Systematics and Phylogeny of Sessile Rotifers (Rotifera, Monogononta, Gnesiotrocha)

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A Thesis Submitted in Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Biology
Prince of Songkla University
2015

Thesis Title	Systematics and phylogeny of sessile rotifers (Rotifera, Monogononta, Gnesiotrocha)
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ชื่อวิทยานิพนธ์ การจัดระบบทางชีววิทยาและสายสัมพันธ์ทางวิวัฒนาการของโรติเฟอร์จำพวกยึดเกาะ (Rotifera, Monogononta, Gnesiotrocha)

ผู้เขียน นายภูริพงศ์ เมฆสุวรรณ

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บทคัดย่อ

การศึกษาครั้งนี้ทดสอบสมมติฐานในการจัดจำแนกทางอนุกรมวิธานและศึกษารูปแบบทางวิวัฒนาการของลักษณะทางสัณฐานวิทยาของโรติเฟอร์จำพวกยึดเกาะโดยใช้สายสัมพันธ์ทางวิวัฒนาการเชิงโมเลกุลและการวิเคราะห์ลักษณะทางสัณฐานวิทยาอื่น ๆ ประกอบการพิจารณา ซูเปอร์ออร์เดอร์ Gnesiotrocha จำนวน 40 สปีชีส์ ใช้เป็น ingroup และสมาชิกของซูเปอร์ออร์เดอร์ Pseudotrocha จำนวน 10 สปีชีส์ ใช้เป็น outgroup ลักษณะทางสัณฐานวิทยาที่ศึกษา ได้แก่ รูปร่างและจำนวนพูของโคโรนา การปรากฏของ modulus และ oviferon รูปแบบของโทรฟี ความสมมาตรของโทรฟี ระดับความแตกต่างของฟัน unci รูปแบบการดำรงชีวิต และรูปแบบการสร้างโคโลนี สำหรับข้อมูลทางโมเลกุล ใช้ลำดับนิวคลีโอไทด์ของยีน 18S rRNA ของทุกชนิดที่ศึกษา โดยลำดับยีนของ 40 สปีชีส์ ได้จากการวิเคราะห์ในการศึกษาครั้งนี้ และอีก 10 สปีชีส์ ได้จากฐานข้อมูล GenBank การสร้างสายสัมพันธ์ทางวิวัฒนาการใช้วิธีวิเคราะห์ Bayesian inference, Maximum likelihood และ Neighbor-joining สายสัมพันธ์ทางวิวัฒนาการของยีนที่ได้ ถูกนำมาใช้ในการอนุมานความสัมพันธ์ทางวิวัฒนาการของโรติเฟอร์จำพวกยึดเกาะที่ศึกษา

จากการเก็บตัวอย่างในแหล่งน้ำจืดจำนวน 18 แหล่ง ในภาคต่างๆ ของประเทศไทย ได้แก่ ภาคเหนือ (3 แหล่ง) ภาคตะวันออกเฉียงเหนือ (5 แหล่ง) ภาคกลาง (1 แหล่ง) และภาคใต้ (9 แหล่ง) พบโรติเฟอร์จำพวกยึดเกาะทั้งสิ้น 41 สปีชีส์ ในจำนวนนี้เป็นสปีชีส์ใหม่ 1 สปีชีส์ ซึ่งได้บรรยายลักษณะและตีพิมพ์เผยแพร่ในการศึกษาครั้งนี้ ส่วนอีก 1 สปีชีส์เป็นสปีชีส์ที่พบครั้งแรกในเขต Oriental และประเทศไทย จากการศึกษาสายสัมพันธ์ทางวิวัฒนาการพบว่า ทุกสปีชีส์ที่ศึกษาในกลุ่ม

gnesiotrochan เป็นวงศ์วานเดี่ยวและมีค่าความเชื่อมั่นสูง สปีชีส์ในออร์เดอร์ Collothecacea เป็นวงศ์วานเดี่ยว โดยในออร์เดอร์นี้ ตัวแทนของแฟมิลี่ Atrochidae เป็นกลุ่มพี่น้อง (sister group) กับสปีชีส์หนึ่งในแฟมิลี่ Collothecidae ในขณะที่ ออร์เดอร์ Flosculariacea ไม่เป็นวงศ์วานเดี่ยว แต่สมาชิกแบ่งออกเป็น 2 เชื้อสาย (lineage) สายแรกประกอบด้วยสมาชิกของจีนัส *Beauchampia*, *Limnias* และสปีชีส์ใน *Ptygura melicerta* group ของแฟมิลี่ Flosculariidae (เรียกสมาชิกในสายนี้ว่ากลุ่ม BLP) นอกจากนี้ สายนี้เป็นกลุ่มพี่น้องกับเชื้อสายของออร์เดอร์ Collothecacea สายที่สองประกอบด้วยสมาชิกจากทุกแฟมิลี่ในออร์เดอร์ Flosculariacea และจีนัสที่เหลือของแฟมิลี่ Flosculariidae ผลการวิเคราะห์ภายในสายนี้ไม่สามารถแสดงความสัมพันธ์ที่ชัดเจนระหว่างแฟมิลี่ต่าง ๆ ได้ ยกเว้นความสัมพันธ์ระหว่าง แฟมิลี่ Conochilidae กับ Flosculariidae โดยที่แฟมิลี่ Conochilidae ใกล้ชิดกับสมาชิกจำนวนหนึ่งของจีนัส *Ptygura* ของแฟมิลี่ Flosculariidae สำหรับการวิเคราะห์รูปแบบทางวิวัฒนาการของลักษณะทางสัณฐานวิทยาพบว่า ลักษณะที่ปรากฏในสายวิวัฒนาการหลายครั้งและไม่ควรนำมาใช้ในการจัดจำแนกทางอนุกรมวิธานในขั้นสูง (higher levels) ได้แก่ รูปร่างและจำนวนพูของโคโรนา โทรฟีประเภท malleoramate ระดับความแตกต่างของฟัน unci รูปแบบการดำรงชีวิต และการสร้างโคโลนี ในขณะที่ลักษณะที่ต้องได้รับการศึกษาเพื่อเติมโดยเฉพาะการใช้สปีชีส์ตัวแทนจำนวนเพิ่มขึ้น ได้แก่ การปรากฏของ modulus และ oviferon และความสมมาตรของโทรฟี

การศึกษาครั้งนี้สรุปได้ว่า ซูเปอร์ออร์เดอร์ Gnesiotrocha เป็นกลุ่มที่แท้จริงในไฟลัม Rotifera ผู้วิจัยเสนอให้ 3 วงศ์วานหลัก ได้แก่ Collothecacea, กลุ่ม BLP และ แฟมิลี่และจีนัสอื่น ๆ ที่เหลือของ Flosculariacea รวมอยู่ในออร์เดอร์เดียวกัน คือ ออร์เดอร์ Flosculariacea สำหรับระดับแฟมิลี่ ได้พิจารณาให้ Atrochidae และ Conochilidae เป็นชื่อพ้องของ Collothecidae และ Flosculariidae ตามลำดับ ส่วนกลุ่ม BLP เป็นแฟมิลี่ใหม่ในออร์เดอร์นี้ สำหรับระดับจีนัส ชื่อจีนัส “*Ptygura*” เป็นชื่อที่ถูกต้องของสปีชีส์ *P. melicerta* group ในกลุ่ม BLP ส่วนสมาชิกสปีชีส์อื่น ๆ ที่เคยอยู่ในจีนัส *Ptygura* รวมทั้ง *P. crystallina* ควรใช้ชื่อ “*Oecistes*” เนื่องจากเป็นชื่อที่เคยใช้ตั้งจีนัสใหม่ให้แก่ *P. crystallina*

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Major Program Biology
Academic Year 2014

ABSTRACT

The present study aims at testing hypotheses in the current classification, as well as interpreting evolutionary patterns of morphology of sessile rotifers. This is performed using molecular phylogenetic approaches, supplemented by analysis of morphological features. Forty species of Superorder Gnesiotrocha are included as ingroup, and 10 species of Superorder Pseudotrocha as outgroup in the present analysis. The morphological characters considered include corona shape and number of its lobes, presence of modulus and oviferon, trophi type, symmetry of trophi, differentiation of unci teeth, life habit and colony formation. Regarding molecular data, nucleotide sequences of 18S rRNA gene of all the species were obtained. The gene sequences of 40 species were sequenced, while of 10 species were acquired from GenBank database. Phylogenetic tree of the gene was reconstructed using different methods including Bayesian inference, Maximum likelihood, and Neighbor-joining analysis. The gene trees obtained were used to infer the phylogeny of the representative taxa.

From specimen collection in 18 freshwater habitats (FHs) in different parts of Thailand including Northern (3 FHs), Northeastern (5 FHs), Central (1 FH) and Southern (9 FHs) part, a total of 41 species of sessile rotifers was identified. Of these, one is new to science and formally described in the present study, and one is new to Oriental region and Thailand. Regarding phylogenetic analysis, the results reveal that all of the gnesiotrochan representatives form a monophyletic clade with strong support. Species of Order Collothecacea form a single clade in which representative of Family Atrochidae forms sister group to a species of Family Collothecidae. In contrast, Order Floscullariacea is not monophyletic but the representatives belong to two different lineages. The first lineage is composed of genus *Beauchampia*, *Limnias*

and species of *Ptygura melicerta* group of Family Flosculariidae (this lineage is called the BLP group). Moreover, the lineage forms sister group to a lineage of Collothecacea. The second lineage consists of all families of Flosculariacea and the remaining genera of Flosculariidae. In this second lineage, relationships among families are not completely resolved in the present study, except for the relationship between Conochilidae and Flosculariidae. A monophyletic clade of Conochilidae is located within a clade formed by some species of genus *Ptygura* of Flosculariidae. Regarding the analysis of evolutionary patterns of morphological characters, the characters that are evolved several times and are therefore homoplastic should not be used for taxonomic grouping at higher levels. These include corona shape and number of its lobes, malleoramate trophi, differentiation of unci teeth, life habit and colony formation. Meanwhile, characters that would benefit from more extensive examination include presence of modulus and oviferon, and symmetry of trophi.

According to the present study, Superorder Gnesiotrocha is a valid taxon in Phylum Rotifera. I propose to recognise a single Order Flosculariacea containing 3 major clades, namely Collothecacea, the BLP group and the remaining families and genera of Flosculariacea. At family level, I consider Atrochidae and Conochilidae as synonyms of Collothecidae and Flosculariidae, respectively, and propose the BLP group as a new family in this order. At the generic level, "*Ptygura*" is the correct name for species of *P. melicerta* group of the BLP group. Meanwhile, the name "*Oecistis*" applies to the remaining species of the traditional "*Ptygura*", as this group includes *P. cystallina* which is the generotype of "*Oecistis*".

ACKNOWLEDGEMENTS

This thesis was accomplished with kindly assistance from the persons and organizations addressed in the following.

I would like to express my gratitude to my advisor, Assoc. Prof. Dr. Pornsilp Pholpunthin, Department of Biology, Faculty of Science, Prince of Songkla University (PSU). He has provided me scientific thinking, as well as ethics awareness in doing biological research. He has brought me to research grants, making the present work possible. Moreover, he has let me see how scope of a research should be and how a smart work is. A good solution of any problem of me is usually suggested by him.

My deep gratefulness goes to my co-advisor, Dr. Hendrik Segers, Royal Belgian Institute of Natural Sciences, Brussels, Belgium, who has sharpened my thought and developed my scientific attitude. He has taught me how a piece of work can be published and how inadequate is. All scientific articles published during my study, as well as this thesis were enriched by him. In addition, he has guided me through challenge research gaps, opening research opportunities to me for the future. His expertise in rotifer taxonomy and biology in general inspires me to be a smart scientist as he is.

My special appreciation goes to the examining committee, Prof. Dr. La-orsri Sanoamuang (chairperson), Department of Biology, Faculty of Science, Khon Kaen University; Prof. Dr. Saowapa Angsupanich, Department of Aquatic Science, Faculty of Natural Resources, PSU; Asst. Prof. Dr. Warapond Wanna, Department of Molecular Biotechnology and Bioinformatics, Faculty of Science, PSU, for their constructive comments and valuable suggestions on this thesis.

I am extremely thankful to Mr. Kittisak Thongpijit, an undergraduate student (at that time) of Department of Molecular Biotechnology and Bioinformatics, Faculty of Science, PSU, who assisted me in doing molecular techniques, especially DNA extraction procedure at the beginning of this work. My first understanding of molecular practice was provided by him.

My special appreciation goes to Assoc. Prof. Dr. Nunta Churngchow and Dr. Nion Chirapongsatonkul, Department of Biochemistry, Faculty of Science, PSU, for a practical training of several molecular techniques, including primer design, PCR, gel electrophoresis, DNA cloning, DNA purification, preparation for DNA sequencing, with the chemicals and facilities during the beginning of this work. The appreciation also goes to their Ph.D. students, including Miss Thitikorn Chanwun, Miss Orawan Bunyatang, Miss Nisaporn Muhamad, Miss Uraiwan Khunjan, and Mr. Kittichon U-taynapun, Department of Aquatic Science, Faculty of Natural Resources, PSU, for their kindly helped along all molecular manipulations mentioned above with useful practical techniques and several essential concepts concerned.

I am extremely thankful to Dr. Russell J. Shiel, Department of Ecology and Evolutionary Biology, University of Adelaide, Australia, and his Ph.D. candidate, Ms. Deborah Furst, who kindly provided me sediment of Australian wetlands.

I feel very much grateful to the Freshwater Laboratory, Royal Belgian Institute of Natural Sciences (RBINS), Brussels, Belgium, and the RBINS museum in providing me working space and new experience of both exhibiting specimens and all necessary facilities. My knowledge of evolutionary history was broadened here. Moreover, I would like to thanks Dr. Valentina Pieri, Mr.Rylan Shearn, Miss Tasnim Patel, Miss Marie Verheye, Miss Severine Fourdrilis, and Mrs. Piyatida Pimvichai, who were working at the RBINS in that time, for their kind suggestions on living in Brussels, for suggestion in English usage, and for cultural exchange.

My special appreciation goes to Dr. Pavel Škaloud, Department of Botany, Charles University, Czech Republic, for his suggestions and comments on the phylogenetic analyses, partly, in this work.

I would like to express my thanks to staff of the Scientific Equipment Centre and the GEO-Informatics Research Center for Natural Resource and Environment (Southern Regional Center of Geo-Informatics and Space Technology) (SouthGIST), especially Mrs. Ratana Tongyoi, for their excellent services.

My deep gratefulness goes to colleagues in laboratory of Ecology and Molecular Evolution Research Unit, including Miss Bongkot Wichachucherd, Mr. Kwan Nualcharoen and Mr. Patamarerk Engsontia, for their useful suggestions during processing all molecular procedures in the lab and spending time for discussion on phylogenetic concepts and evolution.

Special thanks go to colleagues in laboratory of Plankton Research Unit, namely Asst. Prof. Dr. Supiyanit Maiphae, Dr. Rujinard Sriwoon, Miss Phannee Saardrit, Miss Koraon Wongkamhaeng, Mr. Eknarin Rodcharoen, Mrs. Ratana Hirunphan, Miss Wijitra Choedchim, Miss Thanida Saetang, Miss Rapeepan Jaturapruek and Miss Sineepan Sukpol, for their friendly help with anything and encouragement during my study making the lab as a big warm family.

I am extremely thankful to Department of Biology, Faculty of Science, PSU, for the excellent facilities and all departmental administrations for their great services.

I am grateful to Graduate School, PSU, Hat Yai campus, for the financial supports including the PSU-Ph.D. Scholarship, the Graduate School Research Supporting Funding for Thesis, and the Oversea Research Scholarship. I am sincerely thankful Faculty of Science and Prince of Songkla University, Hat Yai campus, for everything shaping me to this position.

I very much respect all animals which were used to develop the knowledge in this research arena (i.e. rotifer biology). The knowledge would be meaningful for mankind in general.

My heart-felt appreciation goes to my parents, Mr. Prasit Meksuwan and Mrs. Urai Meksuwan who have been shaping me to have intellectual maturity as well as moral awareness. All my success is owed to them. I would like to thank my elder sister, Miss Wipawee Meksuwan, my relatives, and Miss Yurachat Yodyotee, for their kind encouragement, care, and love.

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CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Sessile rotifers are aquatic pseudocoelomate micrometazoans that live permanently attached to diverse substrata (Wallace, 1980, 2002). They are important not only for ecosystem functioning, as primary and secondary consumers and nutrient re-cyclers, but also as test organisms in general biological studies. In particular, they are of interest for the study of evolution in fixosessile organisms and of the associated adaptations. In the group, these are the modified corona, elongated body and foot with attachment organ, an ability to discriminate their substratum, and colony formation in many of them (Wallace, 1978, 1980, 1987, 2002; Wallace and Edmondson, 1986). Although they are, for convenience, grouped by their fixosessile mode of life, taxonomically they are assigned to three families of two orders, namely Atrochidae, Collothecidae, and Flosculariidae, each of which is characterised by a number of hypothesized syn- or autapomorphic attributes (Koste, 1978; Segers, 2002a; Segers and Shiel, 2008). However, Wallace (1980) suggested to include the planktonic colonial family Conochilidae in the group, considering his hypothesis that these are advanced planktonic, colonial rotifers that evolved from colonial fixosessile ancestors, a condition that can also be seen in some species within Collothecidae and Flosculariidae. Accordingly, the number of species recorded worldwide in the group of so-called sessile rotifers attains one hundred fourteen valid species (Segers, 2007; Segers and Shiel, 2008; Meksuwan et al., 2011, 2013).

