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Short Communication

Stenostomum cf. *leucops* (Platyhelminthes) in Thailand: a surface observation using scanning electron microscopy and phylogenetic analysis based on 18S ribosomal DNA sequences

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Abstract

The genus *Stenostomum* contains small turbellaria that are widely distributed in freshwater environments worldwide. However, there are only rare reports or studies of this genus from Thailand. Therefore, the objective of this study was to report *S.* cf. *leucops* in Thailand collected from Pathum Thani Province. The worm morphology and surface topography using scanning electron microscopy were determined. Moreover, the phylogenetic tree of *S.* cf. *leucops* was analysed with 17 flatworms based on the 18S ribosomal DNA sequences. The phylogenetic relationship shared a common ancestry of Catenulida species, and *S.* cf. *leucops* displayed a monophyletic pattern within *Stenostomum* spp. The results of the morphological and molecular data are discussed. These results may increase the knowledge of freshwater microturbellarians in Thailand.

Keywords: freshwater turbellarians, epidermal topography, nucleus gene, Thailand

1. Introduction

In Platyhelminthes, flatworms of the genus *Stenostomum* (Schmidt, 1848) are free-living microturbellaria belonging to the family Stenostomidae in the class Catenulida (Nuttycombe, and Waters, 1938). They are widely distributed in freshwater environments of Europe, America, South America, Australia, Africa and Asia (Young and Kolasa, 1974; Larsson and Jondekius, 2008; Damborenea *et al*., 2011; Yamazaki *et al*., 2012). More than 10 species in this genus are recognised, including *S. grande*, *S. simplex*, *S. tuberculosum*, *S. saliens*, *S. heebuktense*, *S. gotlandense*, *S. sphagnetorum*, *S. steveoi*, *S. tenuicauda*, *S. matarazzoi* and *S. leucops* (Noreña *et al*., 2005).

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Stenostomum spp. are organisms used for experimental biology, including morphology, taxonomy, developmental biology and molecular genetic studies (Noreña *et al*., 2005; Larsson *et al*., 2008; Martín-Durán and Egger, 2012). In Thailand, unknown *Stenostomum* and *Rhynchoscolex simplex* of Stenostomidae were found in the Northeastern region (Heckman, 1979). The *S. Leucops*, Dugès, 1828, are a stenostomid species that are found in the flowing streams and temporary ponds (Thorpand and Covich, 2009) in many regions of natural freshwater (Larsson *et al*., 2008; Damborenea *et al*., 2011; Yamazaki *et al*., 2012). However, some record of this species has been reported from Thailand.

The 18S ribosomal DNA (rDNA) is a nuclear gene that is widely used for studying the phylogenetic evolution of many metazoans (Canales-Aguirre *et al*., 2011; Machida and Knowlton, 2012). Larsson and Jondekius (2008) created the catenulid phylogeny based on rDNA sequences supporting platyhelminth taxa.

The objective of this study was to determine the occurrence of *S.* cf. *leucops* in Thailand. The morphology and surface topography using scanning electron microscopy (SEM) were examined, and the phylogenetic relationship of *S.* cf. *leucops* was analysed within selected species of Catenulida based on the 18S rDNA sequences.

2. Materials and Methods

2.1 Turbellarian sampling and morphological study

In May 2014, *Stenostomum* were collected during a survey of freshwater invertebrates in ponds from Pathum Thani Province, Thailand (14° 6'40.20'' N 100°30'18.56''E) (Figure 1A) using a 40-125 µm mesh size net. Those worms were identified as *S.* cf. *leucops* according to the taxonomy guidelines of Larsson (2008) and Damborenea *et al*. (2011). The morphology of *S.* cf. *leucops* 0.2-0.5 mm wide and 1-2 mm long were observed under light microscopy, and the live organisms were then drawn. *S.* cf. *leucops* were cultured in small aquaria with sterile pond water.

