biogeographic realms. In the present study, we generated partial sequences of the nuclear large ribosomal subunit (28S) rDNA and NADH dehydrogenase I (NDI) mitochondrial (mt)DNA gene of a variety of psilostomid taxa collected from the Palearctic and Nearctic. The newly

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generated and previously published 28S sequences were used to infer phylogenetic interrelationships among members of the Psilostomidae, whereas the *ND1* sequences are provided for future comparative studies. In addition, we describe a new species of *Neopsilotrema* from lesser scaup *Aythya affinis* (Eyton) collected in Minnesota.

MATERIALS AND METHODS

Adult and larval psilostomids were collected from intestines of a variety of avian definitive hosts and a snail in the Palearctic and Nearctic between 1997 and 2020 (Table I). Live digeneans were briefly rinsed in saline, heat-killed in hot water, and fixed in 70% ethanol. Dead digeneans from frozen and thawed intestines were immediately fixed in 70% ethanol or 10% buffered formalin. Specimens used for morphological study were stained with Semichon's acetocarmine or aqueous alum carmine, dehydrated in an ethanol series of ascending concentrations, and permanently mounted using Damar gum or Canada balsam (Lutz et al., 2017). Psilostomids were measured using a digital imaging system on a differential interference contrast-equipped Olympus BX40 compound microscope (Olympus, Tokyo, Japan). A Leica DMC 4500 microscope (Leica Corp., Buffalo Grove, Illinois) equipped with a drawing tube was used to prepare drawings. Measurements in the text are given in micrometers. The type series of the new species and new voucher specimens are deposited in the collection of the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum, Lincoln, Nebraska (Table I).

DNA was extracted from partial or whole specimens using a ZR Genomic DNATM tissue micro prep kit (Zymo Research, Irvine, California) following the manufacturer's protocol or according to the protocol described by Tkach and Pawlowski (1999). Additional loci of some psilostomid isolates previously sequenced by Kudlai et al. (2016, 2017) and Tkach et al. (2016) were amplified as well. Polymerase chain reaction (PCR) was performed using a T100TM thermal cycler (Bio-Rad, Hercules, California). A ~1,350 base-pair-long fragment at the 5' end of 28S was amplified using forward primer digL2 (5'-AAG CAT ATC ACT AAG CGG-3') and reverse primer 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') (Tkach et al., 2003). Fragments of the ribosomal internal transcribed spacer region (*ITS1* + 5.8S + *ITS2*) were amplified for 3 previously described *Neopsilotrema* species including isolates of *N. lisitsynae* from the Palearctic and Nearctic; PCR reactions for the *ITS* region used forward primer ITSf (5'-CGC CCG TCG CTA CTA CCG ATT G-3') and reverse primer 300R (5'-CAA CTT TCC CTC ACG GTA CTT G-3') (Littlewood and Olson, 2001; Snyder and Tkach, 2007). A fragment of *ND1* was amplified using forward primer NDJ11 (5'-AGA TTC GTA AGG GGC CTA ATA-3') and reverse primer NDJ2a (5'-CTT CAG CCT CAG CAT AAT-3') (Morgan and Blair, 1998; Kostadinova et al., 2003). PCRs were performed using GoTaq G2 DNA polymerase from Promega (Madison, Wisconsin) according to the manufacturer's protocol with an annealing temperature of 53 C for rDNA and 40 C for mtDNA reactions. Despite our best efforts, we were unable to amplify *ND1* of several species of psilostomids (Table I). In addition, partial sequences of the mt cytochrome *c* oxidase I gene were generated for 2 psilostomid taxa and deposited in GenBank under accession numbers MW959706 and MW959707.

PCR products were purified using an Illustra ExoProStar PCR cleanup enzymatic kit from Cytiva (Marlborough, Massachusetts). Cleaned PCR products were cycle sequenced with MCLab BrightDye[®] terminator chemistry (Molecular Cloning Laboratories, San Francisco, California). PCR primers were used for sequencing reactions; in addition, the reverse primer d58R (5'-CAC GAG CCG AGT GAT CCA CCG C-3') was used for sequencing reactions of *ITS1* (Curran et al., 2006). Sequencing reactions were purified using a MCLab BigDye[®] sequencing cleanup kit and subsequently run on an ABI 3130 automated capillary sequencer (Thermo Fisher Scientific, Waltham, Massachusetts). The new contiguous sequences were assembled using Sequencher ver. 4.2 (GeneCodes Corp., Ann Arbor, Michigan) and submitted to GenBank (Table I).