At present, the diagnosis and classification of families and genera of sessile rotifers is based mostly on external morphology, in particular corona shape, number of corona lobes, corona dorsal gap, and lateral antenna features (e.g., Thorpe, 1893; Harring, 1913; Vidrine et al., 1985; Segers and Shiel, 2008; Meksuwan et al., 2011). However, this approach has been challenged as the biological relevance of the features concerned is unclear, or because there are indications that several taxa are paraphyletic. For instance, Segers and Shiel (2008) reported on a preliminary

molecular analysis that appeared to contradict the results of a morphological analysis in which species belonging to two genera, clearly diagnosed by what appeared to be different autapomorphic corona features, turned out to belong to the same clade. An analysis of the morphological diagnosis of genera in Flosculariidae revealed several instances where the diagnoses are inconsistent, indicating that some of the genera may very well be paraphyletic (Meksuwan et al., 2011). Moreover, although the monophyly of superorder Gnesiotrocha – the taxon that includes all sessile rotifer families and a number of non-sessile, planktonic families – has been accepted by most contemporary authors, there are disagreements regarding relationships among its family- and order-group taxa. For example, a preliminary study based on molecular data found that the two extant sessile families of order Collothecacea, that share ambush predation feeding with uncinate trophi, are unrelated (Segers, H., personal communication). In order Flosculariacea, some findings indicate a sister group of a planktonic and a sessile family, leaving both "planktonic" (Hexarthridae+ Testudinellidae+Trochosphaeridae) and "sessile" (Flosculariidae+Collothecacea) group being arbitrary groupings (Kutikova, 1983; Sørensen and Giribet, 2006). This evidence, of both morphological and molecular nature, illustrates the need for a revision of the phylogeny of sessile rotifers, in particular regarding higher taxonomic levels, in an attempt to come to a classification that reflects the evolutionary history of the group.

Evolutionary history of characters and life styles among sessile rotifers is also a big challenge. There is an ongoing debate on the direction of evolution of life styles, from solitary, fixosessile, colonial sessile and planktonic colonial, in conjunction to the feeding modes of the taxa concerned such as filter feeders and ambush predators. Moreover, although a previous study proposed that the sessile condition is pre-adaptive for planktonic solitary and colonial taxa or even ancestral for all monogononts (Kutikova, 1983; Wallace, 1987), empirical phylogenetic evidences for this conclusion, especially support from molecular data, is lacking.

The present study therefore aims at overcoming the gaps in our knowledge on the taxonomy and the evolution among sessile rotifers. To accomplish this, it will apply the approach of phylogenetic inference, a scientific method that not only aims at uncovering the phylogeny of the investigated organisms but also guides systematicists in grouping and ranking processes to construct a hypothesis reflecting a natural classification as well as to reconstruct character evolution (e.g., Bryant, 2001; Segers and Wallace, 2001; Sørensen and Giribet, 2006). The method's strength lies in the combined use of molecular (gene sequence) data and morphological data. Regarding molecular data, sequences of the same gene will be acquired from representatives of as many as possible genera, families and orders of sessile rotifers, and these will be compared with representatives of the planktonic families of Gnesiotrocha, and with representatives of order Monogononta for outgroup comparison (Lemey et al., 2009). Regarding morphology, I will include external morphological features as well as trophi structure, which will be examined using light and scanning electron microscopy. This combined approach should enable us to formulate new and more robust hypothesis on the taxonomy and the evolution of sessile rotifers.

1.2 Literature review

1.2.1) General features of sessile rotifers

Most species of sessile rotifers carry a relatively large, expanded corona (Figs 1a, 2a) compared to other rotifers (see Koste, 1978). The shape of corona varies among taxa, and can be circular-, kidney- or heart-shaped, or can be expanded to form lobes, which again vary in number: there are two-, three-, four-, five-, seven-, and eight-lobed coronas. In order Collothecacea, the dorsal lobe is generally largest (Fig. 1C: a). In order Flosculariacea, a dorsal interruption of the bands of cilia of the corona (Fig. 2A: b) can be tiny to wide, and a ventral sinus (Fig. 2C: c) between the ventral corona lobes can be shallow to deep. When the animals are disturbed, the corona is invaginated into their trunk (Fig. 1D: f).

Trunk and foot of sessile rotifers are usually clearly separated. Lateral antennae are located on the trunk and can be tiny to remarkably long (Fig. 2C: d). A single dorsal antenna of most species is small except for *Beauchampia crucigera* possessing relatively long dorsal antenna (Koste, 1978), located dorsally on the truck, and serves as a sensory organ that its specific function has not been known. The foot is elongated, it can be short to very long, and is terminated by an attachment organ with a stalk (Figs 1A: c, 2C: e) that can be small and hardly discernible to remarkably long (e.g., *Collotheca campanulata longicaudata*, see Fig. 4I in Meksuwan et al., 2013).

The trophi of sessile rotifers can be attributed to two types, uncinate (Fig. 1E) and malleoramate (Figs 2D-2E). Uncinate trophi comprise of two pairs of unci (Fig. 1E: g) and other components (fulcrum, rami, and manubria) that are very less noticeable comparing to the unci (Fig. 1E: h). The pairs of remaining unci can vary in size, shape, and head structure (Meksuwan et al., 2013). Its hook-like structure serves their ambush predation feeding habit. The species containing uncinate trophi carry a wide, funnel-shaped infundibulum (Fig. 1A: i) – an expansion of buccal region. They wait until mobile prey gets into contact with sensory organs on the inner surface of the open infundibulum. The infundibulum is then closed and the prey is swallowed by pumping action of a membrane supported by the trophi. Some species (e.g., *Acyclus*

inquietus) settle within a colony of a specific target-species of other sessile rotifers (e.g., *Sinantherina socialis* is the target of *A. inquietus*), and feed on eggs in the colony. Moreover, an undescribed relative of *A. inquietus* takes adult specimens in the colony of *Lacinularia flosculosa* as prey (Meksuwan et al., 2011). At present, the species carrying uncinate trophi are united within order Collothecacea (Wallace et al., 2006).

In malleoramate trophi, the unci are composed of transverse rows of teeth which can be thin, several separated teeth (Fig. 2D: i) to a few, robust connected teeth (Fig. 7D). The manubria are connected to the unci (Fig. 2D: j). It is as prominent as the unci and other components but without elongated shape as in some types of trophi such as virgate and forcipate (Wallace et al., 2006). Transverse ridges (Fig. 2E: k) on the manubria form three chambers where are related to the musculature of the mastax. Another major element is the incus which is composed of rami (Fig. 2E: l) and the single fulcrum (Fig. 2E: m). The rami are triangular in appearance and teeth-like scleropili are formed along more or less the distal half of the inner margins (Fig. 2E: n). In many taxa, the proximal tips of the rami are protruded forming alula (Fig. 2o). The fulcrum is conspicuous (Fig. 2E: m). This element supports the rami in a forceps-like functioning. This structure of malleoramate trophi supporting the mastax musculature serves for a crushing function in conjunction with its filter feeding habit. At present, all taxa containing malleoramate trophi are united in order Flosculariacea.

Sessile rotifers grow by two stages, planktonic juvenile (Figs 1B, 2B) and settled adult (Figs 1A, 1C, 2A, 2C). A mobile juvenile hatching from either a parthenogenetic egg (Fig. 1C: d) or a resting egg (explained below) swims using its trochus cilia and finds a proper substratum to attach using sensory apparatus located on the corona region (Wallace, 1980). After permanently attaching to the surface, the juvenile develops to be a mature female. A population is usually dominated by settled, mature females that produce parthenogenetic eggs – the *amictic* females. In certain ambient conditions however, some parthenogenetic eggs (if not all) develop into physiologically-different females so-called the *mictic* females that produce haploid mictic eggs. If the mictic eggs are not fertilized by a male, they develop into haploid males. On the other hand if the male meet the mictic females, it does copulation and transfers its sperms to fertilize the eggs. The fertilized eggs then undergo development

into a particular type of diapausing eggs that possess thick shell and can resist drastic environment for embryo survival as well as for dispersal advantage – the resting eggs. When a certain clue(s) of the "normal" environment is present, the juveniles hatch from the resting eggs and amictic life dominate again. Besides these two cycles, amictic and mictic phase, there is a certain type of female reported which is the *amphoteric* female. This kind of females can produce either of amictic and mictic eggs. The females may be occurred in the way as similar as production of mictic females (i.e. stimulated amictic eggs), but must be a different manner (Wallace et al., 2006). The females are reported in many species, including sessile rotifers (Gilbert, 1977; Wallace et al., 2006). Furthermore, taxonomically all species of sessile rotifers, as well as most other rotifers (i.e. monogonont group) are diagnosed by female characters. The males are present in a short period, and they have never been observed in a majority of the monogonont species including several sessile rotifers (Koste, 1978; Wallace et al., 2006).

Sessile rotifers can create a transparent gelatinous case (Fig. 1C: e), or a dense case in which various kinds of debris are incorporated (Fig. 2C: f), to form an almost opaque construction (fig. 2A: g), or pellet-building tube (Figs 28D-F). Some species live solitary (Figs 1A, 1C, 1D, 2C) while some form a colony which can be pseudocolony (Fig. 2A), or a (true) colony (Fig. 30) (see Table 4 for details).

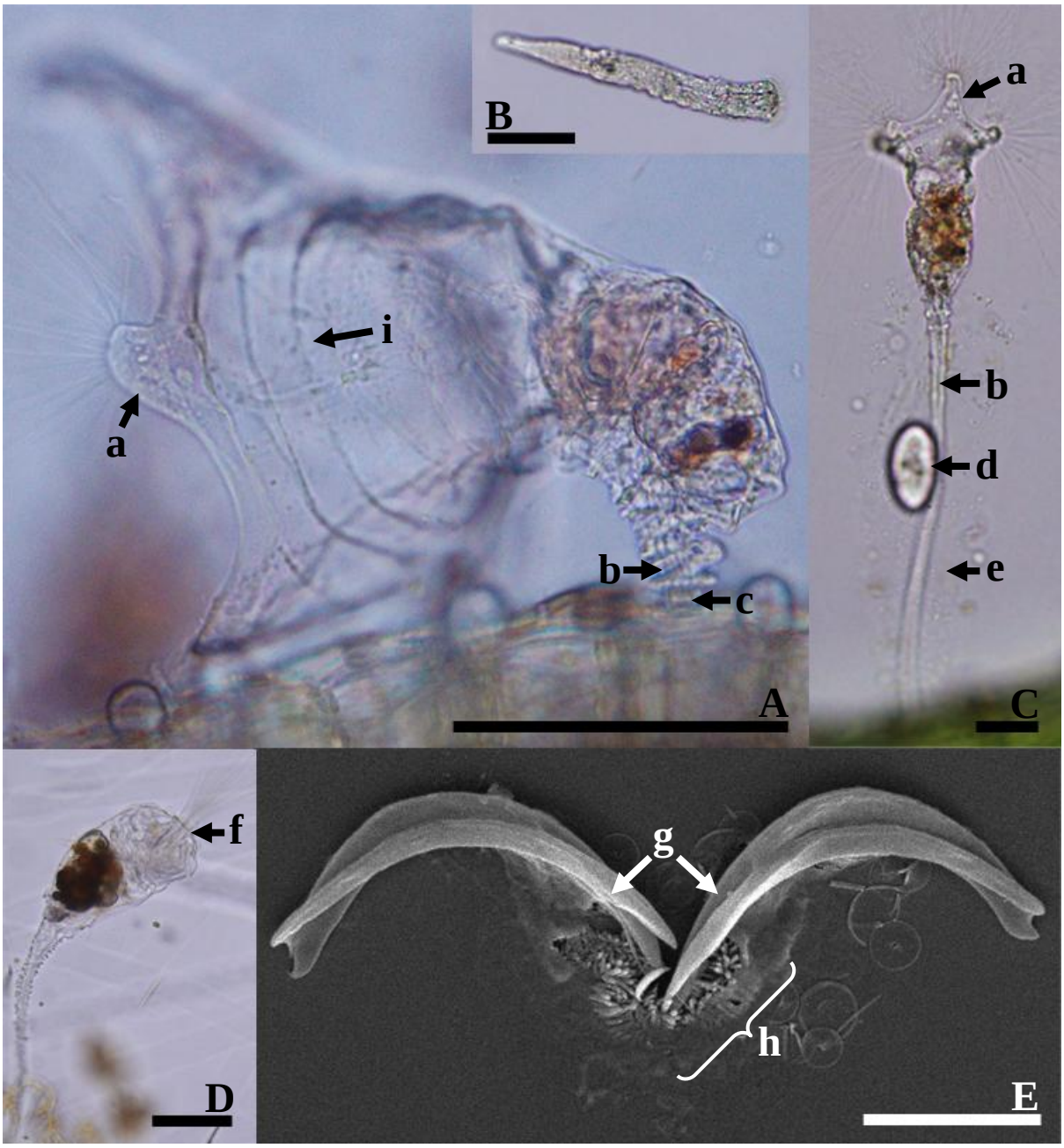


Figure 1 General characters of Collothecacea's species. A: *Collotheca orchidacea* (lateral view); B: a planktonic juvenile of *Acyclus inquietus*; C: *C. ornata* (dorsal view); D: *C. campanulata campanulata* (lateral, contracted); E: uncinate trophi of *C. tenuilobata* (the view could not be specified). The arrows: a: corona lobe; b: foot; c: attachment organ (stalk); d: a parthenogenetic egg; e: inhabiting gelatinous case; f: collar; g: unci teeth; h: other components of the trophi; i: infundibulum. Scale bars: A-B, D = 100 µm., C = 50 µm., E = 5 µm.

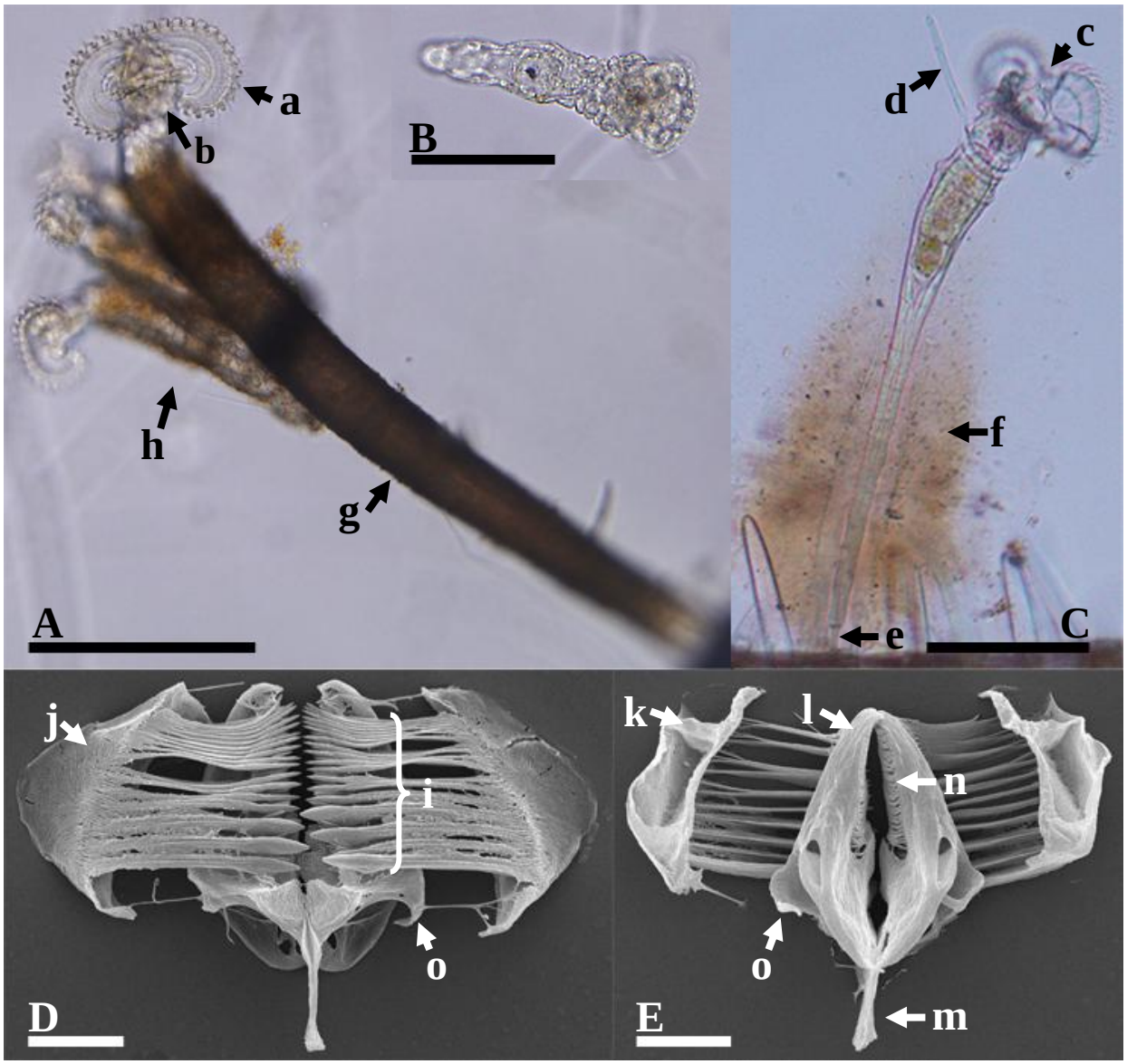


Figure 2 General characters of Flosculariidae's species. A: *Limnias ceratophylli* (dorsal view) with its pseudocolony's members; B: a planktonic juvenile of *Sinantherina socialis*; C: *Ptygura thalenoiensis* (dorsal-lateral view); D-E: malleoramate trophi of *S. socialis* (frontal and caudal view, respectively). The arrows: a: corona; b: dorsal gap of corona; c: ventral sinus of corona; d: lateral antenna; e: attachment stalk; f-g: inhabiting case with debris; h: the pseudocolony's members; i: unci teeth; j: manubrium; k: the transverse ridge; l: ramus; m: fulcrum; n: the teeth-like scleropili; o: the alula. Scale bars: A = 200 µm., B-C = 100 µm., D = 10 µm.