2.2 SEM investigation

Six *S.* cf. *leucops* individuals were fixed with 2.5% glutaraldehyde solution in 0.1 sodium cacodylate buffer (pH 7.4) at 4°C for 12 h. The specimens were washed with 0.05 sodium cacodylate buffer 3 times and post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 30 min. They were washed with distilled water 3 times and then dehydrated through serial concentrations of ethanol. These materials were dried in a Hitachi HCP-2 critical point drying machine. The worms were coated with gold in an ion-sputtering apparatus, SPI-Model sputter coater for 4 min, and examined using a JEOL JSM-5400.

2.3 Phylogenetic analysis

The total DNA was extracted from live samples of *S.* cf. *leucops* using a DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol. The nuclear genes of the 18S rDNA were amplified using Taq DNA polymerase (Takara, Tokyo, Japan) with the forward primer (5'-AMCTGGTTGAT CCTGCCAG-3') and reverse primer (5'-TGATCCATCTGCA GGTTCACCT-3') (Noren and Jondelius, 1999). The PCR cycling conditions were an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, 52°C for 45 s for annealing, 72°C for 1 min for extension, and 10 min for a final extension. The PCR products, approximately 1800 bp, were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and viewed on a UV transilluminator. The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen).

DNA sequencing was performed by Macrogen DNA Sequencing Service, Korea. The nucleotide sequences of *S.* cf. *leucops* were deposited into GenBank (Benson *et al*., 2005) with the accession numbers listed in Table 1. The software package, Molecular Evolutionary Genetics Analysis (MEGA) version 5.10, was used for creating the neighbour-joining phylogenetic tree with 1000 bootstrap replicates (Tamura *et al*. 2011).

Table 1. Lists of *S.* cf. *leucops* and selected species with accession numbers for 18S rDNA sequences

Species	Accession number	References
S. leucops	FJ384828	Larsson *et al*. (2008)
S. grande	AB665104	Yamazaki *et al*. (2012)
S. bryophilum	FJ196319	Larsson and Jondelius (2008)
S. arevaloi	FJ384833	Larsson *et al*. (2008)
S. cf. *leucops* 1	KP113675	This study
S. cf. *leucops* 2	KP113676	This study
Catenula lemnae	FJ196324	Larsson and Jondelius (2008)
Paracatenula galateia	HQ231344	Dirks *et al*. (2011)
Diceratocephala boschmai	KC517073	Ngamniyom *et al*. (2014)
Castrella truncata	AY775777	Willems *et al*. (2006)
Strongylostoma elongatum	AY775771	Willems *et al*. (2006)
Indosolenorchis hirudinaceus	AY222110	Olson *et al*. (2003)
Heronimus mollis	AY222118	Olson *et al*. (2003)
Dasyrhynchus giganteus	FJ788112	Olson *et al*. (2010)
Poecilancistrium caryophyllum	FJ788111	Olson *et al*. (2010)
Geocentrophora wagini	AJ012509	Littlewood *et al*. (1999)
Caenorhabditis elegans	EU196001	Kiontke *et al*. (2007)

3. Results

The turbellarian body of the *S.* cf. *leucops* was cylindrical in shape, slight and yellowish in colour. The anterior end was blunt and rounded. The middle region of the body was constricted, and the posterior end formed a tail. Two ciliated pits were anterolateral at the anterior side. Short cilia were observed around the body. Two anterior lobes of the brain were dentate. The posterior brain lobes connected to the light-refracting corpuscles. Protonephridium appeared between the right and left anterior lobes. The worm presented 2 zooid chains. The mouth opening was variable, quite rounded and associated with the pharynx. The ellipse pharynx with a simple muscle was large, positioned at the anterior part to a beginning of the middle part and was adored with numerous pharyngeal glands. The intestine presented from the middle to the posterior end and was associated with the nephridiopore in subterminal. Minot cells were found scattering the intestinal region (Figure 1B).