An alignment of 28S sequences was used for phylogenetic inference to study the interrelationships within the Psilostomidae. Sequences were initially aligned with the assistance of ClustalW implemented in MEGA7 (Kumar et al., 2016) and trimmed to the length of the shortest sequence. *Stephanoprora pseudoechinata* (Olsson, 1876) was selected as the outgroup for the 28S analysis on the basis of the topology presented by Tkach et al. (2016). The 28S alignment included 6 new sequences and 12 previously published sequences of psilostomids, including a previously published sequence from a larval isolate misidentified as a species of *Echinoparyphium* Dietz, 1909 (GenBank AY395577). MEGA7 software identified the general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + G) as the best-fitting nucleotide substitution model (Kumar et al., 2016). The present phylogenetic analysis was conducted utilizing Bayesian inference (BI) implemented in MrBayes Ver. 3.2.6 software (Ronquist and Huelsenbeck, 2003). The BI analysis was conducted as follows: Markov chain Monte Carlo chains were run for 3,000,000 generations, log-likelihood scores were plotted, and only the final 75% of trees were used to produce the consensus trees. The number of generations was considered sufficient as the average standard deviation of split frequencies stabilized below 0.01.

The *ND1* sequences of psilostomids are provided for future comparative studies. Pairwise comparisons were performed with assistance of MEGA7.

DESCRIPTION

Neopsilotrema itasca n. sp. Achatz, Bennett and Sorensen (Figs. 1–3)

Description (on the basis of 10 fully mature specimens. Measurements of the holotype are given in the text; measurements of the entire series are given in Table II): Body elongate, 1,457 long, maximum width at level of ventral sucker, 345; body width:length ratio 0.24. Forebody 263 long, forebody length:body length ratio 0.18. Tegument armed with fine spines. Oral sucker terminal, subspherical, 93 × 83. Ventral sucker protuberant with deep cavity, consisting of strongly muscular portion and extensive surrounding tegument, 290 × 289; larger than oral sucker, ventral sucker:oral sucker width ratio 3.48. Prepharynx short, 15. Pharynx muscular, subspherical, smaller than oral sucker, 58 × 54. Esophagus short. Cecal bifurcation anterior to ventral sucker, often obscured by protruding ventral

Table 1. List of psilostomid taxa sequenced in the present study. HCIP: Helminthological Collection of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic; ISEA: Zoological Museum at the Institute of Systematics and Ecology of Animals, Novosibirsk, Russia; HWML: Harold W. Manter Laboratory, Lincoln, Nebraska. rDNA: ribosomal deoxyribonucleic acid; *ND1*, NADH dehydrogenase gene.

Taxa	Host species	Geographic origin	Museum no.	Accession numbers		Reference
				rDNA	<i>ND1</i>	
<i>Byrdtrema sponsae</i>	<i>Aix sponsa</i>	Minnesota	HWML 216465	MW962214*	—	Present study
<i>Byrdtrema sponsae</i>	<i>Aix sponsa</i>	Wisconsin	HWML 216466	MW962215*, MW962216*	—	Present study
<i>Byrdtrema sponsae</i>	<i>Aix sponsa</i>	Mississippi	HWML 103030, 103031	KT956955*; MW962217*	MW963174	Kudlai et al., 2017; Present study
<i>Longisaccus elvirae</i>	<i>Aix sponsa</i>	Mississippi	HWML 103026, 103027	KT956954*	MW963175	Kudlai et al., 2017; Present study
<i>Longisaccus</i> sp.	<i>Aix sponsa</i>	Minnesota	HWML 216467	MW962218*	—	Present study
<i>Macracetabulum albeolae</i>	<i>Bucephala albeola</i>	North Dakota	HWML 103028, 103029	KT956953*, MW962219*	MW963176– MW963178	Kudlai et al., 2017; Present study
<i>Macracetabulum albeolae</i>	<i>Bucephala albeola</i>	Minnesota	HWML 216468	MW962220*	—	Present study
<i>Neopsilotrema affine</i>	<i>Aythya affinis</i>	Minnesota	HWML 101952, 101953	MW962221†, MW962222* MW969680‡	MW963179	Kudlai et al., 2016; Present study
<i>Neopsilotrema affine</i>	<i>Bucephala clangula</i>	Minnesota	HWML 216469	MW962223– MW962225*	—	Present study
<i>Neopsilotrema itascae</i> n. sp.	<i>Aythya affinis</i>	Minnesota	HWML 216470, 216471	MW962226*, MW962227*	—	Present study
<i>Neopsilotrema lakotae</i>	<i>Aythya americana</i>	North Dakota	HWML 101954, 101955	MW962228†	MW963180	Kudlai et al., 2016; Present study
<i>Neopsilotrema lisitsynae</i>	<i>Aythya affinis</i>	Minnesota	—	MW962229*	—	Present study
<i>Neopsilotrema lisitsynae</i>	<i>Aythya collaris</i>	Minnesota	—	MW962230*	—	Present study
<i>Neopsilotrema lisitsynae</i>	<i>Anas crecca</i>	Ukraine	HWML 101956, 101957	MW962231†	MW963181	Kudlai et al., 2016; Present study
<i>Neopsilotrema lisitsynae</i>	<i>Anas platyrhynchos</i>	Minnesota	HWML 216472	MW962232– MW962237*, MW969681§	—	Present study
<i>Neopsilotrema lisitsynae</i>	<i>Spatula discors</i>	Minnesota	—	MW962238*	—	Present study
<i>Psilotrema</i> cf. <i>simillimum</i>	<i>Bithynia tentaculata</i>	Novosibirsk oblast, Russia	ISEA NoNe2002P_ <i>simillimum</i> 1554B_ <i>tent</i>	MW962239*	—	Present study
<i>Sphaeridiotrema pseudoglobulus</i>	<i>Aythya affinis</i>	Minnesota	HCIP D-722; HWML 101872, 216473	KT956957*, MW962240*, MW962241*	MW963182, MW963183	Tkach et al., 2016; Present study

* Partial 28S sequence.