1.2.2) Taxonomic research on sessile rotifers

The number of sessile rotifer species described has increased gradually by rotifer taxonomists since more than a couple of century ago (e.g., Hudson and Gosse, 1886; Thorpe, 1893; Edmondson, 1939, 1940; Segers, 1997; Segers and Shiel, 2008; Meksuwan et al., 2011, 2013). A major progress on classification and nomenclature of the group was made by Harring (1913) who recognized order Flosculariacea, order Collothecacea, a number of sessile families as well as planktonic malleoramate families, and many sessile genera which are still accepted nowadays (Segers, 2002a; Wallace et al., 2006). The Flosculariacea and Collothecacea are characterized by their members possessing malleoramate and uncinate trophi, respectively. The former taxon is composed of five families of which only two, Conochilidae and Flosculariidae, are defined as sessile families, while the latter taxon consists of the two sessile families only, Atrochidae and Collothecidae. These families are different in their morphology and ecology, in particular regarding the structural orientation of filtering corona (i.e. Conochilidae), complex case building (i.e. Flosculariidae), modified funnel-shaped buccal region (the infundibulum) with corona lacking of cilia (i.e. Atrochidae) or with long cilia on the corona rim (i.e. Collothecidae) (Koste, 1978; Segers and Wallace, 2001; Wallace et al., 2006; Meksuwan et al., 2013).

Based on external morphology, Koste (1978) summarized the generic diagnosis in the family Flosculariidae to corona features, including shapes, number of lobes and dorsal gap size, and some distinct shared features such as presence of an egg-carrying organ (the oviferon). The genera in Conochilidae are also diagnosed by corona feature, and location of dorsal antennae and mouth (Segers and Wallace, 2001). Corona morphology is also considered taxonomically significant for the genera of family Atrochidae and Collothecidae, but life style is important as well (Koste, 1978). For example, *Collotheca* contains broad modified cup-shaped corona with long setae along a number of corona projections and lobes, while *Acyclus* has a modified large dorsal lobe without setae and inhabits colonies of specific species of their sessile rotifer prey (Koste, 1978; Meksuwan et al., 2011).

Recently, another major progress on classification and nomenclature was by Segers (2002a, 2007) who revised all valid name of Phylum Rotifera including sessile rotifers, with their synonymy and proposed a classification of the Phylum that has been widely accepted (Wallace et al., 2006).

As early as 2008, however, Segers and Shiel analyzed SSU rDNA gene of some sessile rotifer species. They found that the species of the new genus *Pentatrocha* (five-lobed corona) fell within the clade formed by the two species of genus *Sinantherina* (circular or heart-shaped corona). Moreover, the two genera share the presence of an oviferon, which the authors interpreted as being synapomorphic. This result casts doubt on the current taxonomic approach among sessile rotifer taxa, least at the generic level, where the diagnosis largely refers to corona shape and number of corona lobes (Koste, 1978). In addition, Meksuwan et al. (2011) recognized some characters of the *Ptygura melicerta* group, such as presence of a stiffened tegumental plate located anterior-dorsally of the body, that seem indicating that they are not ingroup of the genus, meanwhile at present they are included in the genus by the similar corona appearance. In genus *Floscularia*, moreover, *F. melicerta* has been included into the genus by its four-lobed corona, but the species lacks of a modulus – the organ that is used to build the pellet tube and that is hypothesized as being syn- and autapomorphy of this genus. These observations indicate that the taxonomic approach in the group, relying mostly on external morphology, may need to be reconsidered especially in the way that other independent characters, such as molecular data, are included before delimiting and ranking of sessile rotifer taxa at any level.

The current classification of sessile rotifers according to Segers (2002a, 2007), Segers and Shiel (2008) and Meksuwan et al. (2011) is revealed below (* = families of sessile rotifers).

Subclass Monogononta Plate, 1889

Superorder Gnesiotrocha Kutikova, 1970

Order Flosculariacea Harring, 1913

*Conochilidae Harring, 1913

Conochilopsis Segers and Wallace, 2001 (1 sp.)

Conochilus Ehrenberg, 1834 (6 spp.)

*Flosculariidae Ehrenberg, 1838

Beauchampia Harring, 1913 (1 sp.)

Floscularia Cuvier, 1798 (9 spp.)

Lacinularia Schweigger, 1820 (3 spp.)

Lacinularoides Meksuwan et al., 2011 (1 sp.)

Limnias Schrank, 1803 (5 spp.)

Octotrocha Thorpe, 1893 (1 sp.)

Pentatrocha Segers & Shiel, 2008 (1 sp.)

Ptygura Ehrenberg, 1832 (28 spp.)

Sinantherina Bory de St. Vincent, 1826 (6 spp.)

Hexarthridae Bartos, 1959

Testudinellidae Harring, 1913

Trochosphaeridae Harring, 1913

Order Collothecacea Harring, 1913

*Atrochidae Harring, 1913

Acyclus Leidy, 1882 (2 spp.)

Atrochus Wierzejski, 1893 (1 sp.)

Cupelopagis Forbes, 1882 (1 sp.)

*Collothecidae Harring, 1913

Collotheca Harring, 1913 (47 spp.)

Stephanoceros Ehrenberg, 1832 (1 sp.)

1.2.3) Research on phylogeny and evolution of sessile rotifers

Based on morphological data such as feeding type, locomotion strategy, direction of cilia movement and trophi structure and function, Kutikova (1983) proposed that family Flosculariidae and Conochilidae are sister group and she interpreted that the latter probably originated from a Floscullariidae sessile ancestor. She placed the two taxa in a clade forming order Floscullariacea (but used the term Monimotrochida) which forms a sister group of Bdelloidea. In Kutikova's (1983) hypothesis, order Collothecacea (or her Paedotrocha) is sister taxon to the clade of Floscullariacea and Bdelloidea, and these three lines together constituted Superorder Gnesiotrocha. Superorder Pseudotrocha is then proposed as sister group to the Gnesiotrocha, and these two together are considered member descendants of the Eurotatoria. According to different analyses, not only the sister group relation between Floscullariacea and Bdelloidea, but also the inclusion of bdelloids for all within the Gnesiotrocha has been rejected by all contemporary rotiferogists (e.g., Segers, 2002a; Sørensen, 2002; Sørensen and Giribet, 2006), including in subsequent works of the author herself (see below).

Wallace and Colburn (1989) firstly performed a cladistic analysis of the order-group taxa of the Rotifera. In their analysis, several anatomical data such as number of gonads, presence of vitellarium and prostate glands and trophi type were included. The result revealed that order Floscullariacea and Collothecacea are sister taxa (forming the Gnesiotrocha group). This clade then connects to Ploima, and the three orders together form a sister group to Bdelloidea, establishing the Eurototoria. The relationship can be represented by this formula:

(((Floscullariacea,Collothecacea),Ploima),Bdelloidea)

In 1993, Kutikova and Markevich proposed an alternative evolutionary scheme among sessile rotifer taxa based on the sclerite mastax system. The Floscullariidae (they also included all planktonic malleoramate families: e.g., Hexarthridae, Testudinellidae, in this taxon) and the Conochilidae were sister group, but both taxa were raised to suborder category. The two new suborders established a new order-level taxon Protoramida, in accordance with their hypothesis of shared

primitive-organized rami in this taxon. In their view, the name Floscullariacea (or their Monimotrochida) was rejected since it doesn't represent the identity of the taxon which was replaced by Protoramida. Moreover, they considered the Protoramida to be closest to order Ploima (or their Ploimida) and this clade formed a sister group to a few representatives of Collothecacea (or their Paedotrochida). Accordingly, Gnesiotrocha is lost in the subclass Eurotatoria where the major taxa and their relationship can be represented by this formula:

(((Protoramida,Ploima),Collothecacea),Bdelloidea)

As far as the English publications on rotifer systematics are concerned, the name Protoramida has not been used after the proposal. Moreover, based on cladistic analysis using detail morphology of the trophi, Segers and Wallace (2001) opposed the view of Kutikova and Markevich (1993), that ranked the Conochilidae at suborder level, but formally redescribed the taxon and re-established it at family level.

Segers (2002a) revised all valid family- and generic-group names and reflected the relationship among rotifer taxa by his classification that exactly reflected the phylogeny as reconstructed by Wallace and Colburn (1989):

(((Floscullariacea,Collothecacea),Ploima),Bdelloidea)

Sørensen and Giribet (2006) tried to formally evidence the relationship between Floscullariacea and Collothecacea by using DNA information in combination with morphological and anatomical data. However, they mentioned that because only a few representatives of both Floscullariacea and Collothecacea, in particular a single species of Collothecacea, was analyzed, the results end up with sister taxa between the planktonic Floscullariacea, *Filinia longiseta*, and the single representative of Collothecacea, *Collotheca campanulata*. Moreover, paraphyly of Conochilidae resulted from turning out to be sister taxa between *Conochilus hippocrepis* and *Sinantherina ariprepes*. Theses outcomes were questioned even by the authors. The results lead the present study to add more representatives of the two orders in the molecular phylogenetic analysis.

Knowledge of character evolution among sessile rotifers has developed slowly. Wallace (1987) proposed that once sessile rotifers acquired sessile condition (note: this could be implied that he believed the ancestor of the group was mobile), several important events concerning the adaptation should have evolved, such as, development to permanent attachment, evolution of the planktonic juvenile stage, and development of substrate discrimination ability (see also Wallace, 1980). Nevertheless, at present there is no an alternative proposal of the life habit of the Gnesiotrocha ancestor.

One other aspect that has been studied is evolution of colony formation. The colonial forms in phylum Rotifera are found only in family Flosculariidae and Conochilidae. Sessile Flosculariidae species form colonies to different degrees, but some species have been observed only solitary, while most Conochilidae species form planktonic colony but also in different degrees. There are three major alternative hypotheses to explain the adaptive advantage of colony formation. These are Energetic advantage, Predator avoidance, and Sexuality hypothesis. Wallace (1987) hypothesized that sessile life style is preadaptive for the evolution of colony formation. Moreover, to explain the evolution of the planktonic colonial species as well as several planktonic solitary in all of the sessile families, he further proposed that predation pressure is the driving factor forcing some sessile ancestors gave rise to the planktonic life again. There has been no an argument among rotiferogists regarding the evolution of the planktonic species in the sessile families except for the life habit of the ancestor of the Gnesiotrocha including all sessile rotifers as addressed above.

1.2.4) Major schools of taxonomy and the science of phylogenetics

The following concepts and methodologies in taxonomy and phylogenetics are drawn from the literature, including Mayr and Ashlock (1991), Lipscomb (1998), Page and Holmes (1998), Mayr (1999), Futuyma (2005), and Lemey et al. (2009). However, all of the following words and sentences are written based on my understanding of these fields. If there is a misconception, it is mine.

Since the book *The Origin of Species* of Charles Darwin was published in 1859, the concept of taxonomic classification has changed from arbitrary classes of fixed organismal forms to natural groups (taxa) of modifiable forms originating by countless variation and adaptation (and also other processes such as neutral evolution) of an isolated unit of organisms. Hence, one of the major tasks of taxonomy is how to recognize such groups and classify (rank) them according to their evolutionary relationship. There are major three schools in taxonomy, which are different in their philosophical basis and methodology to recognize the natural taxa and reconstruct their historical relatedness, which are Numerical Phenetics, Cladistics, and Evolutionary Taxonomy.

Numerical Phenetics recognizes taxa by degree of similarity using *overall similarity* of an as large as possible number of organismal features. They claim that to be objective, all features applied must be treated as being of equal importance – not weighted by investigators, and the classification is more natural when more characters are used under analysis. The strength of this school is that the classification seems repeatable when it is done by different examiners, reflecting objectivity (they claim) and reaching stability property of a desire classification. However, the weakness is that they ignore the fact of similarity caused by homoplasy (e.g., convergent and reversal evolution) that is evidently abundant in nature. This appears regardless character sharing by common descent of the evolutionary theory. As a result, their grouping and ranking are theory-free approaches, and naturalness of their taxa is hardly defensible. In addition, in this approach, phylogenetic relationship is reconstructed by distance methods (see below) and is represented by phenogram. Peter H. A. Sneath and Robert R. Sokal are two of the well-known authors in this taxonomic school.

The concept of Cladistics was proposed by Willi Hennig. Originally, the concept was called “phylogenetic systematics”. This school recognizes (groups) taxa as well as their historical relationship using weighted features which are considered shared derived character(s) (synapomorphy). Ranking process is based on the branching pattern of their cladogram which is reconstructed using the synapomorphic characters under certain inference methods (classically it is done manually). Moreover, sister taxa (two monophyletic groups that derived from the same, nearest common ancestor) in the cladogram should be ranked in the same category. The strength point of Cladistics is that it discriminates homologous and analogous features as well as apomorphy and plesiomorphy, and applies only apomorphic homology that is relevant to organismal evolution, delimiting and reconstructing the historical relationships among the taxa. Thus, this reflects a theory-based approach, and the resulting classification is claimed to be natural, by being basing on the reconstructed cladogram. However, other schools argue that Cladistics considers only a half part of a hypothesized phylogeny (represented by their cladogram), which is cladogenesis (branching pattern). While, anagenesis (relative amount of subsequent character differentiation), the other half part of a phylogeny, is disregarded. Accordingly, the Cladistics classification lost several evolutionary information (e.g., plesiomorphic characters) and is less applicable. For example, Cladistics recognize crocodiles and birds as a taxon, by possessing several synapomorphic characters, although crocodiles retain several ancestral features shared also by other reptilians and look similar to other reptilians, while birds gain many modified characters which make them much different from crocodiles and other reptile groups. This recognition is argued being incomplete classification by other schools (e.g., Evolutionary Taxonomy).

Evolutionary Taxonomy is the third school. It recognizes taxa by two processes by more or less combining phenetic and cladistic approaches. Firstly, they group individuals using similarity of all available homologous features, irrespective of whether the character state is derived or primitive. Finally, these groups are tested to fit defined concept of monophyletic group – which is different from Cladistics's monophyly – before adopting them as taxa. In this school, the concept of monophyly is as follows "*A group is monophyletic if all the included species and their ancestors are derived from the most recent common ancestral species, which is also included in this taxon*". *This definition does not required that ex-groups be included in a monophyletic taxon*. In Cladistics on the other hand, a group forming by only stem taxa but not includes crown groups (i.e. ex-groups of Evolutionary Taxonomy) is not monophyletic, but paraphyletic group. In other words, paraphyletic group is accepted in Evolutionary Taxonomy as long as they share similar feature (even it is primitive characters) and came from the same common ancestor. In the example mentioned above, Evolutionary Taxonomy considers crocodiles and other reptilians as a (monophyletic) taxon (but it is paraphyletic in Cladistics, then rejecting as a taxon), while birds is a separate group to crocodiles, although phylogenetically birds and crocodiles are more related to each other than other retiles. However, it is argued that such group is still not natural if all descendants of the same ancestor are not included. Here, a phylogeny is made by both phenetic clustering and cladistic analysis (to test monophyletic group), but it represented by a phylogram, which is a branching diagram that includes degree of divergence on each branch. This approach is also known as Simpson-Mayr school.

Although phylogenetic reconstruction methods are originated from the different schools of taxonomy (e.g., Hennigian Argumentation of Cladistic analysis), at present the science of phylogenetics seem to have its own separate discipline since several methods are developed not by reference to a certain school of taxonomy, rather they are classified into *Distance* and *Character state* (or *discrete-character*) methods which are supplemented by statistical algorithms and computer power and software. Nevertheless, the modern phylogenetics and taxonomic schools are connected according to the characters used (i.e. unweighted – Phenetics – or weighted characters – Cladistics and Evolutionary taxonomy).