In the SEM topography, the surface of the ventral side was irregularly swollen on the anterior region and around the oral areas (Figures 1C and D). The surface was bumpy and rough at the middle to posterior part, including the ciliated pit areas (Figures 1E-G). No cilia were observed on the ventral side of the body. On the dorsal side, the epidermal surface was covered by cilia from the anterior to posterior end of the body (Figure 1H). The cilia in the anterior and middle regions appeared denser than those in the posterior region (Figures 1I-L).

For the 18S rDNA phylogenetic relationship of *S.* cf. *leucops* and 18 selected species, the catenulid taxa showed common ancestry with the genus *Stenostomum*, *Paracatenula* and *Catenula* (*S.* cf. *leucops* 1, *S.* cf. *leucops* 2, *S. leucops*, *S. grande*, *S. arevaloi*, *S. bryophilum*, *P. galateia* and *C. lemnae*). For genus *Stenostomum*, *S.* cf. *leucops* 1, *S.* cf. *leucops* 2, *S. leucops*, *S. grande*, *S. arevaloi* and *S. bryophilum* were monophyletic. The phylogeny of *S.* cf. *leucops* 1 and 2 was the sister group to the tree containing *Stenostomum* spp., *P. galateia* and *C. lemnae*. The phylogenetic result clearly isolated *S.* cf. *leucops* from Rhabdocoela (*Castrella truncate*, *Strongylostoma elongatum* and *Diceratocephala boschmai*), Seriata (*Pseudomonocelis ophiocephala*), Lecithoepitheliata (*Geocentrophora wagini*), Trematoda (*Indosolenorchis hirudinaceus* and *Heronimus mollis*) and Cestoda (*Dasyrhynchus giganteus* and *Poecilancistrium caryophyllum*). Those ingroup species were exactly distant from the outgroup species (*Caenorhabditis elegans*) (Figure 2).

4. Discussion

The findings of *S.* cf. *leucops* from the natural environment of Thailand are supported by the previous study of Noreña *et al*. (2005), suggesting that the distribution of