† Partial internal transcribed spacer 1 gene (*ITS1*), complete 5.8S + *ITS2* and partial 28S sequence.

‡ Partial *ITS1* sequence.

§ Partial *ITS1* and complete 5.8S + *ITS2* sequence.

sucker. Ceca thin-walled, extending to near posterior extremity of body.

Testes 2, entire, slightly oblique or tandem, in the second quarter of body; anterior testis transversely oval or somewhat triangular, positioned some distance posterior to ventral sucker, ventral to and slightly overlapping posterior testis, 120 × 147; posterior testis subspherical, 149 × 145. Posttesticular field 660 long, posttesticular field:body length ratio 0.45. Cirrus sac elongate-oval, dorsal to ventral sucker, 164 × 94. Internal seminal vesicle bipartite, saccular. Pars prostatica poorly developed; prostatic cells small, few. Cirrus small. Genital pore median, weakly muscular, immediately anterior to ventral sucker, near level of cecal bifurcation.

Ovary subspherical or transversely oval, entire, immediately posterior to ventral sucker, positioned oblique to anterior testis, dextral, slightly overlapping anterior testis ventrally, 98 × 122.

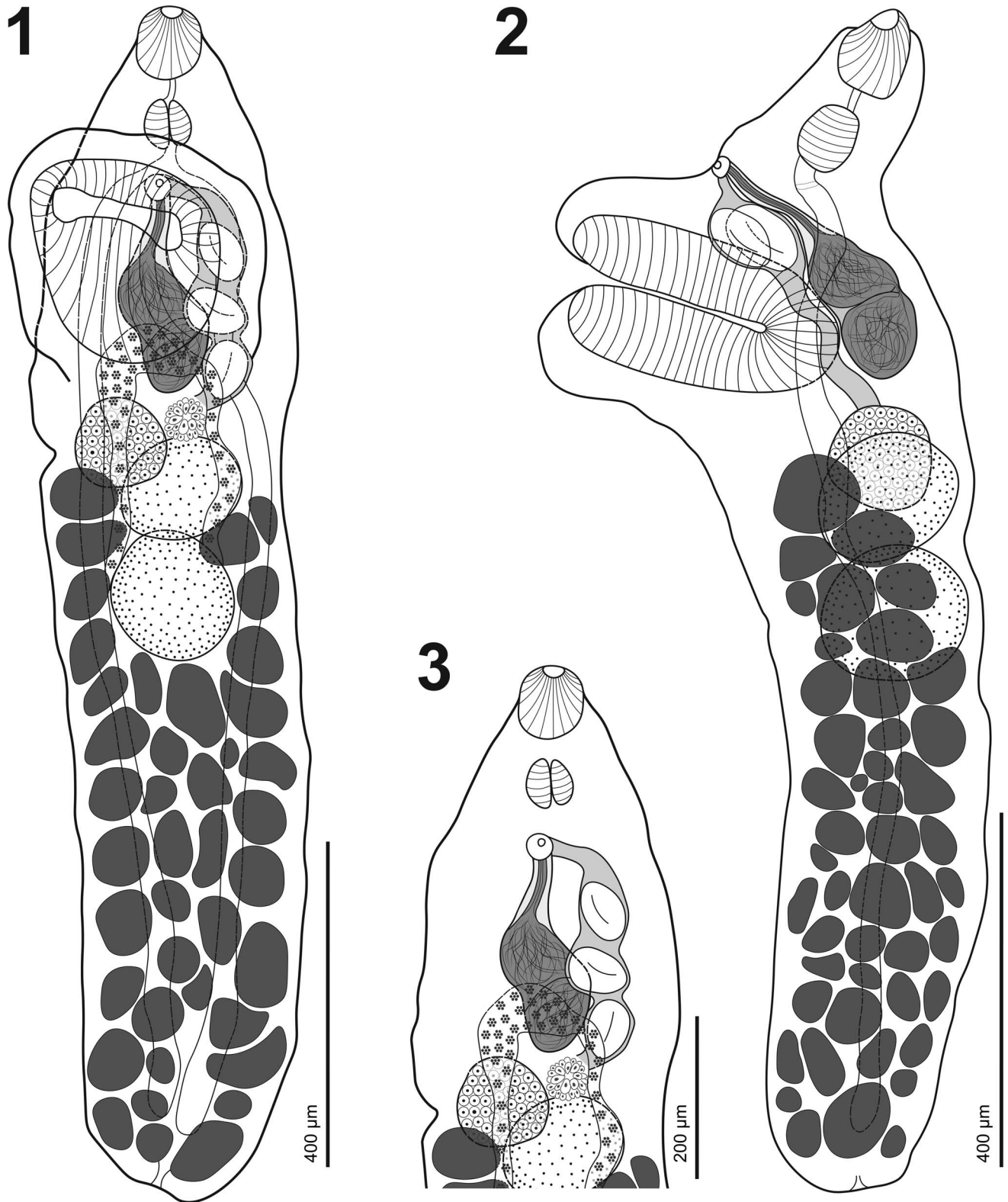
Mehlis' gland at level of ovary, sinistral, ventral to gonads. Seminal receptacle and Laurer's canal not well-observed. Vitellarium in 2 lateral bands, distributed between posterior margin of the ventral sucker or level of ovary and near posterior extremity of body, bands sporadically confluent in posttesticular field. Vitelline reservoir well defined, anterior to gonads, immediately posterior to or dorsal of ventral sucker. Uterus with short metraterm (not well-observed), contains few eggs (up to 7). Eggs 75–88 × 45–57.