In distance methods, data matrix of characters of taxa in analysis is transformed into distance matrix by pairwise calculations. The distance matrix is processed by cluster analysis with different algorithms that result in a phenogram. There are two widely used distance methods including Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) and Neighbor-Joining (NJ). Pheneticists develop and use distance approach for their phylogenetic inference.

There are three widely used character state methods including Maximum parsimony, Maximum likelihood, and Bayesian inference. In these methods firstly, all possible trees are constructed (e.g., 4 taxa give 3 possible unrooted trees, 8 taxa give 10,395 trees, 10 taxa give 2,027,025 trees and so on). Then, a tree(s) which is fit to the optimal criterion of each method (below) is search using available tree-searching methods (e.g., Branch-and-Bound search, Heuristic search). The tree(s) which reach the optimal criterion is selected to be the most plausible phylogeny.

Similar to the classical cladistic analysis, Maximum parsimony (MP) method seeks for the tree(s) whose topology contains fewest steps of character changes – the most parsimonious tree(s). The MP method usually offers more than one most parsimonious trees, and this situation is resolved by tree-consensus strategies.

The other two methods are usually used with molecular data especially DNA sequences, and different models explaining how nucleotide sequences evolved have to be chosen first before starting the analysis in each method. Maximum likelihood determines structure of the tree (topology), branch lengths, and the evolutionary model that maximize the probability of our observed data set (i.e. aligned DNA sequences). The tree that contains highest likelihood score is selected to explain the phylogeny. On the other hand, Bayesian inference estimates the prior probability distribution (the *prior*) from our observed data and the fitted evolutionary model. The *prior* value then is used to determine the posterior probability distribution (the *posterior*) using the function of the Bayes' theorem. The *posterior* specifies the probability of the trees given the observed data, the model, and the prior. The tree containing the highest probability represents the most plausible phylogeny. These approaches usually produce a single tree.

Although taxonomy and phylogenetics has their own major function as demonstrated above, there is relationship between these two disciplines especially between classification and a phylogeny, for which Mayr and Ashlock (1991) concluded that *"It is not true that classification gives phylogeny but rather that an analysis of characters permits inferences on phylogeny that are used in the construction of a classification."*

1.3 Research questions

1) Does molecular phylogeny support sessile rotifer taxa at different categories (Genera, Families, Orders) in the current classification as monophyletic groups?
2) What are the evolutionary acquisition patterns of major characters and life habits among sessile rotifers?

1.4 Objectives

1) To reconstruct the phylogeny of sessile rotifer species using gene sequence data
2) To interpret the evolutionary acquisition patterns of major characters and life habits of sessile rotifers
3) To reconsider the taxonomic grouping and ranking of the current taxa of sessile rotifers

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Approach adopted in the present study

I reconstructed the phylogeny of a selected gene (below) acquired from representatives of sessile rotifers and several potential outgroups and used the gene tree to infer the phylogeny of the rotifers. To uncover evolutionary acquisition pattern of major morphological characters and life habits, each character was mapped on the gene tree by replacing species name at the terminal nodes of the tree by light and SEM photographs of morphological features. Then, the number of character state change as well as possible apomorphic and plesiomorphic states are inferred. In addition, the monophyly of taxa of sessile rotifers at different levels (genera, families and orders) was tested by concordance between the taxa and the clades recovered by the gene tree.

2.2 Freshwater habitats in Thailand explored during the present study and the sampling localities at global scale

Eighteen freshwater habitats in different parts of Thailand (Fig. 3) were explored to obtain the target species of sessile rotifers. The habitats explored ranging from small ponds (~ 10x10 m), medium-sized swamps (~ 150x150 m), relatively large lakes (~ 5x5 km), to very large lakes (~ 12x5 km). In each habitat, submerged parts of growing aquatic plants were collected and investigated to search for the sessile rotifers. Details of the freshwater habitats are provided in Table 1.

Although most specimens of species examined were from Thailand (see below), an additional material of a single species, that was also observed in Thailand, was from Australia. It was obtained by hatching sediment collected from the country by colleagues (see acknowledgements). Moreover, data on some species were obtained from GenBank database (http://www.ncbi.nlm.nih.gov). The localities of all species examined were mapped in Figs 3 and 4 and the details are provided in Tables 1 and 2.

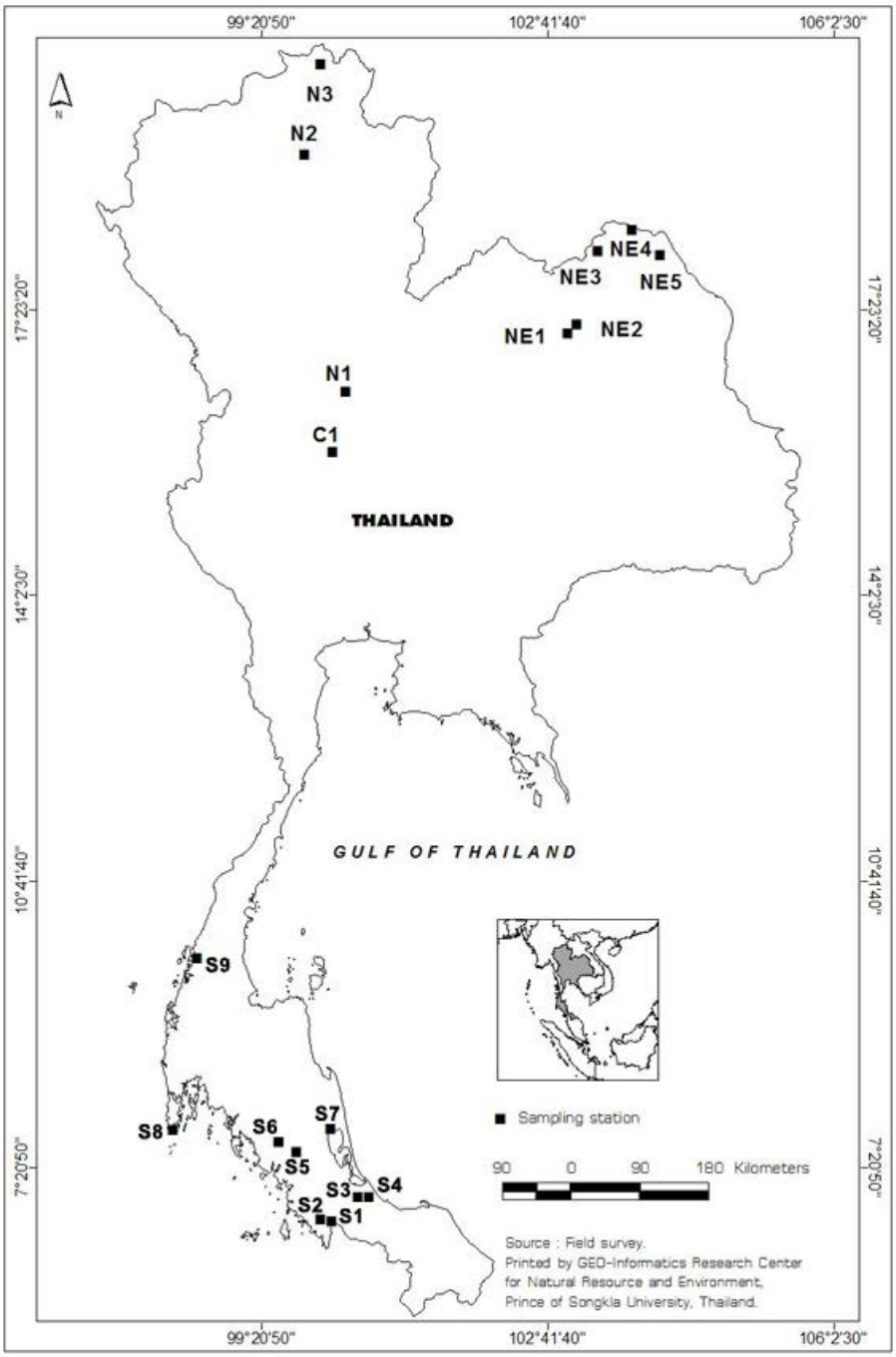


Figure 3 Sampling sites in Northern (N), Northeastern (NE), Central (C), and Southern (S) part of Thailand explored during the present study. The map acquired from SouthGIST, PSU. Details of each sampling site are in Table 1.

Table 1 Code and detail of sampling sites in Thailand explored in this study. Unit of GPS location is in decimal degree.

Code	Sampling site	Province	GPS location
N1	Si Fai swamp	Phichit	16.427092 N, 100.333439 E
N2	Phayao Lake	Phayao	19.191558 N, 99.858751 E
N3	Chiang Saen Lake	Chiang Rai	20.253490 N, 100.047571 E
NE1	Pond	Udon Thani	17.105041 N, 102.936004 E
NE2	Nong Han Lake	Udon Thani	17.213663 N, 103.037183 E
NE3	Pond	Nong Khai	18.245314 N, 103.195554 E
NE4	Kud Thing Lake	Bueng Kan	18.317214 N, 103.679003 E
NE5	Khong Long swamp	Bueng Kan	18.023656 N, 104.012994 E
C1	Pond	Nakhon Sawan	15.714422 N, 100.177809 E
S1	Thale Bun Lake	Satun	6.710378 N, 100.168881 E
S2	Pluk Paya swamp	Satun	6.742623 N, 100.042921 E
S3	Pond1 in PSU	Songkhla	7.002158 N, 100.492489 E
S4	Pond2 in PSU	Songkhla	7.007100 N, 100.498533 E
S5	Khlong Lam Chan swamp	Trang	7.529812 N, 99.754674 E
S6	Swamp	Trang	7.617150 N, 99.560467 E
S7	Thale Noi Lake	Phatthalung	7.796882 N, 100.155961 E
S8	Pond	Phuket	7.782038 N, 98.310735 E
S9	Pond	Ranong	9.794058 N, 98.593006 E

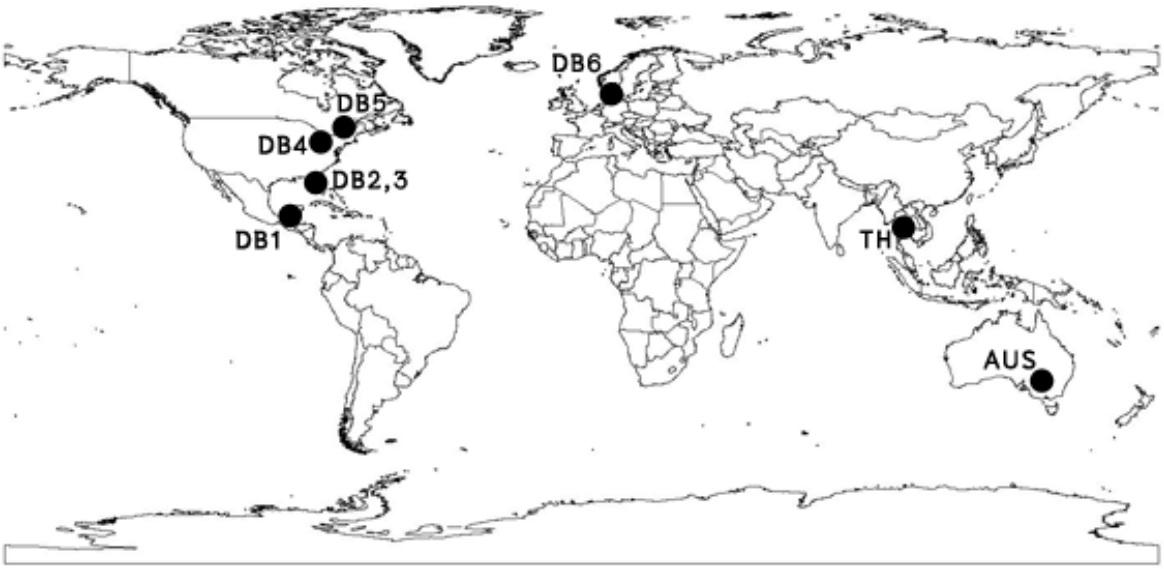


Figure 4 Sampling localities of the species examined at global scale (DB1-DB6 = the estimated localities of the species of which data were acquired from GenBank database according to published literature; TH = Thailand; AUS = Australia). Details of the localities are provided in Table 2. The map acquired from SouthGIST, PSU.

Table 2 Code and detail of sampling localities at global scale. Unit of GPS location is in decimal degree.

Code	Locality	GPS location/Reference
AUS	Southern part**,** Australia	33.939983 S, 140.865804 E
DB1	Belize	Giribet et al., 2004
DB2	Florida, USA	Sørensen et al., 2006
DB3	Everglades, FL, USA	Sørensen and Giribet, 2006
DB4	White Mountains, NH, USA	Sørensen and Giribet, 2006
DB5	Mount Desert Isl., MA, USA	Sørensen and Giribet, 2006
DB6	Denmark	Sørensen and Giribet, 2006

2.3 Species examined and their sampling localities

Thirty-six species of almost all genera, all families and orders of the sessile rotifers were obtained for phylogenetic analysis. Moreover, species that are suspected of being misattributed to a genus in the current classification (e.g., Meksuwan et al., 2011), were also included (e.g, *P. furcillata*, *P. mucicola*) (Table 3).

Four species of planktonic families within superorder Gnesiotrocha and nine species of superorder Pseudotrocha were included as outgroup taxa. The planktonic families were used to test the relationship of all fixosessile taxa while the pseudotrochan species tested monophyly of all Gnesiotrochans (Table 3).

Table 3 List of species examined, collecting localities, and accession numbers (AN) of the data acquired from GenBank (* = details of the codes of collecting locality, see Table 1 and 2, **= uncompletely identified species, see appendix A).

Superorder and order	**Family**	**Species**	**Locality* & AN**
Superorder Gnesiotrocha			
Order Collothecacea	Atrochidae	*Acyclus inquietus*	S7
	Collothecidae	*Collotheca campanulata* f. *campanulata*	S1
		C. campanulata f. *longicaudata*	S7
		C. ferox	S4
		C. ornata	S1
		C. stephanochaeta	S7
		C. tenuilobata	S7
		C. trilobata	S7
		Stephanoceros millsii	S7
Order Flosculariacea	Conochilidae	*C. (Conochilus) hippocrepis*	DB4 (DQ297688)
		C. (Conochilus) unicornis	DB4 (DQ297687)
	Flosculariidae	*Beauchampia crucigera*	S1
		Floscularia armata	S7
		F. bifida	S7
		F. conifera	NE2
		F. pedunculata	S6
		Lacinularia flosculosa	S7
		Lacinularia* cf. *pedunculata	S7
		Lacinularoides coloniensis	S7

Table 3 (continued)

Superorder and order	Family	Species	Locality & AN
		***Limnias ceratophylli* group sp.1	S7
		***L. ceratophylli* group sp.2	S7
		L. melicerta	S7
		Octotrocha speciosa	S7
		Pentatrocha gigantea	S7
		Ptygura furcillata	S7
		P. mucicola	S7
		P. beauchampi	S7
		P. crystallina	S7
		P. longicornis	S7
		P. pedunculata	S7
		P. pilula	S9
		P. tacita	S5
		P. noodti	N2
		P. thalenoiensis	S7
		Sinantherina semibullata	S7
		S. socialis	S6
		S. socialis	AUS
		S. spinosa	S7
	Hexarthridae	*Hexarthra brasiliensis*	S3
	Testudinellidae	*Testudinella dendradena*	S3
		Testudinella sp.	DB1 (AY218113)
	Trochosphaeridae	*Filinia longiseta*	DB2 (DQ079914)

Table 3 (continued)

Superorder and order	**Family**	**Species**	**Locality & AN**
Superorder Pseudotrocha			
Order Ploima	Asplanchnidae	*Asplanchnopus* sp.	S7
	Lecanidae	*Lecane bulla*	S7
		L. elsa	DB6 (DQ297699)
		L. leontina	DB3 (DQ297700)
		Lecane ungulate	S4
	Mytilinidae	*Mytilina mucronata*	DB6 (DQ297708)
	Notommatidae	*Cephalodella gibba*	DB1 (AY218114)
	Trichocercidae	*Trichocerca elongata*	DB5 (DQ297721)
		T. rattus	DB5 (DQ297722)
	Trichotriidae	*Trichotria tetractis tetractis*	S7

2.4 Specimen acquisition

1) Specimens from natural habitats

In the field, local water was filtrated with a 60 μm mesh size plankton net and placed into a 10 liter plastic jar and a collecting container (e.g., plastic bag, glass jar, plastic jar). Submerged parts of aquatic plants growing within the lake were collected, rinsed within the 10-liter plastic jar filled with local filtrated water, and placed into the collecting container. After that, the collected plants were brought to the laboratory as fast as possible. At arrival, the plants were placed into aquariums which were placed under day light and oxygenated.

In the laboratory, plant materials were observed under a SZ51 stereo microscope (Olympus) to search for sessile rotifers. Any sessile rotifer observed was picked up using forceps (held at its surrounding substratum near the specimens), transferred immediately into a small chamber, and observed under a CX21 compound microscope (Olympus) for identification. The identified specimens were preserved in absolute ethanol (Merck, Germany) for further study.