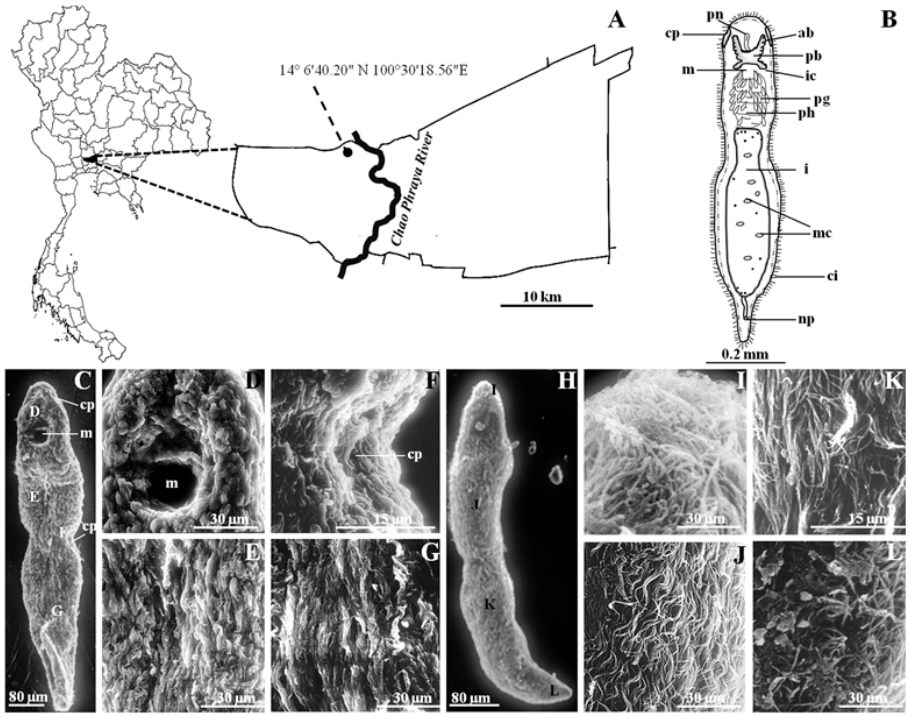

Figure 1. (A) Site collection of *S.* cf. *leucops* from natural ponds in Pathum Thani Province, Thailand. (B) Morphological description of *S.* cf. *leucops*. (C-G) Ventral views and dorsal views (H-L) of *S.* cf. *leucops* under SEM investigation. Capital letters in C and H indicate the high magnification areas, consequently. ab, anterior brain lobe; ci cilia; cp, ciliated pit; i, intestine; ic, light-refracting corpuscles; m, mouth opening; mc, minot cell; np, nephridiopore; pb, posterior brain lobe; ph, pharynx; pg, pharyngeal gland; pn, protonephridium.

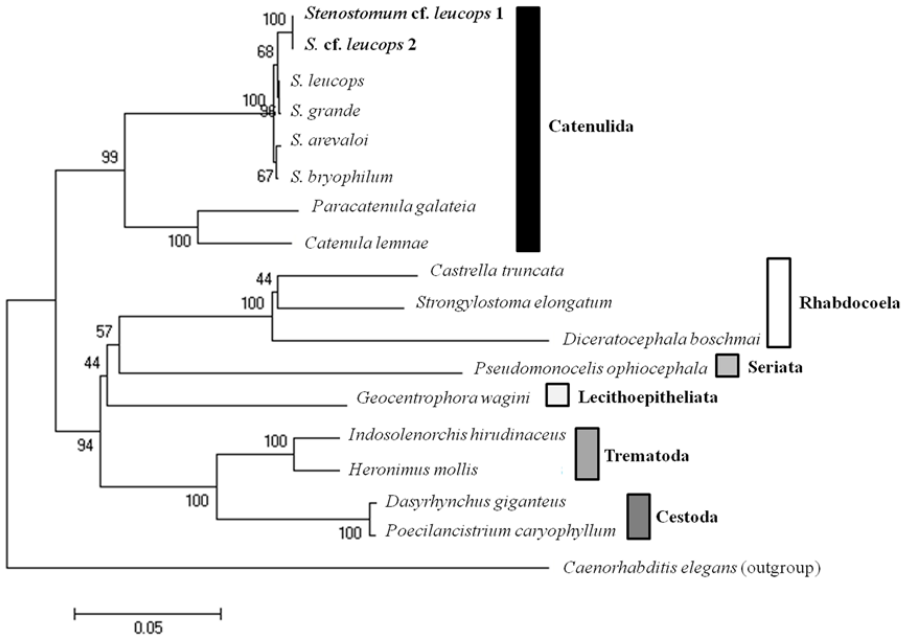

Figure 2. Molecular phylogenetics of 18S rDNA sequences between *S.* cf. *leucops*, 17 turbellarians and 1 nematode.

S. leucops is cosmopolitan. The morphology of *S.* cf. *leucops* is similar to the reports of Gamo and Leal-Zanchet (2004), Larsson and Jondekius (2008), Damborenea *et al*. (2011) and Yamazaki *et al*. (2012), in which *S. leucops* were described from Brazil, Sweden, Peru and Japan, respectively.

For epidermal topography, our results are supported by the preliminary SEM report of Larsson (2008) showing ciliated pits and distributed ciliation on a dorsal view of *S. leucops*. However, cilia in the ventral part were absent in *S.* cf. *leucops*, which was not consistent with the findings of Antoniazzi and Silveira (1996), who observed ventral ciliated grooves in *S. grande*.