Excretory vesicle not well-observed. Excretory pore terminal.

Taxonomic summary

Type host: *Aythya affinis* (Eyton) (Anseriformes: Anatidae).

Site of infection: Small intestine.



Figures 1–3. *Neopsilotrema itasca* n. sp. from *Aythya affinis*. (1) Holotype, ventral view. (2) Paratype, lateral view. (3) Holotype with ventral sucker and ceca omitted, ventral view.

Table II. Morphometric characters of *Neopsilotrema itasca* n. sp. (n = 10). Measurements are in micrometers.

Character	Mean	Range	Standard deviation	Coefficient of variation
Body length	1,380	1,225–1,560	109.2	7.9
Body width	313	267–364	38.9	12.4
Body width:length ratio	0.23	0.19–0.25	0.02	9.2
Forebody length	288	198–355	50.4	17.5
Forebody length:body length ratio	0.21	0.15–0.28	0.04	18.7
Oral sucker length	86	75–97	8.2	9.5
Oral sucker width	77	70–83	5.6	7.3
Ventral sucker length	243	198–290	32.1	13.2
Ventral sucker width	272	230–315	30.4	11.1
Ventral sucker:oral sucker width ratio	3.6	3–4	0.4	11
Prepharynx	19	11–23	4.4	24
Pharynx length	60	54–70	5	8.5
Pharynx width	55	53–57	1.4	2.5
Anterior testis length	133	115–161	15	11.2
Anterior testis width	162	141–180	17.2	10.6
Posterior testis length	152	131–178	15.7	10.3
Posterior testis width	157	145–174	10.9	7
Posttesticular field length	602	510–736	76.8	12.8
Posttesticular field:body length ratio	0.44	0.4–0.47	0.02	5.7
Cirrus sac length	171	137–213	25.4	14.8
Cirrus sac width	85	72–96	9.2	10.9
Ovary length	102	74–122	15.9	15.7
Ovary width	109	86–122	15.7	14.5
Egg length	81	75–88	4	5
Egg width	52	45–57	3.6	6.9

Locality: Lake Winnibigoshish, Minnesota; 47°25'38.8"N, 94°18'54.3"W.

Type specimens deposited: The type series consists of 10 fully mature specimens deposited in the HWML. Holotype: HWML 216470, labeled ex. *A. affinis*, Lake Winnibigoshish in Minnesota, 9 October 2012, coll. R. E. Sorensen. Paratypes: HWML 216471 (lot of 9), labels identical to the holotype.

Representative DNA sequences: MW962226, MW962227 (28S)

ZooBank registration: urn:lsid:zoobank.org:act:7FD802DE-26E0-44F4-9AD5-7D1F788F858C.

Etymology: The species is named after the county of the type locality.

Remarks

Morphological characters, such as the presence of a bipartite seminal vesicle, muscular pharynx, massive ventral sucker, and position of the genital pore, along with genetic comparisons demonstrate that the digeneans described should be considered members of *Neopsilotrema*.

The new species can be differentiated from *N. affine*, *N. lakotae*, and *N. marilae* on the basis of a relatively narrower body (body width:length ratio 0.19–0.25 in the new species vs. body width:length ratio 0.47–0.56 in *N. affine*, 0.38–0.57 in *N. lakotae*, and 0.52 in *N. marilae*), relatively longer posttesticular field (posttesticular field:body length ratio 0.4–0.47 in *N. itasca* vs. posttesticular field:body length ratio 0.14–0.22 in *N. affine*, 0.17–0.22 in *N. lakotae*, and 0.17 in *N. marilae*), and a substantially longer body (body length 1,225–1,560 in *N. itasca* vs. 550–648 in *N. affine*, 622–803 in *N. lakotae*, and 510–630 in *N. marilae*). In addition, *N. itasca* can be easily distinguished from the other members of *Neopsilotrema* on the basis of a generally larger

ventral sucker:oral sucker width ratio (ventral sucker:oral sucker width ratio 3–4 in the new species vs. ventral sucker:oral sucker width ratio 2.3–3.3 in *N. affine*, 1.9–3 in *N. lakotae*, 2–2.5 in *N. lisitsynae*, and 2 in *N. marilae*). Furthermore, 28S sequences of *N. itasca* differ from *N. affine* by 0.5% and from *N. lakotae* by 0.2%.