2) Resting egg hatching method

A small quantity of dried, fragmented sediment (ca. 10-20 g.) was placed into a 250-ml container, half-filled with distilled water. A 22x60-mm cover glass was hung in the containers under the water surface. The containers were placed under 12 h light-12 h dark conditions. Every four days, the immersed cover glasses as well as the water were placed into a chamber, and filled back with new cover glasses and water in the containers. The immersed cover glasses and the water were observed under the microscopes for searching and identifying the target sessile rotifer species.

The major literature used for identification of the sessile rotifers were Koste (1978), Segers (1997), Segers and Shiel (2008), and Meksuwan et al. (2011, 2013).

2.5 Acquisition of character data

2.5.1 Morphological data

1) The selected characters

Seven morphological characters (including life habits) of sessile rotifers which are relevant to taxonomic grouping and ranking as well as discussion on evolutionary adaptations within the group (e.g., Wallace, 1987; Segers and Wallace, 2001) were selected. These characters were used for analysis of the character evolutionary pattern as well as for supplementing inference of sessile rotifer phylogeny. The characters are listed and summarized in Table 5, and their relevance is outlined below.

1.1) Corona (shape and number of lobes)

About seven states of characters related to the corona in term of shape and number of lobes are recognized. These including circular, two-lobed with heart-shaped, two-lobed with kidney-shaped, three-lobed, four-lobed, five-lobed, seven-lobed and eight-lobed corona. In Order Flosculariacea, these character states are used (in combination with some characters) to diagnose genera (Koste, 1978; Segers and Shiel, 2008; Meksuwan et al., 2011), whereas the number of lobes appear to indicate species diagnosis in order Collothecacea (e.g., Meksuwan et al., 2013). Therefore, only those corona features in Flosculariacea which are involved in taxonomic grouping of higher taxa (i.e. generic category) were investigated.

1.2) Modulus

The modulus is a ciliated, cup-shaped organ that is located on the ventral region of the head, posterior of the mouth (Fig. 5, arrows). It produces gelatinous secretions as well as collects small particles or debris. This combination is used for constructing components of the case or tube, in which the components can be varied in shape and degree of hardness depending on kinds and amount of the debris (e.g., Fontaneto et al., 2003). While this structure is found only in sessile, filter-feeding species and is not very widespread, phylogenetic relationships of those species carrying modulus has to date not been addressed in particular across genera.

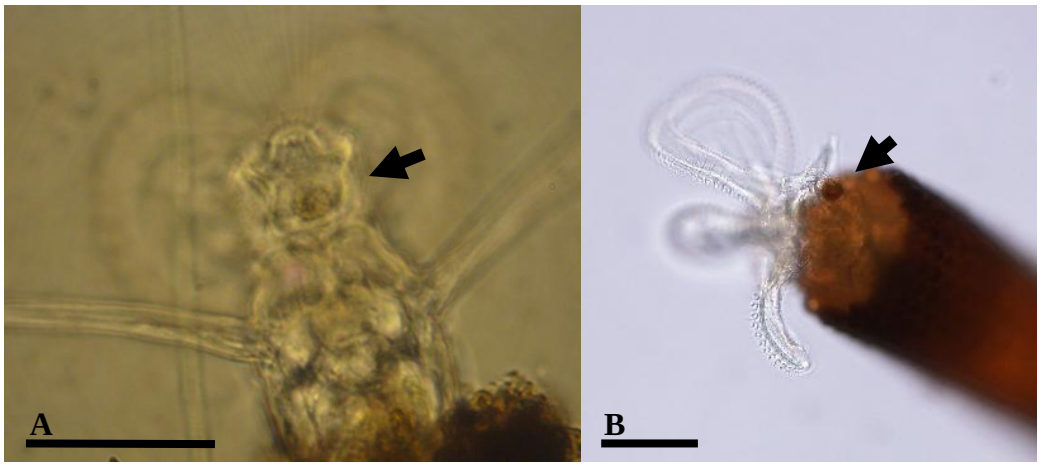


Figure 5 Modulus features (arrows). A: *Ptygura noodti*; B: *Floscularia bifida*. Scale bars: A = 50 µm., B = 100 µm.

1.3) Oviferon (egg-carrier structure)

The oviferon is an organ located on the trunk, near the cloacal aperture, to which egg(s) are attached (Fig. 6A, an arrow). This structure is found only in two genera of sessile rotifers, *Pentatrocha* (1 sp) and *Sinantherina* (5 spp) (Koste, 1978; Segers, 2007; Segers and Shiel, 2008). Phylogenetic relationships among the species possessing the oviferon have not been considered yet.

Figure 6 Comparison of species in which an oviferon is present (A, arrow) and absent (B). A: *Pentatrocha gigantea* (an egg is attached to the oviferon), B: *Lacinularia flosculosa* (eggs are not attached to any organ but deposited in the gelatinous case inhabited by the maternal specimen). Scale bars: A-B = 200 µm.

1.4) Trophi type

There are two types of trophi recorded in sessile rotifers, namely malleoramate (Figs 2D, 2E) and uncinate (Fig. 1E). In the taxonomy of the group, malleoramate trophi is considered diagnostic for members of Order Flosculariacea while uncinate trophi characterize Order Collothecacea.

1.5) Differentiation of unci teeth

In malleoramate trophi, it is recognized that the unci teeth can be divided into two groups, a proximal group and a distal group. The teeth of proximal group (Fig. 7A: a) are always relatively stronger than those of the distal group (Fig. 7A: b) (e.g., Koste, 1978; Meksuwan et al., 2011). Thus, the difference in size between teeth in the two groups is recognized as character "Differentiation". In a number of species, this differentiation is weak and the features of unci teeth in the two groups are relatively similar. Hence, this characteristic state is defined as weakly differentiated (Figs 7A-B). In other species on the other hand, the proximal teeth are more strongly developed while the distal teeth are reduced, giving an appearance of unci teeth of the two groups being distinctly different. Therefore, this state is defined as strongly differentiated state (Figs 7C-D). The character states, weakly and strongly differentiated, are used successfully to discriminate closely related species in many taxa (e.g., Segers, 1997). This character is not present in uncinate trophi (Meksuwan et al., 2013).

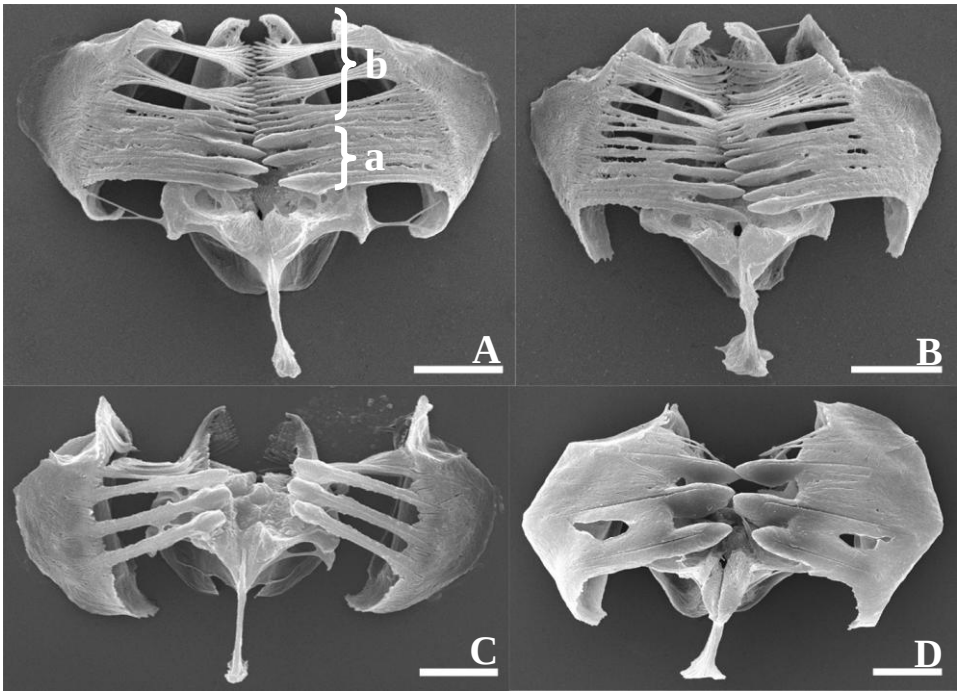


Figure 7 Differentiation of malleoramate unci teeth. A (*Sinantherina socialis*) and B (*Lacinularia flosculosa*) show weakly differentiated unci (a = proximal group, b = distal group); C (*S. semibullata*) and D (*Floscularia bifida*) show strongly differentiated unci. Scale bars: A-D = 10 µm.

1.6 Symmetry of trophi

A majority of trophi of sessile rotifers is symmetrical or nearly so. This holds for all species of order Collothecacea investigated so far (e.g., Koste, 1978; Segers, 1997; Meksuwan et al. 2013). They can be asymmetrical in other taxa (e.g., Segers and Wallace, 2001). The asymmetrical trophi can be found in different genera of order Flosculariacea, for example in *Sinantherina* (e.g., *S. spinosa*), *Ptygura* (e.g., *P. cystallina*), or in all species of family Conochilidae (e.g., Segers and Wallace, 2001). Although symmetry of trophi is a character that has been used to diagnose Conochilidae (Segers and Wallace, 2001), the relationship among taxa carrying asymmetrical state of sessile rotifers has not been addressed.

1.7 Life habit and colony formation

While a majority of sessile rotifers is solitary, several species in different genera have the ability to form colonies (Wallace, 1987). Thus, two life habits, namely sessile solitary and sessile colony, are recognized. However, a sessile colony, forming by immature individuals swimming and attaching itself to the case of a mature female that already settled, is considered pseudocolonial habit (i.e. Allorecruitive type of colony formation – AlCF (Wallace et al., 2006)). In addition, some of both solitary and colonial species, in which morphological and anatomical features indicating that they are monophyletic congeners of sessile taxa, are planktonic (e.g., *Collotheca pelagica*, *C. libera*, *Sinantherina semibullata*) (Koste, 1978; Wallace et al. 2006; Segers, 2007). Hence, within sessile families, two additional habits, planktonic solitary and planktonic colony, are also known. To date, the phylogenetic relationships among these life styles have not been investigated. Characteristics of each state are summarized in Table 4.

Table 4 Characteristics of the categories of life habit and colony formation.

Life habit and colony formation	**Characteristics**
Planktonic solitary	After hatching, a mobile, immature individual can develop to maturity without attaching a substratum (but mostly secretes a gelatinous case covering its body).
Planktonic colony	After hatching, mobile, immature individuals from the same clone settle (Autorecruitive type of colony formation, AtCF) or form a colony comprising only the newly hatched ones (Geminative type, GCF) with their maternal colony. Eventually, part(s) of the colony is(are) split into free-living colony(ies), or the newly formed colony swims away from the maternal colony, and the young of the two types develop to adult stage without attaching a substratum.
Sessile solitary	After hatching, a mobile, immature individual swims to find an available substratum for attachment and development (maturity).
Sessile pseudocolony	After hatching, mobile, young individuals swim before attaching itself to the case of an immature/mature female that already settled (AlCF or AtCF).
Sessile colony	After hatching, mobile, immature individuals of some species settle with their sessile, maternal individual or colony (AtCF). Alternatively, young colonies that formed by breaking away from a maternal colony (see above, GCF), attach to a substratum and develop to maturity.

Table 5 Summary of the morphological characters examined.

Characters	Character states	References
1. Corona (shape and lobe)	Circular	Koste, 1978; Segers and Shiel, 2008; Meksuwan et al., 2011, 2013
	Two-lobed, heart-shaped	
	Two-lobed, kidney-shaped	
	Four-lobed	
	Five-lobed	
	Eight-lobed	
2. Modulus	Absent	Koste, 1978; Fontaneto et al., 2003
	Present	
3. Oviferon	Absent	Koste, 1978; Segers and Shiel, 2008
	Present	
4. Trophi type	Malleoramate	Wallace et al., 2006; Meksuwan et al. 2013
	Uncinate	
5. Differentiation of unci teeth	Undifferentiated	Segers, 1997; Segers and Shiel, 2008
	Differentiated	
6. Symmetry of trophi	Symmetric	Segers and Wallace, 2001
	Asymmetric	
7. Life habit and colony formation	Planktonic solitary	Koste, 1978; Wallace, 1987; Hochberg, 2010
	Planktonic colony	
	Sessile solitary	
	Sessile pseudocolony	
	Sessile colony	

2) Character collecting procedure

To obtain the morphological data, several photographs and movies of living specimens of the target species were taken using BX 51 Olympus compound microscope with DP 71 photographic apparatus linked to the computer of Department of Biology, Faculty of Science, PSU.

Preparation of trophi of scanning electron microscopy (SEM) was performed by modified method of Segers (1993) and De Smet (1998) which included 1) tissue digestion in about final 5% commercial bleach on a small cover glass, 2) cleaning the remain trophi in distilled water 3-5 times, 3) air-drying the cleaned trophi, 4) placing and sticking the cover glass on a stub, 5) coating the trophi on the cover glass with gold, 6) taking a SEM photograph. All SEM photographs were taken by Scientific Equipment Centre, PSU, using FEI Quanta 400 and JEOL JSM-5800 LV.

2.5.2 Molecular data

The molecular data of most species were newly sequenced for the present study. Data of the remaining species analyzed were acquired from GenBank via the National Center for Biotechnology Information (NCBI). Accession numbers of these are showed in Table 3.

Almost all of the following processes, including DNA extraction, polymerase chain reaction (PCR), gel electrophoresis, except DNA sequencing were carried out using equipment in the laboratory of Ecology and Molecular Evolution Research Unit, Department of Biology, PSU. However at the beginning of this work, I consulted with, and tested processes by other labs outside the department (see acknowledgements).

1) Selected gene

I selected the 18S rRNA gene (or 18S rDNA which transcribes into small subunit ribosomal RNA, SSU rRNA) to be the source of genetic data. The gene (about 1,800 base pairs, bp) is located in the nucleus and usually has a number of repeated copies within the same and different chromosomes. Sequence data of 18S rDNA have been used successfully to reflect evolutionary relationship of organisms at different levels of classification, especially higher ranks which indicate deep evolutionary divisions, because of its slow nucleotide substitution rate and high capacity of data preservation by relatively long sequence (Hillis and Dixon, 1991; Halanych et al., 1995; Aguinaldo et al., 1997; Herlyn et al., 2003).

2) DNA extraction

A female with its parthenogenetic egg(s) or only several eggs produced by the same maternal individual of the target species which preserved in absolute ethanol was/were used for DNA extraction. A DNA isolation kit of Agilent technologies (USA), AGL-R-200600, was applied to extract the animal genomes (see appendix B for the extraction protocol). The isolated DNA in deionized (DI) water was store at -20^{o}C until utilization.

3) Primers and polymerase chain reaction (PCR)

A standard procedure of DNA sequencing applied by this study provided reliable sequence signals only in the length about 700 nucleotides, while an expected length of the 18S rRNA sequence is about 1,700 bases. To obtain the desired sequence length, I therefore used three pairs of primers (Table 6) which amplify the gene at the beginning, the middle, and the end along the gene, reaching the desired length.

The 18SF1 and 18SR3 primer were used in the first amplification obtaining about 1,700 bp of DNA fragment (extension time (ET) was 1.50 min, see the PCR profile below). This first PCR product was used as template for further re-amplification. The first PCR product was re-amplified using three pairs of primers (amplified separately) including 18SF1-18SR1, 18SF2-18SR2, and 18SF3-18SR3 which gave product sizes about of 550 bp (ET: 1 min), 1,000 bp (ET: 1.10 min), and 700 bp (ET: 1 min), respectively. These three different products contained overlapping regions of the gene resulting in a desired length (> 1,600 bp, see part 3.2.1) of the gene.

Table 6 Primers used in the present study.

Name	Primer sequence	Source
18SF1	5'-AGATTAAGCCATGCATGCGTAAG-3’	Forward primer of Garey et al. (1996)
18SR1	5'-GAATTACCGCGGCTGCTGG-3’	4R primer of Giribet et al. (1996)
18SF2	5'-GTTCGATTCCGGAGAGGG-3’	Modified 3F primer of Giribet et al. (1996)
18SR2	5'-CTAGAGTCTCGTTCGTTATCGG-3’	Modified 18Sbi primer of Whiting (1997)
18SF3	5'-AGTATGGTTGCAAAGCTGAAAC-3’	Modified 18Sa2.0 primer of Whiting (1997)
18SR3	5'-TGATCCTTCTGCAGGTTCACCTAC-3’	Reverse primer of Garey et al., 1996

PCR was performed using an EmeraldAmp GT PCR master mix (Takara, Japan). PCR reaction applied here was composed of (the example is of 20-µl reaction):

1) EmeraldAmp GT PCR master mix	10	µl
2) DNase/RNase-free water	5	µl
3) Forward primer	0.5	µl
4) Reverse primer	0.5	µl
5) Template (first PCR product, 1,700 bp)	4	µl
Total volume of PCR reaction	20	µl

PCR profile used is in the following:

(1) Initial denaturation: 94^{o}C 5 min
(2) Denaturation: 94^{o}C 1 min
(3) Annealing: 55^{o}C 1 min
(4) Extension: 72^{o}C 1/1.10/1.50 min (depending on fragment sizes, above)
Cycle: 39 times from (2) to (4)
(5) Final extension: 72^{o}C 10 min

4) PCR product purification

PCR products were purified using a Gel/PCR DNA fragments extraction kit (DF100, Geneaid, Taiwan). Protocol of the purification is demonstrated in appendix C.