For the phylogenetic framework based on the 18S rDNA gene, the result confirms the molecular relationship in which Catenulida is monophyletic within the Platyhelminthes and is consistent with a report by Larsson and Jondelius (2008), who determined that flatworm species were a monophyletic clade using the 18S, 28S rDNA sequences. Furthermore, there is congruence with the DNA taxonomy of Larsson *et al*. (2008), who evaluated the phylogenetic classification among catenulid species based on combined sequences of 18S, 28S, COI and ITS-5.8S. Therefore, the molecular information of *S.* cf. *leucops* from Thailand is added to the phylogeny. In this study, *S.* cf. *leucops* were closely related to *S. leucops* from other locations, although the morphological characteristics were similar. This result is supported by the recent report of Yamazaki *et al*. (2012), who phylogenetically analysed the relationship of 4 species in Stenostomidae from 10 Japanese rice fields based on the 18S rDNA sequences.

During two decades, the cryptic diversities have been described in many studies of freshwater invertebrates including turbellaria (Belyaeva and Taylor, 2009). Those evidences are two or more distinct genetics that are identified by morphological similarity as a single species (Jörger and Schröd 2013). In this study, there was a little distinction in molecular data between *S.* cf. *leucops* and *S. leucops*, although they had almost similar morphology. It is so far to precisely determine *S.* cf. *leucops* as a cryptic or cosmopolitan species, since other molecular and morphological data of our study are not sufficient.

To the best of our knowledge, this study is the report of *S.* cf. *leucops* in natural ponds from Thailand and introduces morphological and molecular information for this microturbellarian. However, the morphological and molecular analyses remain to be performed for a precise identification of *S.* cf. *leucops* inhabiting the freshwater environment of Thailand.

References

Antoniazzi, M.M. and Silveira, M. 1996. Studies on *Stenostomum grande* Child, 1902 (Platyhelminthes, Catenulida): fine structure of the digestive tract and the endocytotic activity of the gastrodermis. Acta Zoologica. 77, 25-32.

Belyaeva, M. and Taylor, D.J. 2009. Cryptic species within the Chydorus sphaericus species complex (Crustacea: Cladocera) revealed by molecular markers and sexual stage morphology. Molecular Phylogenetics and Evolution. 50(3), 534-46.

Canales-Aguirre, B., Rozbaczylo, N. and Hernández, C.E. 2011. Genetic identification of benthic polychaetes in a biodiversity hotspot in the southeast Pacific", Revista de Biología Marina y Oceanografia. 46, 89-94.

Damborenea, C., Brusa, F., Almagro, I. and Noreña, C. 2011. A phylogenetic analysis of *Stenostomum* and its neotropical congeners, with a description of a new species from the Peruvian Amazon Basin. Invertebrate Systematics. 25, 155-169.

Dirks, U., Gruber-Vodicka, H.R., Leisch, N., Sterrer, W. and Ott, J.A. 2011. A new species of symbiotic flatworms, *Paracatenula galateia* sp. nov. (Platyhelminthes: Catenulida: Retronectidae) from Belize (Central America). Marine Biology Research. 7, 769-777.

Duges, A. 1828. Recherches sur l'organisation et les moeurs des planariees. Annales des Sciences Naturelles 15, 139-182.

Gamo, J and Leal-Zanchet, A. 2004. Freshwater microturbellarians (Platyhelminthes) from Rio Grande do Sul, Brazil. Revista Brasileira de Zoologia. 21, 897-903.

Heckman, C.W. 1979. Rice Field Ecology in Northeastern Thailand, Dr. W. Junk bv Publishers, The Hague, The Netherlands.

Jörger, K.M. and Schröd, M. 2013. How to describe a cryptic species? Practical challengesof molecular taxonomy. Frontiers in Zoology. 10(1), 59.

Kiontke, K., Barriere, A., Kolotuev, I., Podbilewicz, B., Sommer, R., Fitch, D.H. and Felix, M.A. 2007. Trends, stasis, and drift in the evolution of nematode vulva development. Current Biology. 17, 1925-1937.