Neopsilotrema itasca further differs from *N. lisitsynae* on the basis of a larger body size (body 1,225–1,560 × 267–364 in the new species vs. 810–875 × 238–263 in *N. lisitsynae*) and a typically shorter cirrus sac (cirrus sac length 137–213 in *N. itasca* vs. 182–220 in *N. lisitsynae*). The 2 species also differ by 0.2% in 28S sequences. A detailed morphometric comparison of *Neopsilotrema* spp. is provided in Table III.

Molecular phylogenies

After trimming to the length of the shortest sequence, the alignment of 28S sequences was 1,049 bases long. The overall topology and nodal supports of the phylogeny resulting from the analysis of 28S (Fig. 4) were similar to results presented by Kudlai et al. (2016, 2017) and Schwelm et al. (2020). A clade (100% supported) of *Psilostomum brevicolle* (Creplin, 1829) + a subclade (100% supported) of *Psilochasmus* spp. was positioned as a sister group to a weakly supported clade that contained the remaining Psilostomidae. This clade consisted of 2 strongly supported clades. The first of them (100% support) included a 100% supported clade containing species of *Psilotrema* Odhner, 1913 and a 93% supported clade consisting *Longisaccus* + *Macrace-tabulum* + *Byrdtrema* + *Neopsilotrema*. Within the 93% supported clade, the 2 sequences of *Longisaccus* spp. (100% supported clade) were positioned as a sister group to a 100% supported clade that contained 2 additional subclades (Fig. 4). The first weakly

Table III. Metric characters of *Neopsilotrema* spp. Range values are followed by mean in parentheses.

Taxa	<i>N. itasca</i> n. sp.	<i>N. affine</i>	<i>N. lakotae</i>	<i>N. lisitsynae</i>	<i>N. marilae</i>
Reference	Present study	Kudlai et al. (2016)	Kudlai et al. (2016)	Kudlai et al. (2016)	Price (1942)
Locality	United States	United States	United States	Ukraine	United States
Host	<i>Aythya affinis</i>	<i>Aythya affinis</i>	<i>Aythya americana</i>	<i>Anas crecca</i>	<i>Aythya affinis</i>
Body length	1,225–1,560 (1,380)	550–648 (596)	622–803 (688)	810–875 (833)	510–630
Body width	267–364 (313)	264–362 (302)	264–354 (316)	238–263 (250)	250–340
Body width:length ratio	0.19–0.25 (0.23)	0.47–0.56 (0.51)	0.38–0.57 (0.46)	0.27–0.33 (0.30)	0.52*
Forebody:body length ratio	0.15–0.28 (0.21)	0.31–0.38 (0.34)	0.21–0.37 (0.30)	0.23–0.27 (0.25)	0.31*
Oral sucker length	75–97 (86)	63–101 (72)	68–80 (74)	69–76 (72)	—
Oral sucker width	70–83 (77)	66–91 (73)	68–85 (75)	71–81 (75)	60
Ventral sucker length	198–290 (243)	147–187 (160)	136–206 (177)	154–180 (163)	115
Ventral sucker width	230–315 (272)	171–240 (200)	161–220 (190)	159–187 (169)	150
Ventral sucker:oral sucker width ratio	3–4 (3.6)	2.3–3.3 (2.8)	1.9–3 (2.5)	2–2.5 (2.2)	2*
Pharynx length	54–70 (60)	51–76 (60)	48–68 (54)	52–56 (54)	45
Pharynx width	53–57 (55)	48–70 (55)	48–60 (54)	53–56 (55)	35
Anterior testis length	115–161 (133)	58–96 (74)	97–120 (107)	81–96 (89)	—
Anterior testis width	141–180 (162)	78–119 (91)	80–125 (98)	105–119 (110)	75
Posterior testis length	131–178 (152)	72–106 (82)	86–126 (107)	76–96 (87)	—
Posterior testis width	145–174 (157)	76–111 (92)	83–122 (101)	85–116 (99)	75
Posttesticular field:body length ratio	0.40–0.47 (0.44)	0.14–0.22 (0.18)	0.17–0.22 (0.19)	0.37–0.40 (0.39)	0.17*
Cirrus sac length	137–213 (171)	121–198 (163)	136–220 (186)	182–220 (204)	115–150
Cirrus sac width	72–96 (85)	61–99 (76)	66–101 (83)	80–98 (87)	65–75
Ovary length	74–122 (102)	51–76 (61)	68–75 (71)	61–89 (78)	—
Ovary width	86–122 (109)	43–63 (53)	60–81 (70)	55–76 (66)	55
Egg length	75–88 (81)	74–96 (83)	70–78 (74)	76–88 (82)	85–90
Egg width	45–57 (52)	52–56 (54)	43–50 (46)	46–50 (48)	50–60