5) Gel electrophoresis

Size, quality (i.e. a sharp single-band product) and estimated amount of DNA fragments (either unpurified or purified PCR products) were checked by gel electrophoresis technique. This study used 1.2% agarose gel (Molecular Biology Grade, Vivantis) and 0.5X TAE buffer. DNA ladder was 100 bp DNA ladder (DL100), Biosciences.

6) DNA sequencing

The purified PCR products with its specific primers were sent to Bio Basic Inc. (Canada) via Pacific Science Co., Ltd. (Thailand) for nucleotide sequencing. The analysis was performed by multiple 3730XL sequencers with Fluorescent dye-terminator sequencing.

2.6 Data analyses

2.6.1 Sequence alignment

All sequences of 18S rRNA gene obtained were aligned by MAFFT version 7 (http://mafft.cbrc.jp/alignment/server) (Katoh & Standley, 2013), and checked manually using GENEDOC program (Nicholas et al., 1997). The aligned data were saved in MNS file before converting to other formats required for other analyses (below).

2.6.2 Statistics of obtained nucleotide sequence

Statistical quantities including conserved sites, variable sites and parsimonious-informative sites were qualified using MEGA 4.0 program (Tamura et al., 2007). In this study, the MNS file was converted to PHYLIP format (.phylip) and the PHYLIP was converted again to MEGA file (.meg) by the MEGA program before the analysis.

2.6.3 Qualification of nucleotide substitution model

jModelTest 0.1 program (Posada, 2008) with the Akaike Information Criterion (AIC) was used to qualify a fitted nucleotide substitution model and other parameter involved in phylogenetic analysis such as nucleotide frequency, proportion of invariable sites and gamma distribution of rate heterogeneity. PHYLIP file was used for the qualification.

2.6.4 Substitution saturation test

Saturation of nucleotide substitution was tested by applying DAMBE 5 program (Xia, 2013). Xia's method was performed to qualify saturation of 18S rRNA sequence obtained. In Xia's approach, the index of substitution saturation (*Iss*) is significantly lower than the critical *Iss* value (*Iss.c*) indicating weak substitution saturation which means the sequence data are full of phylogenetic information. Although Xia's method limits OTUs (i.e. species) simulated at 32 taxa, DAMBE can achieve the test by randomly sample subsets (4, 8, 16 and 32 taxa) several times and tests substitution saturation in these subsets (Lemey et al., 2009).

2.6.5 Phylogenetic reconstruction

1) Bayesain inference analysis

Bayesian analysis was run by MrBayes3.2.2 program via the CIPRES Science Gateway (http://www.phylo.org/index.php/portal) on the Extreme Science and Engineering Discovery Environment (XSEDE) (https://www.xsede.org) (Miller et al., 2010). The MNS file of aligned sequence data was changed to NEXUS file format before uploading into the interface. The analysis was carried out with 10 million running generations. The parameters acquired from the jModelTest were used in the analysis which are 1) model = GTR, 2) rate variation across sites = gamma-shaped rate variation with a proportion of invariable sites (invgamma), 3) substitution rates of the GTR model: A/C = 2.1677, A/G = 3.2983, A/T = 4.0259, C/G = 0.6945, C/T = 9.5028 and G/T = 1.0000, 4) gamma shape parameter = 0.6130, and 5) proportion of invariable sites = 0.7710. Burn-in was set at 25% of tree sampled generations as well as in summarizing the sampled parameter values and the tree and branch length information (e.g., sump burnin=25000, sumt burnin=25000). The analysis was stopped when reaching the running generations and the average standard deviation of split frequency < 0.01. The phylogenetic tree acquired was rooted using a Figtree1.3.1 program. The posterior probabilities (PP) are indicated on any branch that obtained ≥ 0.9 of the value (Alfaro et al., 2003; Zander, 2004).

2) Maximum likelihood analysis

PhyML 3.0 (www.atgc-montpellier.fr/phyml) (Guindon et al., 2010) was accessed for Maximum likelihood analysis. The parameters which were changed from the program default including substitution model = GTR, proportion of invariable sites = 0.7710, and gamma shape parameter = 0.6130. Bootstrapping was performed 1,000 replications and indicated on a tree branch which carried the bootstrap supports ≥ 70% (Zander, 2004; Lemey et al., 2009).

3) Neighbor-joining analysis

DAMBE 5 program (Xia, 2013) was used for Neighbor-joining analysis (Saitou and Nei, 1987). Number of times to jumble was 1 and the model selected for genetic distance was GTR. Either of bootstrapping and jackknifing were performed 1,000 replications. The two branch supports were indicated on the tree branches containing the values ≥ 70% (Zander, 2004; Lemey et al., 2009).

2.6.6 Analysis of the character evolution

Character states of the selected morphological characters, including corona (shape and number of lobes), trophi type, differentiation of unci teeth, symmetry of the trophi, life habits and colony formation were mapped on terminal branches of a rooted gene tree obtained. This method can be used to interpret gain/loss as well as apomorphy and plesiomorphy of the character states among the sessile rotifer representatives according to the outgroup comparison approach of character polarity determination (Stevens, 1980; Watrous and Wheeler, 1981; Bryant, 2001; Futuyma, 2005).

CHAPTER 3

RESULTS

3.1 Diversity and distribution of sessile rotifers in Thailand

A total of forty-one species and two infraspecific variants of sessile rotifers was identified from various freshwater habitats in Thailand (Fig. 3 and Table 1). The species belong to two orders, three families and twelve genera. The most diverse genera were *Collotheca* (11 spp.) and *Ptygura* (11 spp.), and followed by *Floscularia* (4 spp.) (Table 7).

Of the species identified, one is new to science. It was formally described during the present study (Meksuwan et al., 2013). The other one is new to Oriental region and Thailand, and one infraspecific variant is new in a scientific paper produced to Thailand (Table 7). Differential diagnosis of the new species and taxonomic remarks of the new taxa on record are provided in the following.

Collotheca orchidacea Meksuwan, Pholpunthin & Segers, 2013
(Fig. 8)

Differential diagnosis. The presence of a five-lobed corona separates the new species from most of the known members of genus *Collotheca*. In comparison with other *Collotheca* species having a five-lobed corona (*C. algicola* (Hudson), *C. ambigua* (Hudson), *C. annulata* (Hood), *C. bilfingeri* Bērziņš, *C. ferox* and *C. campanulata* (Dobie)), *C. orchidacea* can be distinguished by its uniquely well-developed thumb-shaped lateral and semi-circular ventral corona lobes. It has a relatively broad infundibulum, and short foot and trunk, similar only to *C. ambigua* and *C. ferox*. In addition, *C. orchidacea* and *C. ferox* hold their infundibulum and corona towards the substratum, whereas most other species including *C. ambigua* and *C. campanulata* normally hold their body and corona upright.

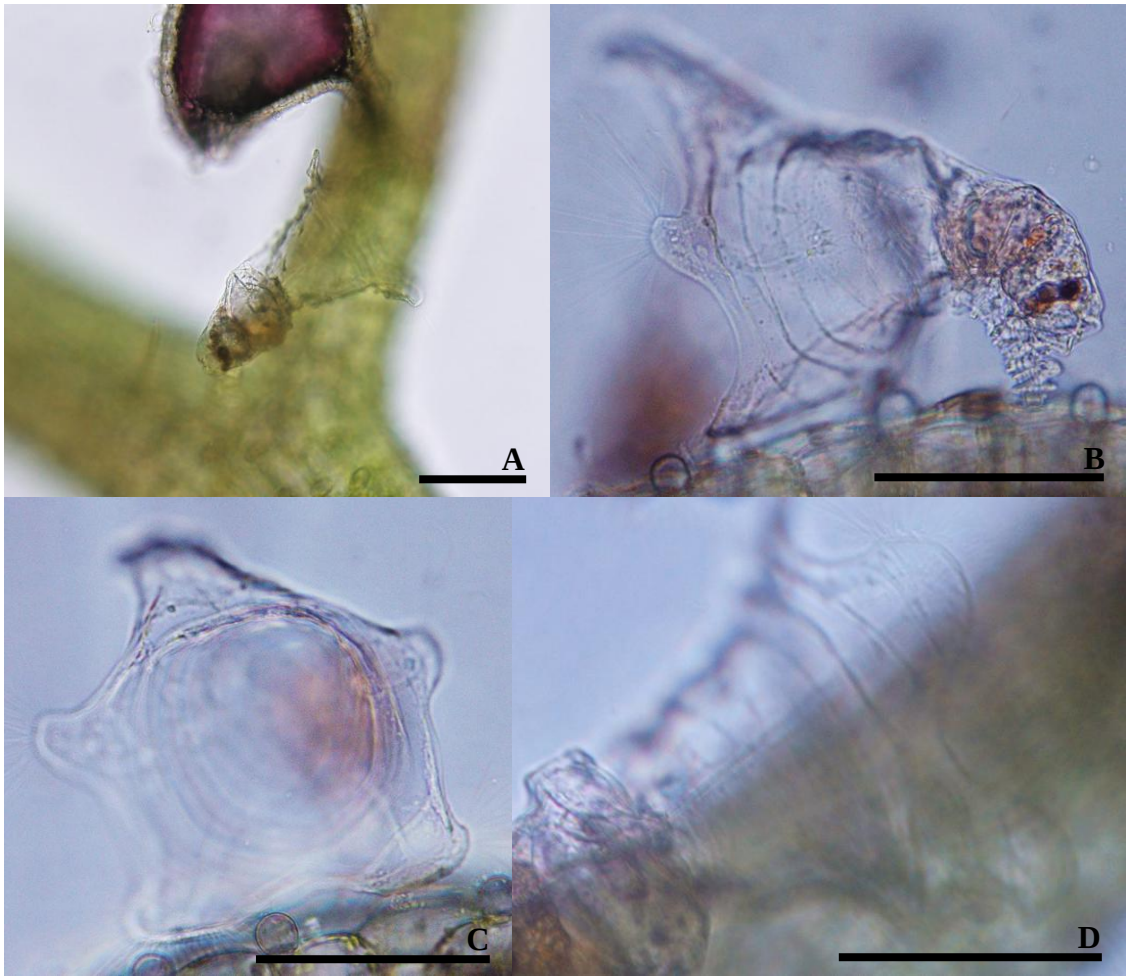


Figure 8 *Collotheca orchidacea* Meksuwan, Pholpunthin & Segers, 2013. A: a female attached on *Utricularia* sp. (dorsal view); B: feature of lateral corona lobe; C: anterior view of the infundibulum; D: feature of ventral corona lobes. Scale bars: A-D = 100 µm.

Collotheca ferox (Penard, 1914)

(Fig. 9)

Remarks. The morphological characters of our specimens agree closely with the description of the species by Penard (1914): the corona of the specimens is more than twice as broad as its trunk and bears five broad lobes. The dorsal lobe tip is relatively large and rounded anteriorly; the lateral lobes are intermediate in size whereas the triangular ventral lobes are relatively small and are set close together. The features of the ventral lobe are unique to this species and prevent confusion with other five-lobed species of the genus. Our photographs of living specimens and trophi of *C. ferox* confirm, in particular, the unique features of the ventral corona lobes illustrated by Penard (1914).

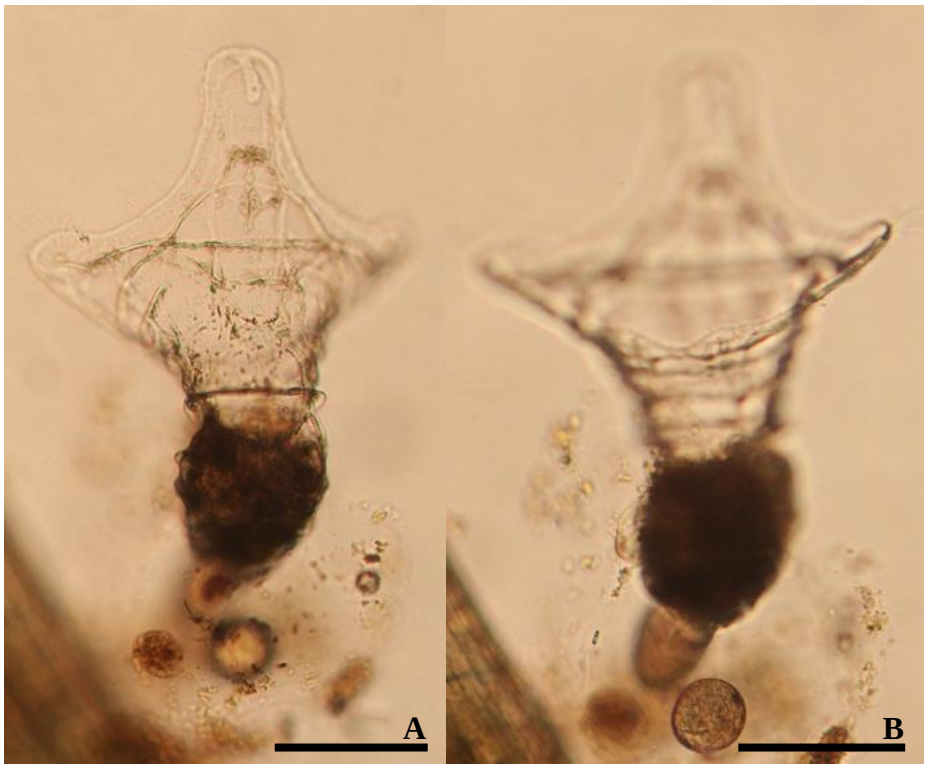


Figure 9 *Collotheca ferox* (Penard, 1914). A: corona characters in dorsal view; B: corona characters and the ventral lobes in ventral view. Scale bars: A-B = 100 µm. The photographs were taken by Miss Rapeepan Jaturapruek, Biology, PSU.

Collotheca ornata f. *cornuta* (Dobie, 1849)

(Fig. 10)

Remarks. This taxon is differentiated from the nominal form by the corona bearing an elongate projection dorsally to the dorsal lobe. The presence/absence of this projection has classically been interpreted as of infrasubspecific relevance only. In the absence of additional data (morphological, molecular or behavioural), we prefer to be cautious and record the taxon separately. The present is the first Thai record of the taxon.

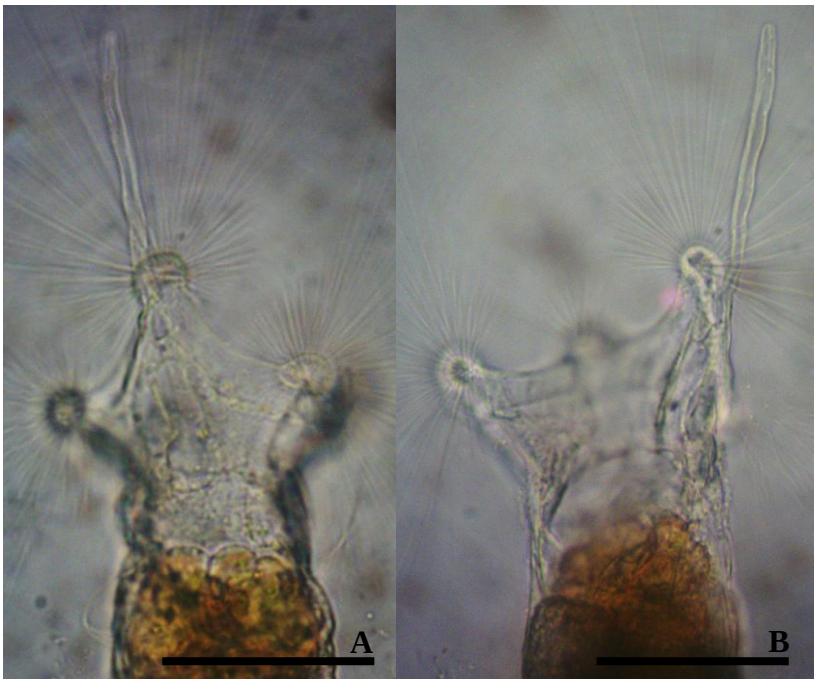


Figure 10 *Collotheca ornata* f. *cornuta* (Dobie, 1849). A: corona feature and the dorsal projection in dorsal view; B: corona feature and the dorsal projection in lateral view. Scale bars: A-B = 50 µm.

Table 7 List of sessile rotifers identified in Thailand during the present study (* = new to Oriental region and Thailand, ** = new to Thailand, *** = taxa not included in the phylogenetic analysis, see below).