Larsson, K. 2008. Taxonomy and Phylogeny of Catenulida (Platyhelminthes) with Emphasis on the Swedish Fauna. Acta Universitatis Upsaliensis. Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology, 395. pp. 13-30.

Larsson, K. and Jondekius, U. 2008. Phylogeny of Catenulida and support for Platyhelminthes. Organisms Diversity and Evolution. 8, 378-387.

Larsson, K., Ahmadzadeh, A. and Jondekius, U. 2008. DNA taxonomy of Swedish Catenulida (Platyhelminthes) and a phylogenetic framework for catenulid classification. Organisms Diversity and Evolution. 8, 399-412.

Littlewood, D.T.J., Rohde, K. and Clough, K.A. 1999. The interrelationships of all major groups of Platyhelminthes: phylogenetic evidence from morphology and molecules. Biological Journal of the Linnean Society. 66, 75-114.

Machida, R.J. and Knowlton, N. 2012. PCR Primers for Metazoan Nuclear 18S and 28S Ribosomal DNA Sequences. PLoS ONE. 7, e46180.

Martín-Durán, J.M. and Egger, B. 2012. Developmental diversity in free-living flatworms. *EvoDevo*. 3, 7.

Ngamniyom, A., Sriyapai, T. and Silprasit, K. 2014. *Diceratocephala boschmai* (Platyhelminthes: Temnocephalida) from crayfish farms in Thailand: investigation of the topographic surface and analysis of 18S ribosomal DNA sequences. Turkish Journal of Zoology. 38, 471-478.

Noren, M. and Jondelius, U. 1999. Phylogeny of the Prolecithophora (Platyhelminthes) inferred from 18S rDNA sequences. Cladistics.15, 103-112.

Noreña, C. Damborenea, C. and Brusa, F., 2005. A taxonomic revision of South American species of the genus *Stenostomum* O. Schmidt (Platyhelminthes: Catenulida) based on morphological characters. Zoological Journal of the Linnean Society. 144, 37-58.

Nuttycombe, J. and Waters, A. 1938. The American species of the genus *Stenostomum*. American Philosophical Society. 79, 213-301.

Olson, P.D., Cribb, T.H., Tkach, V.V., Bray, R.A. and Littlewood, D.T. 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). International Journal for Parasitology. 33, 733-755.

Olson, P.D., Caira, J.N., Jensen, K., Overstreet, R.M., Palm, H.W. and Beveridge, I. 2010. Evolution of the trypanorhynch tapeworms: parasite phylogeny supports independent lineages of sharks and rays. International Journal for Parasitology. 40, 223-242.

Schmidt, E.O. 1848. Die rhabdocoelen Strudelwürmer des süssen Wassers. (Fr. Mauke: Jena.)

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Phylogenetics and Evolution. 28, 2731-2739.

Thorp, J.H. and Covich A.P. 2009. Ecology and Classification of North American Freshwater Invertebrates, Academic Press, Academic Press, San Diego, U.S.A., pp. 157.

Willems, W.R., Wallberg, A., Jondelius, U., Littlewood, D.T.J., Backeljau, T., Schockaert, E.R. and Artois, T.J. 2006. Filling a gap in the phylogeny of flatworms: relationships within the Rhabdocoela (Platyhelminthes), inferred from 18S ribosomal DNA sequences. Zoologica Scripta. 35, 1-17.

Yamazaki, M., Susumu, A., Murase, J. and Kimura, M. 2012. Phylogenetic diversity of microturbellarians in Japanese rice paddy fields, with special attention to the genus *Stenostomum*. Soil Science and Plant Nutrition. 58, 11-23.

Young, J.O. and Kolasa, J. 1974. Studies on the genus *Stenostomum* O. Schmidt (Turbellaria; Catenulida). Freshwater Biology. 4, 167-176.