* Values provided by Kudlai et al. (2016) on the basis of measurement of original illustration.

supported subclade included *Byrdtrema sponsae* Kudlai, Kostadinova, Pulis and Tkach, 2017 + *Macracetabulum albeolae* Kudlai, Kostadinova, Pulis and Tkach, 2017. The second subclade (100% support) comprised the 4 *Neopsilotrema* species included in the analysis. *Neopsilotrema affine* was positioned as a sister group to an 82% supported clade of *N. lisitsynae* + a weakly supported clade of [*N. lakotae* + *N. itasca*]. The 100% supported clade containing 4 species of *Sphaeridiotrema* Odhner, 1913 formed a sister group to the clade of *Psilotrema* + *Longisaccus* + *Macracetabulum* + *Byrdtrema* + *Neopsilotrema* (Fig. 4).

Genetic variation

Intergeneric divergence among psilostomid genera in the 28S fragment was 1–7.9%. *Psilotrema* vs. *Psilochasmus* Lühe, 1909 had the greatest divergence, whereas *Byrdtrema* and *Macracetabulum* showed the greatest similarity. The 28S sequences of the 2 *Longisaccus* spp. differed by 0.1%, whereas the 2 *Psilochasmus* spp. differed by 0.5%. The 3 *Psilotrema* spp. had 1.8–2.9% difference among sequences of 28S. The level of interspecific divergence of 28S sequences was 0.2–0.5% among *Neopsilotrema* spp. and 1–3.6% among *Sphaeridiotrema* spp. No intraspecific variation was detected among 28S sequences (newly generated and previously published) of *B. sponsae* (n = 5), *M. albeolae* (n = 2), *N. affine* (n = 5), *N. itasca* (n = 2), *N. lisitsynae* (n = 10), and *S. pseudoglobulus* (n = 3). Complete pairwise comparison of unique 28S sequences is provided in Table S1.

The divergence among partial ITS region (partial ITS1 and complete 5.8S + ITS2) sequences of *Neopsilotrema* spp. (0.3–0.4%) was similar to the level of divergence observed among 28S sequences of the members of the genus. *Neopsilotrema affine* and

N. lisitsynae were the most divergent species, whereas *N. affine* vs. *N. lakotae* and *N. lakotae* vs. *N. lisitsynae* were the most similar. No intraspecific variation was detected in partial ITS1 sequences of the 2 isolates of *N. affine*. A single ambiguous nucleotide site was detected in the ITS1 sequence of *N. lisitsynae* from Europe (adenine or thymine); the ITS1 sequence of *N. lisitsynae* from North America had an adenine at the same position. Complete pairwise comparisons of the unique partial ITS region sequences are provided in Table S2.

Intergeneric divergence among *ND1* sequences of psilostomid genera was 17.7–34.1%. *Byrdtrema* and *Neopsilotrema* were the most divergent, whereas *Macracetabulum* vs. *Neopsilotrema* showed the lowest level of divergence. The level of interspecific divergence among *ND1* sequences of *Neopsilotrema* species was 12–15.7%. Up to 0.5% of intraspecific variation was detected among the partial *ND1* sequences of *M. albeolae* (n = 3). No intraspecific variation was detected among *ND1* sequences of *S. pseudoglobulus* (n = 2). Complete pairwise comparisons of unique *ND1* sequences are provided in Table S3.

DISCUSSION

Neopsilotrema itasca is the fifth member of the genus and fourth species described from the Nearctic (Price, 1942; Kudlai et al., 2016). Although we were unable to generate *ND1* sequences of the new *Neopsilotrema* species, its partial sequences of 28S demonstrated at least 0.2% from other *Neopsilotrema* species. Although *N. lakotae* and *N. lisitsynae* had the most similar 28S sequences to our new species, *N. lakotae*, *N. lisitsynae*, and *N. itasca* are substantially different morphologically (see remarks above and Table III). Furthermore, *N. lakotae* and *N. lisitsynae*