Order Collothecacea

Family Atrochidae

Acyclus inquietus

Family Collothecidae

*Collotheca algicola****

*C. ambigua****

C. campanulata f. *campanulata*

C. campanulata f. *longicaudata*

*C. ferox**

*C. heptabrachiata****

C. orchidacea **sp. n.*****

C. ornata

C. ornata f. *cornuta*****, ****

C. stephanochaeta

C. tenuilobata

C. trilobata

Collotheca sp.****

*Stephanoceros fimbriatus****

S. millsii

Order Floscullariacea

Family Flosculariidae

Beauchampia crucigera

Floscularia armata

F. bifida

F. conifera

F. pedunculata

Lacinularia flosculosa

Lacinularia cf. *pedunculata*

Lacinularoides coloniensis

Limnias ceratophylli group sp.1

L. ceratophylli group sp.2

L. melicerta

Octotrocha speciosa

Pentatrocha gigantea

Ptygura barbata

P. beauchampi

P. crystallina

P. furcillata

P. longicornis

P. mucicola

P. noodti

P. pedunculata

P. pilula

P. tacita

P. thalenoiensis

Sinantherina semibullata

S. socialis

S. spinosa

The species most frequently observed were *Ptygura cystallina* (9 sampling sites), followed by *Beauchampia crucigera* (8), *Limnias ceratophylli* group sp.2 (8) and *Ptygura beauchampi* (7), respectively (appendix D). Distribution of the species and infraspecific variants recorded in different parts of Thailand is shown in Table 8.

Table 8 Diversity and distribution of sessile rotifers in different parts of Thailand recorded (S, C, N and NE = Southern, Central, Northern and Northeastern part, respectively).

Species	**S**	**C**	**N**	**NE**
Acyclus inquietus	+			
Beauchampia crucigera	+		+	+
Collotheca algicola	+			
C. ambigua	+			
C. campanulata f. *campanulata*	+			+
C. campanulata f. *longicaudata*	+			+
C. ferox	+			
C. heptabrachiata	+			
C. orchidacea	+			
C. ornata f. *ornata*	+			
C. ornata f. *cornuta*	+			
C. stephanochaeta	+			+
C. tenuilobata	+			+
C. trilobata	+			+
Collotheca sp.	+			
Floscularia armata	+			
F. bifida	+			

Table 8 (continued)

Species	**S**	**C**	**N**	**NE**
F. conifera	+			+
F. pedunculata	+			
Lacinularia flosculosa	+		+	+
Lacinularia cf. *pedunculata*	+		+	+
Lacinularoides coloniensis	+		+	+
Limnias ceratophylli group sp.1	+	+	+	
L. ceratophylli group sp.2	+		+	+
L. melicerta	+	+		
Octotrocha speciosa	+		+	+
Pentatrocha gigantea	+			+
Ptygura barbata	+	+	+	
P. beauchampi	+			+
P. crystallina	+		+	+
P. furcillata	+			
P. longicornis	+	+		+
P. mucicola	+			
P. noodti	+		+	+
P. pedunculata	+			
P. pilula	+			+
P. tacita	+			+
P. thalenoiensis	+			+
Sinantherina semibullata	+			
S. socialis	+		+	+
S. spinosa	+			
Stephanoceros fimbriatus	+			
S. millsii	+			+
Number of species	41	4	11	21
Number of sampling sites	9	1	3	5

3.2 Phylogenetic analyses

3.2.1 Molecular data obtained

1) Sequence size

Nucleotides of 18S rRNA gene sequenced from the target species ranged from 1,655-1,749 bases. However, to analyze the phylogenetic relationship among the taxa with nearly equal characters, the beginning and end of the sequences of some species were removed (Appendix E). As a result, the data set using for phylogenetic analysis contained 1,636-1,694 nucleotides. Analytically, however, the performing programs recognized a total of 1,695 characters to be analyzed since all gaps were include and treated as missing data.

2) Data alignment

The aligned gene sequences contain 1,469 conserved sites. Average nucleotide frequencies of A, C, G and T were 25.9%, 20.7%, 26.8% and 26.5%, respectively, with 47.5% GC content. Variable site is 225 positions.

3) Test of substitution saturation

Analysis of nucleotide substitution saturation revealed that *Iss* were significantly lower than *Iss.c* ($P < 0.05$) in all sampling subsets of both symmetrical and asymmetrical trees. This indicated that the sequences of 18S rRNA gene obtained are not saturated (Table 9).

Table 9 Test of nucleotide substitution saturation of 18S rRNA gene.

NumOTU	Iss	Iss.cSym	T	DF	P	Iss.cAsym	T	DF	P
4	.181	.836	31.016	387	.0000	.808	29.659	387	.0000
8	.174	.815	30.718	387	.0000	.717	26.046	387	.0000
16	.179	.797	30.386	387	.0000	.620	21.641	387	.0000
32	.184	.781	30.336	387	.0000	.505	16.293	387	.0000

Note: two-tailed tests are used.

3.2.2 Phylogenetic reconstruction

1) Optimality search criterion methods

1.1) Bayesian inference

In the gene tree inferred by Bayesian analysis with 10 million running generations, all species of superorder Pseudotrocha that were used to root the tree formed a single clade with 1 posterior probability (PP) (Fig. 11a). Rooting with this group, all representatives of superorder Gnesiotrocha including all sessile rotifers formed monophyletic group with 1 PP (Fig. 11b). Within this gnesiotrochan clade, a monophyletic clade of members of order Collothecacea – representatives of genera *Acyclus*, *Collotheca* and *Stephanoceros* – was revealed with high support (1 PP, Fig. 11c). In this clade, *Acyclus inquietus* was located in between some *Collotheca* species (0.99 PP, Fig 11d), while the position of *Stephanoceros millsii* was not resolved (Fig. 11e).

Among representatives of order Floscullariacea on the other hand, two different lineages were uncovered. A first clade (1 PP, Fig. 11f) formed sister group to the Collothecacea clade mentioned above, with 1 PP (Fig. 11g). This group comprised three genera, namely *Beauchampia*, *Limnias*, and species of the so-called *Ptygura melicerta* group (Koste, 1972). In the present study, this lineage is called the BLP group. In this group, a clade of species of *P. melicerta* group (1 PP, Fig. 11h) formed sister group to a clade of *Limnias* species (1 PP, Fig. 11i) with 0.99 PP (Fig. 11j). This group then connected to the single species of *Beauchampia* with a high support (Fig. 11f). The second Floscullariacea clade was made up of the Conochilidae, a majority of the members of Flosculariidae (excluding the BLP), and the planktonic families (0.95 PP, Fig. 11k). In this clade, members of the Conochilidae and Flosculariidae comprised a monophyletic lineage (1 PP, Fig. 11L). The relationship of this lineage to the planktonic families, as well as among the planktonic families themselves, were not resolved.

Within the clade formed by the Conochilidae and the Flosculariidae (Fig. 11L), three groups were revealed. Group 1 (1 PP, Fig. 11m) was composed of three species of genus *Ptygura*, including *P. noodti*, *P. tacita*, and *P. thalenoiensis*, and all representatives of genus *Floscularia* (0.90 PP, Fig. 11n). Group 2 (1 PP, Fig. 11o) was formed by members of four genera, namely *Lacinularia flosculosa*, *Lacinularoides coloniensis*, *Pentatrocha gigantea*, and some *Sinantherina* species. In this group, *P. gigantea* formed sister group to *S. socialis* (1 PP, Fig. 11p), while the position of *S. semibullata* contained rather weak support (0.89 PP, Fig. 11q). These species together were closest to *L. flosculosa* (0.98 PP, Fig. 11r), and all of them together connected to *L. coloniensis* (Fig. 11o). Group 3 (0.94 PP, Fig. 11s) made up of some species of *Ptygura* and a clade of Conochilidae representatives (1 PP, Fig. 11t). In this group however, the position of the Conochilidae was not resolved. In additional to the three groups, positions of the remaining species within this major clade, namely *Lacinularia* cf. *pedunculata*, *Octotrocha speciosa*, *Ptygura pilula*, and *Sinantherina spinosa*, were uncertain.

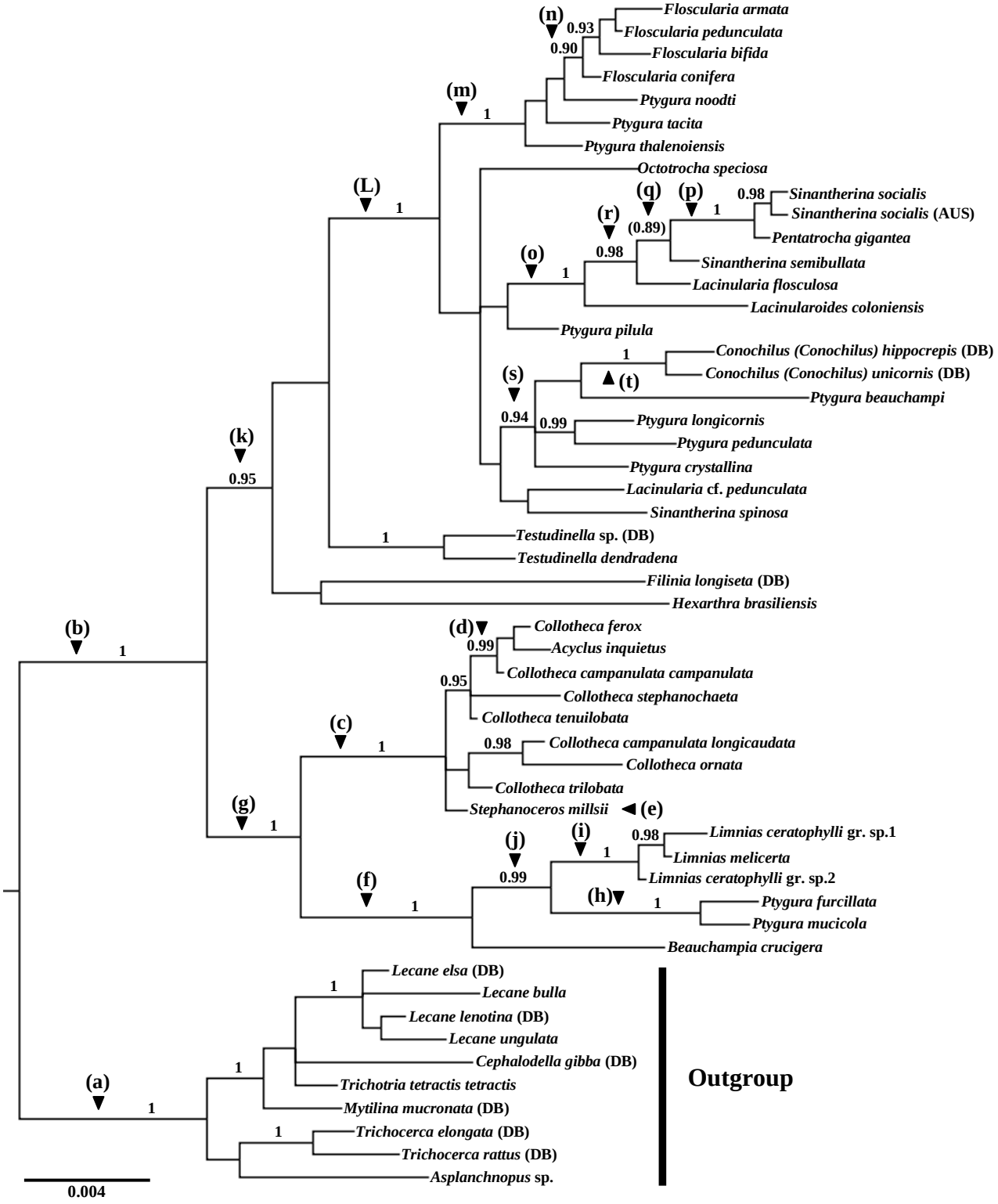


Figure 11 Bayesian inference tree. The posterior probability (PP) values are indicated only on the tree branches that contained ≥ 0.9 PP. The letters represent clades and species that are referred in the text. (AUS) and (DB) are the data from Australian specimens and GenBank database, respectively. Scale bar is degree of divergence (%).

1.2) Maximum likelihood

Rooting with representatives of the Pseudotrocha (100% bootstrap (BT), Fig. 12a), Gnesiotrocha was monophyletic with 100% BT (Fig. 12b). The two major clades of sessile rotifers, namely the Collothecacea (98.9% BT, Fig. 12c) and the BLP group (96.7% BT, Fig. 12d) were also revealed in the ML analysis. In the Collothecacea, only one clade was resolved, where *Acyclus inquietus* was again located in between some *Collotheca* species (81.2% BT, Fig. 12e). In the BLP group on the other hand, all clades were resolved, and relationships among the taxa were identical to the BI tree (compare clades in Figs 11f and 12d). Moreover, these two clades formed sister group (82.1% BT, Fig. 12f) as revealed also in the BI tree (Fig. 11g).

However, relationships among Conochilidae, a majority of Flosculariidae, and the planktonic families were not settled. Other clades revealed in the ML tree, were 1) the clade formed by the three *Ptygura* species (*P. noodti*, *P. tacita*, and *P. thalenoiensis*) and *Floscularia* species (80.7% BT, Fig. 12g), and 2) the clade composed four genera including *Lacinularia*, *Lacinularoides* and *Pentatrocha*, and some *Sinantherina* species (78.5% BT, Fig. 12h). In the former group, no relationships among the taxa were resolved. In the latter group on the other hand, the relationships were identical to the BI tree, in which *P. gigantea* and *S. socialis* formed sister group (99.5% BT, Fig. 12i), a close relationship among *L. flosculosa*, *P. gigantea* and the two *Sinantherina* species, and all of them together were closest to *L. coloniensis* (Fig. 12h).

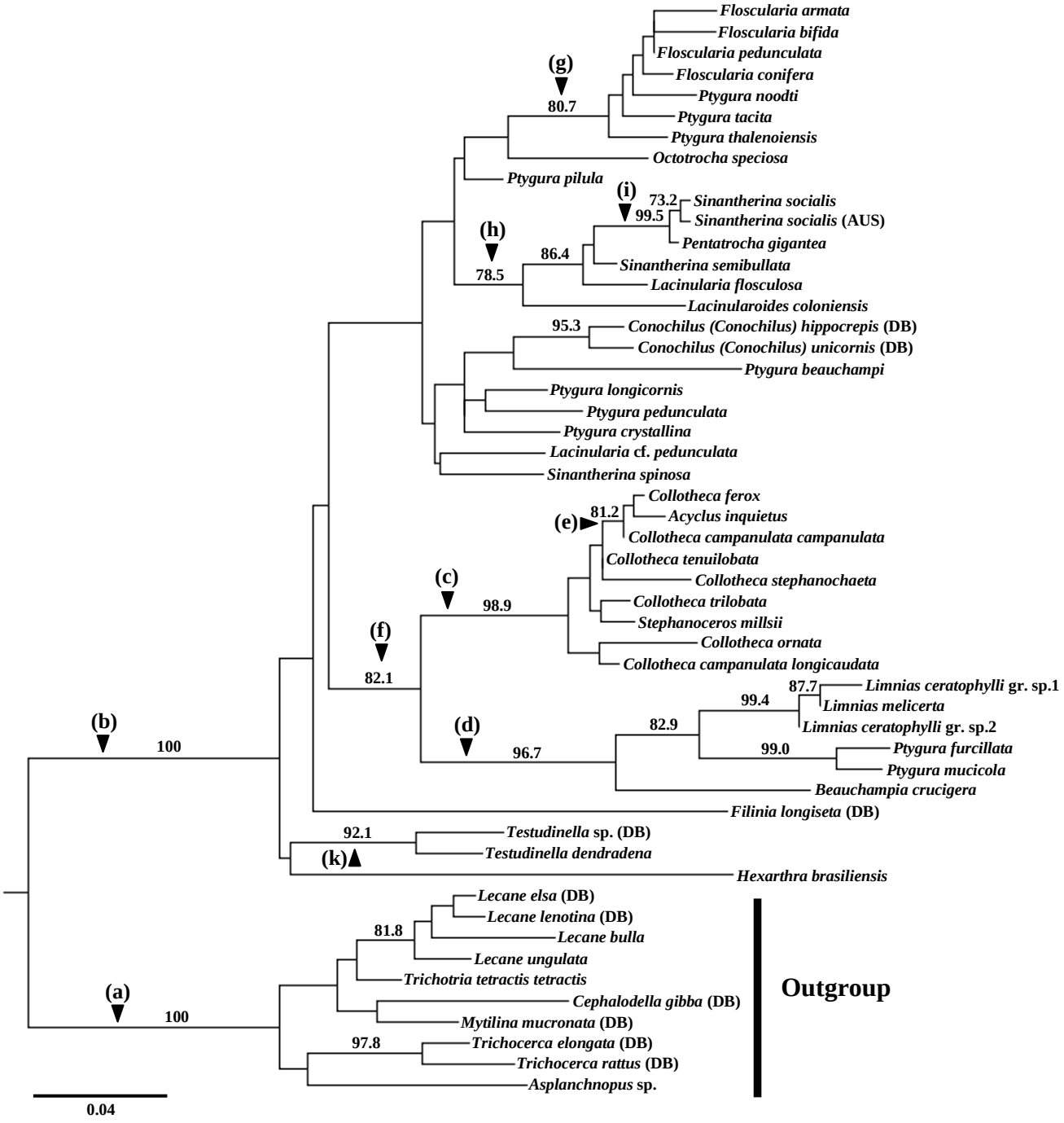


Figure 12 Maximum likelihood tree. The bootstrapping values (1,000 replications) are showed only on the tree branches that contained ≥ 70% of the bootstraps. The letters represent clades and species that are referred in the text. (AUS) and (DB) are the data from Australian specimens and GenBank database, respectively. Scale bar is degree of divergence (%).