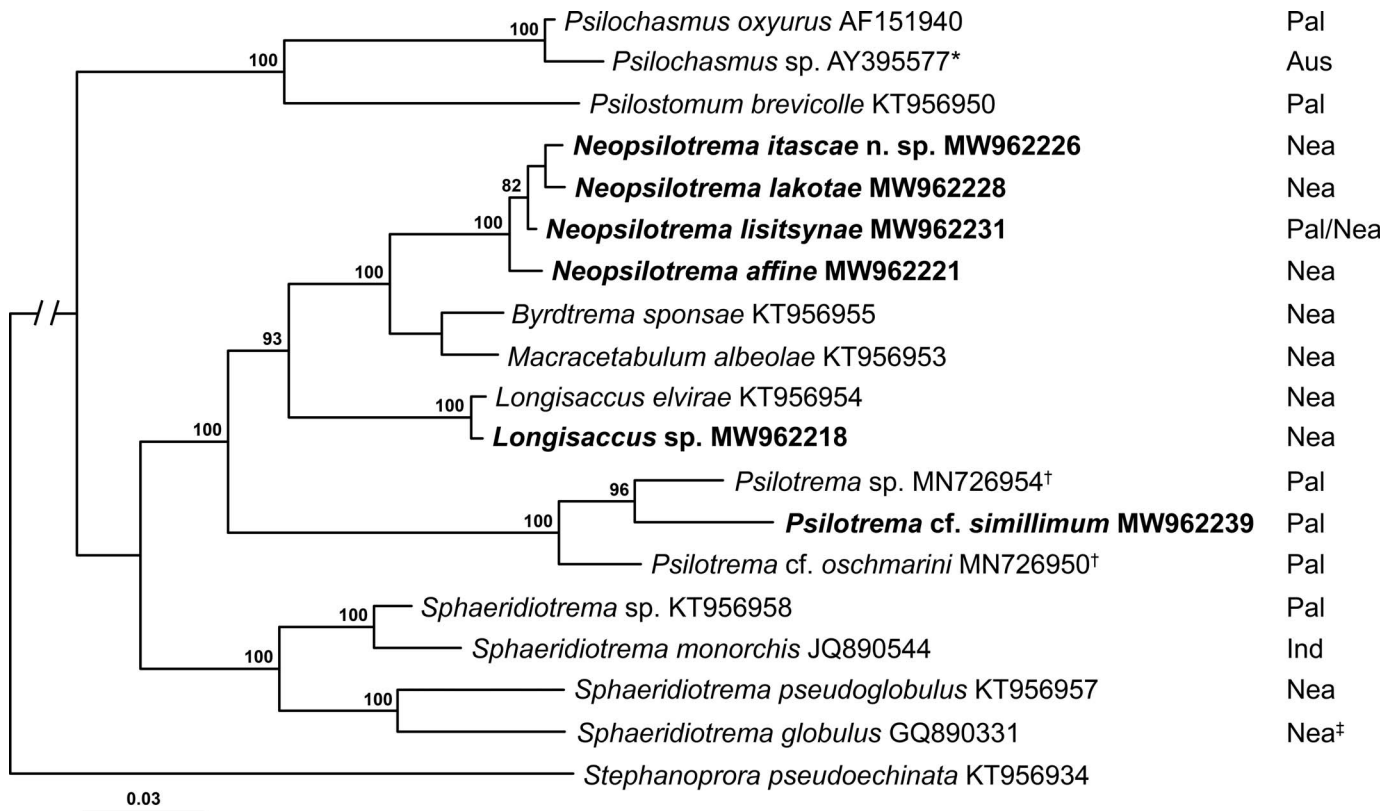


Figure 4. Phylogenetic interrelationships among 18 psilostomid taxa including *Neopsilotrema itasca* n. sp. based on Bayesian inference analysis of partial 28S ribosomal deoxyribonucleic acid sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences obtained in this study are in bold. Scale bar indicates number of substitutions per site. GenBank accession numbers are provided after the names of all species. Biogeographic realms where isolates were collected are indicated next to names of taxa. Abbreviations for biogeographic realms: Aus = Australasian, Ind = Indomalayan, Nea = Nearctic, Pal = Palearctic. * Taxon previously misidentified as a species of *Echinoparyphium*. † Taxa previously not identified to genus-level. ‡ The *Sphaeridiotrema globulus* sequence originates from an isolate collected in the Nearctic; however, the species was originally described from the Palearctic.

(which also have 0.2% divergence between sequences of 28S) differ by 0.3% in partial *ITS* region sequences and 15.7% in partial *ND1* sequences. The combination of morphological differences between *N. lakotae*, *N. lisitsynae*, and *N. itasca* along with the comparison of faster mutating genes (i.e., partial *ITS* region and *ND1*) of *N. lakotae* and *N. lisitsynae* suggests that as few as 2 nucleotide differences in 28S sequences may be informative for species differentiation within the genus.

Our specimens of *Longisaccus* sp. differed by a single base from *Longisaccus elvirae* Kudlai, Kostadinova, Pulis, and Tkach, 2017 in 28S sequences. Unfortunately, the quality of our specimens of *Longisaccus* sp. was poor; therefore, we were unable to confidently identify it to species. As with *N. itasca*, we were unable to generate *ND1* sequences of our *Longisaccus* sp. Most specimens collected from Lake Winnibigoshish in Minnesota were fixed in 10% buffered formalin and stored at room temperature for a long time before being studied.

The morphology of *N. lisitsynae* specimens collected in the Nearctic closely conforms to the original description of the species from the Palearctic (Kudlai et al., 2016). No difference was detected between 28S and partial *ITS* region sequences of *N. lisitsynae* from the Nearctic and Palearctic. At the same time, *Neopsilotrema* spp. demonstrated 0.2–0.5% interspecific diver-

gence in the 28S region (Table S1) and 0.3–0.4% interspecific divergence in the *ITS* region (Table S2). The lack of differences in ribosomal sequences of *N. lisitsynae*, along with morphological similarity, suggests that the specimens collected from the Palearctic and Nearctic are conspecific. This is the first report of *N. lisitsynae* from *A. affinis*, ring-necked duck *Aythya collaris* (Donovan), blue-winged teal *Spatula discors* (Linnaeus), and mallard *Anas platyrhynchos* Linnaeus; and *N. affine* from common goldeneye *Bucephala clangula* (Linnaeus). In addition, this is the first report of *Byrdtrema sponsae* and a member of *Longisaccus* in a host collected north of Mississippi State.

Members of *Psilotrema* and *Neopsilotrema* are morphologically similar but can be distinguished, in part, on the basis of the structure of the seminal vesicle (simple in *Psilotrema* vs. bipartite in *Neopsilotrema*) (Kostadinova, 2005; Kudlai et al., 2016). Atopkin (2011) previously published 28S sequences of adult *Psilotrema* specimens obtained from experimental life-cycle studies conducted by Besprozvanynkh (2003, 2007). Besprozvanynkh (2003) described specimens of *Psilotrema acutirostris* Oschmarin, 1963 with a bipartite seminal vesicle. Later, Besprozvanynkh (2007) described his material of *Psilotrema simillimum* (Mühling, 1898), the type species of *Psilotrema*, with a simple seminal vesicle; at the same time, he described his

specimens of *Psilotrema oschmarini* Besprozvannykh, 2007 with “internal” and “external” seminal vesicles. As noted by Kudlai et al. (2016), the sequences provided by Atopkin (2011) were short and lacked associated vouchers. Our sequences of *P. simillimum* originate from cercariae that morphologically conformed to the description of *P. simillimum* cercariae and match the short 28S sequence of *P. simillimum* published by Atopkin (2011). It is noteworthy that the short 28S sequences of *P. simillimum* and *P. acutirostris* from Atopkin (2011) did not have any differences. Unfortunately, we did not collect any adult *Psilotrema* specimens. The study of newly collected, well-fixed adult *Psilotrema* specimens is critical to evaluate the nature of the seminal vesicle among members of the genus; we anticipate the seminal vesicle of *Psilotrema* spp. to be simple, as originally described. The 28S sequences of ‘*Psilostomidae* gen sp. 1’ from Schwelm et al. (2020) are identical to the short sequence of *P. oschmarini* previously published by Atopkin (2011). We tentatively consider ‘*Psilostomidae* gen sp. 1’ to be conspecific with *P. oschmarini*; however, collection of new well-fixed adult specimens and comparison of faster mutating genes is required for confirmation of this suggestion. ‘*Psilostomidae* gen sp. 2’ of Schwelm et al. (2020) was positioned among the 2 other *Psilotrema* species, thus placing it in *Psilotrema* (Fig. 4).

The level of intergeneric divergence among 28S sequences of psilostomids (1–7.9%) was slightly greater than previously reported by Kudlai et al. (2017) (0.9–6.5%). The interspecific variation of 28S sequences within *Psilotrema* spp. (1.8–2.9%) and *Sphaeridiotrema* spp. (1–3.6%) exceeds the level of divergence between 28S sequences among psilostomid genera (e.g., *Byrdotrema* + *Macracetabulum*). It is worth noting that all sequences of *Psilotrema* spp., *Sphaeridiotrema monorchis* Lin and Chen, 1983, and *Sphaeridiotrema* sp. (GenBank KT956958) originate from larval specimens. The level of divergence among published *Psilotrema* and *Sphaeridiotrema* spp. may suggest that some sequences belong to other genera. On the other hand, *Sphaeridiotrema globulus* (Rudolphi, 1814) and *S. pseudoglobulus* differ by 2.7% in 28S sequences and were both identified on the basis of adult specimens. The position of *Neopsilotrema* in the 28S phylogeny (Fig. 4) and lower interspecific divergence in the 28S gene may indicate relatively recent speciation within the genus. Unsurprisingly, the intergeneric divergence among psilostomids in the sequenced fragment of the *ND1* gene (17.7–34.1%) was much greater than the intergeneric divergence observed among 28S sequences (1–7.9%).

The *Psilotrema* species included in the present analysis originated from the Palearctic and formed a sister group to a clade containing most psilostomids from the Nearctic. *Neopsilotrema lisitsynae*, which is likely distributed in the Nearctic and Palearctic on the basis of the present data, was nested among *Neopsilotrema* spp. from the Nearctic. This suggests a secondary dispersion from the Nearctic into the Palearctic. It is worth noting that *Psilochasmus* sp. (GenBank AY395577) was collected from a bithyniid snail in Australia and was originally misidentified as a new species of *Echinoparyphium* (Koch, 2004). Inclusion of DNA sequences (including faster mutating genes) of several psilostomid taxa from mammals (e.g., *Grysonema* Byrd, Bogitsh and Maples, 1961 and *Mehlisia* Johnston, 1913) into future studies will allow further clarification of the interrelationship of various lineages and patterns of host switching within this group of digeneans.

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