2) Distance method

2.1) Neighbor-joining

In the Neighbor-joining (NJ) tree, the Gnesiotrocha (Fig. 13a) as well as the outgroup (Fig. 13b) were monophyletic group, supported by 100% of both bootstrapping (BT) and jackknifing (JK) analysis. In the Gnesiotrocha, the three major clades, similar to the ones in the BI tree, were found, namely the Collothecacea (100% BT/100% JK, Fig. 13c), the BLP group (99/100, Fig. 13d), and the clade formed by the Conochilidae, the Flosculariidae, and the planktonic families (76/78, Fig. 13e).

Here, no clade were resolved within Collothecacea (Fig. 13c), whereas relationships among taxa within the BLP group were the same as in both the BI tree and ML tree, in which *Limnias* species were closest to the *Ptygura* species and the clade of these species was connected to *Beauchampia crucigera* (Figs 11f, 12d, 13d). A clade composing of the Conochilidae and the Flosculariidae was revealed (72/79, Fig. 13f), while its relationship to the planktonic families, as well as among the planktonic families were not resolved.

In the clade formed by the Flosculariidae and the Conochilidae (Fig. 13f), the two major groups as in the ML tree were also uncovered. Group 1 (97/96, Fig. 13g) was composed of the three *Ptygura* species (*P. noodti*, *P. tacita*, and *P. thalenoiensis*) and *Floscularia* species. Group 2 (74/72, Fig. 13h) was of four genera namely *Lacinularia*, *Lacinularoides* and *Pentatrocha*, and some of *Sinantherina* species. A sister group formed by *P. gigantea* and *S. socialis* was also revealed (Fig. 13i). Positions of the rest taxa including a clade of the Conochilidae (99/99, Fig. 13j) were not resolved.

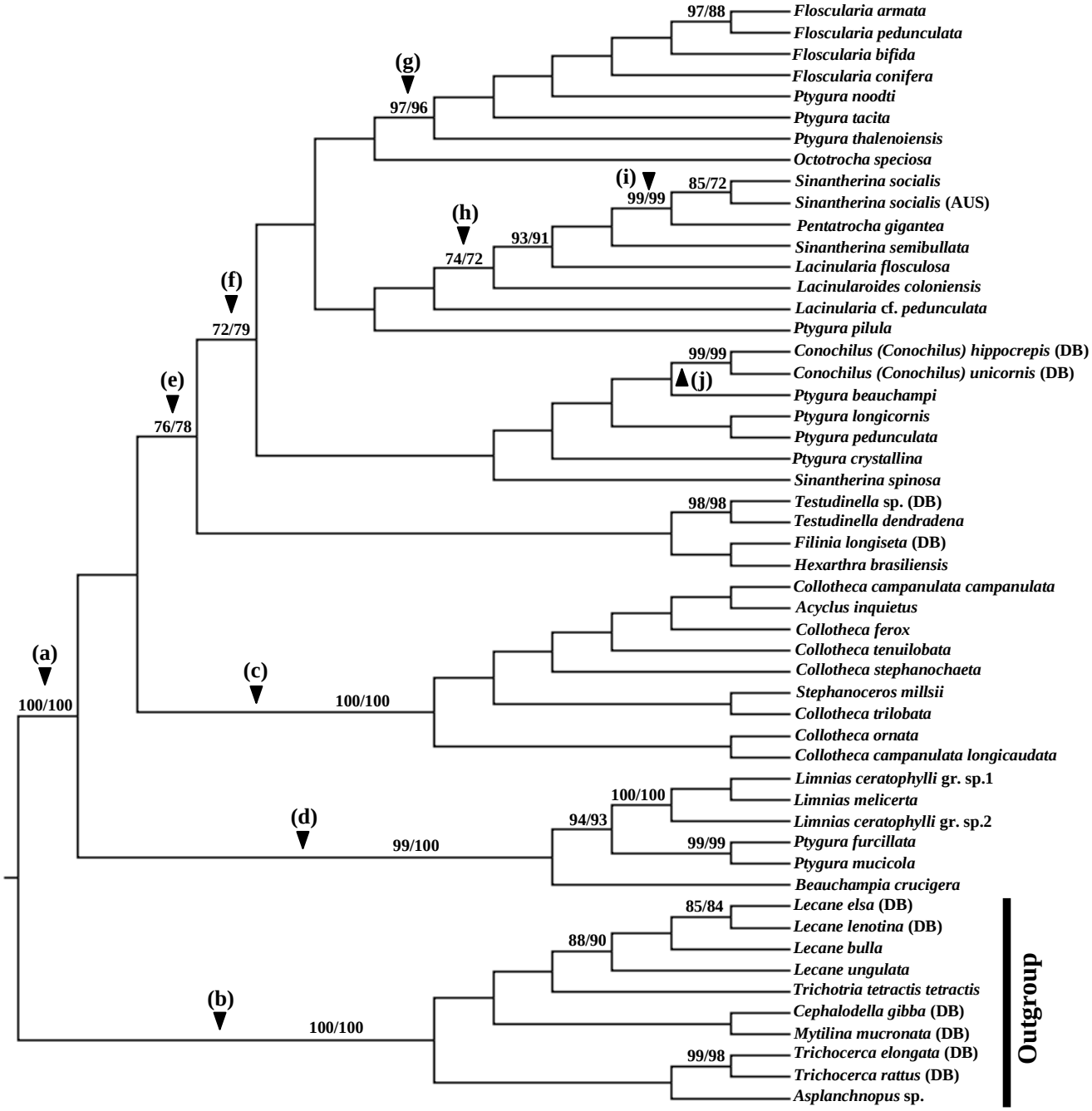


Figure 13 Neighbor-joining tree. The bootstrap and jackknife values, respectively, are indicated on the braches that contain ≥ 70% supports. The letters represent clades and species that are referred in the text. (AUS) and (DB) are the data from Australian specimens and GenBank database, respectively.

3.3 Analysis of evolution of selected morphological characters and life habits

1) Corona (shape and number of lobes)

Mapping the corona character states on the tree illustrated that most corona states were not restricted to a single clade. For instance, circular coronas occurred in different, distantly related clades (Fig. 14a). Species carrying two-lobed, heart-shaped coronas (Fig. 14b), as well as the two representatives bearing five-lobed corona (Fig. 14c), were not directly related.

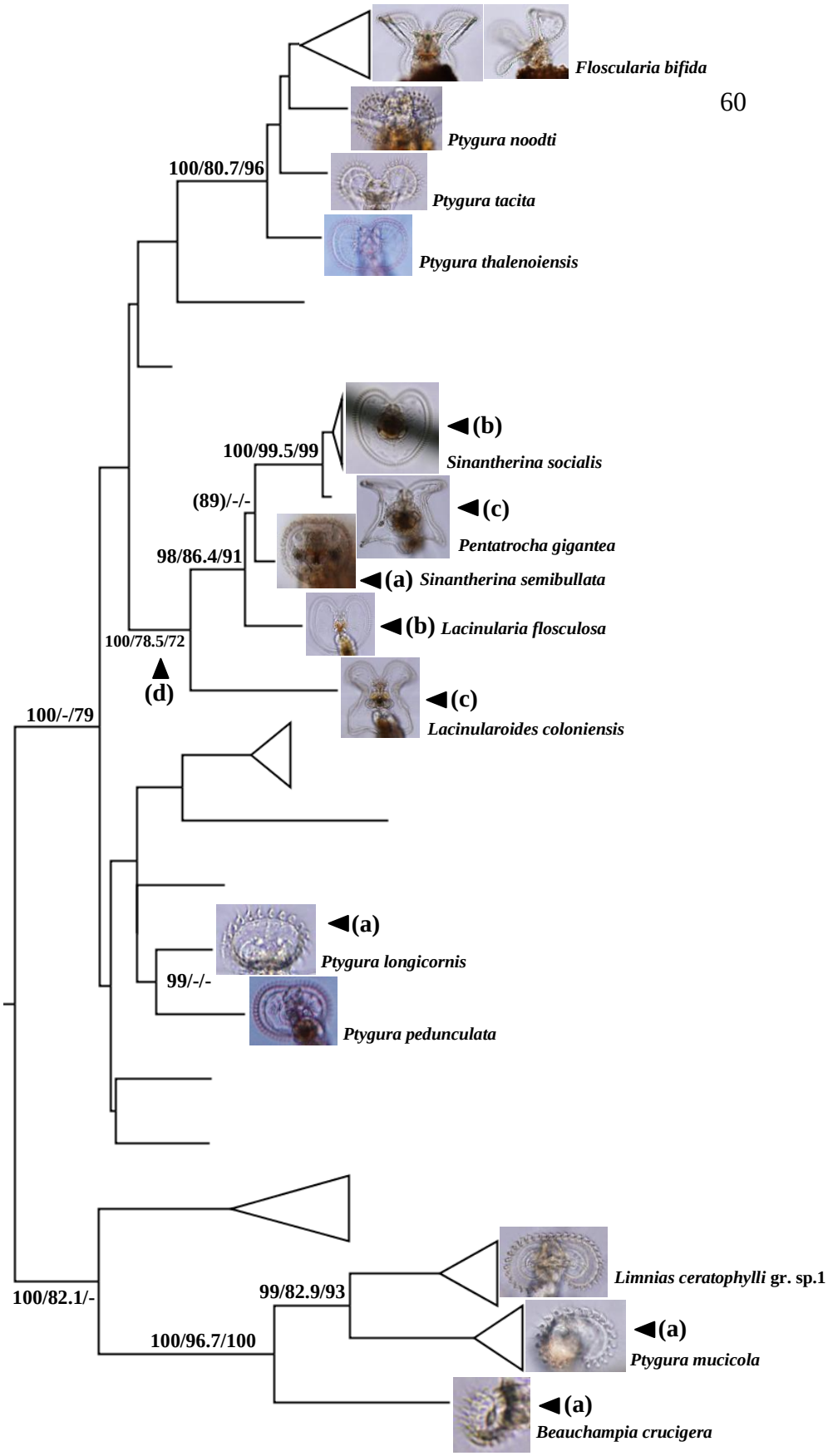


Figure 14 Distribution of the corona feature states on the tree. The three supporting values – percent of the posterior probability (≥ 90%), bootstrapping (ML analysis: 1,000 replications, ≥70%) and jackknifing (NJ analysis: 1,000 replications, ≥70%), respectively – are indicated on the branches. The letters are referred in the text.

2) Modulus

The result revealed that all species carrying modulus belonged to a sing clade (Fig. 15).

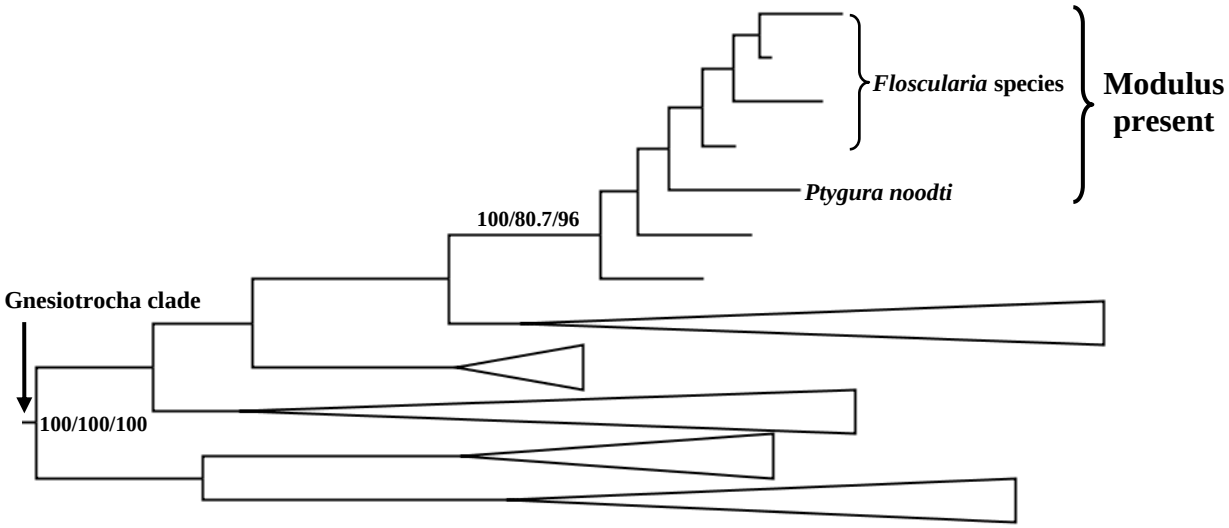


Figure 15 Distribution of modulus on the tree. The three supporting values – percent of the posterior probability (≥ 90%), bootstrapping (1,000 reps, ≥70%) and jackknifing (1,000 reps, ≥70%), respectively – are indicated on the branches.

3) Oviferon

In the present analysis, most representatives possessing oviferon were closely related (Fig. 16a) except for *Sinantherina spinosa*. However, position of this species received weak supports (16b).

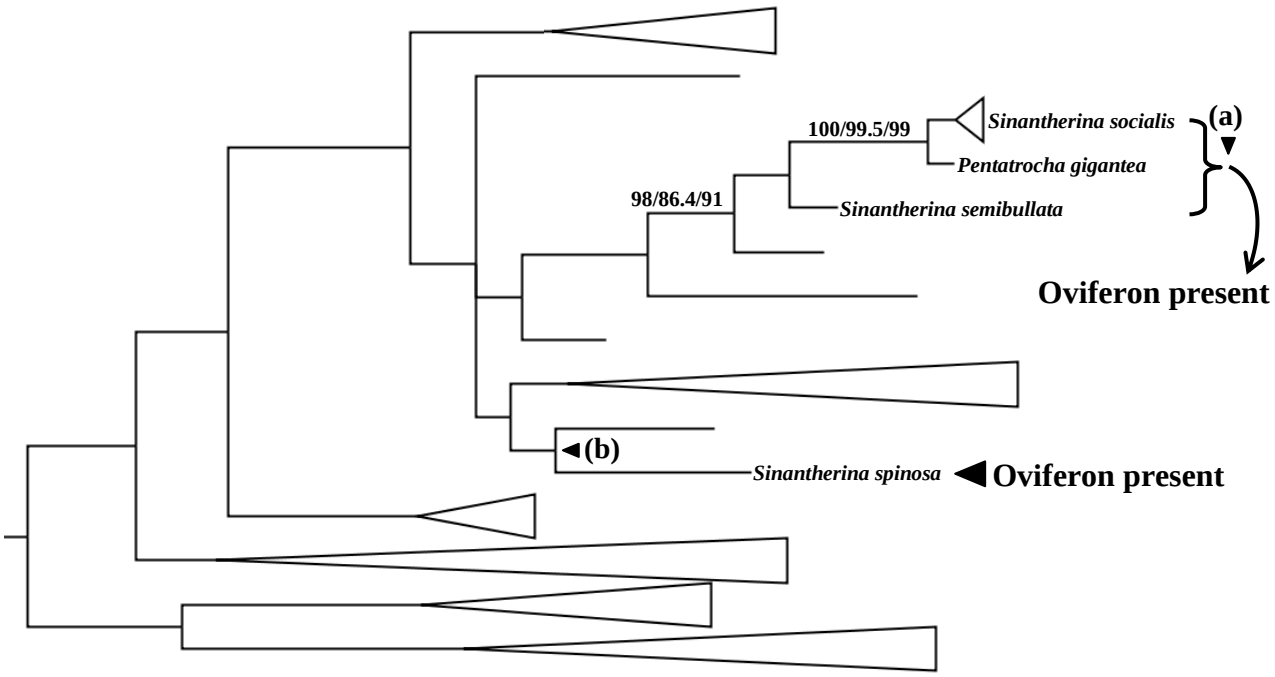


Figure 16 Distribution of oviferon on the tree. The three supporting values – percent of the posterior probability (≥ 90%), bootstrapping (1,000 reps, ≥70%) and jackknifing (1,000 reps, ≥70%), respectively – are indicated on the branches. The letters are referred in the text.

4) Trophi type

The result revealed that malleoramate trophi belonged to two different clades. One clade was supported by 0.95 PP (or 95%) and 78% JK but not for BT support (Fig. 17a). The other one – the BLP group – which received high supports in all analyses (100/96.7/100% of PP, BT, and JK, respectively, Fig. 17b) formed sister group to the monophyletic clade of species carrying uncinate trophi (100/98.9/100, Fig. 17c). These sister clades were connected by high supports of the PP and BT analyses except for the JK (100/82.1/-, Fig. 17d).

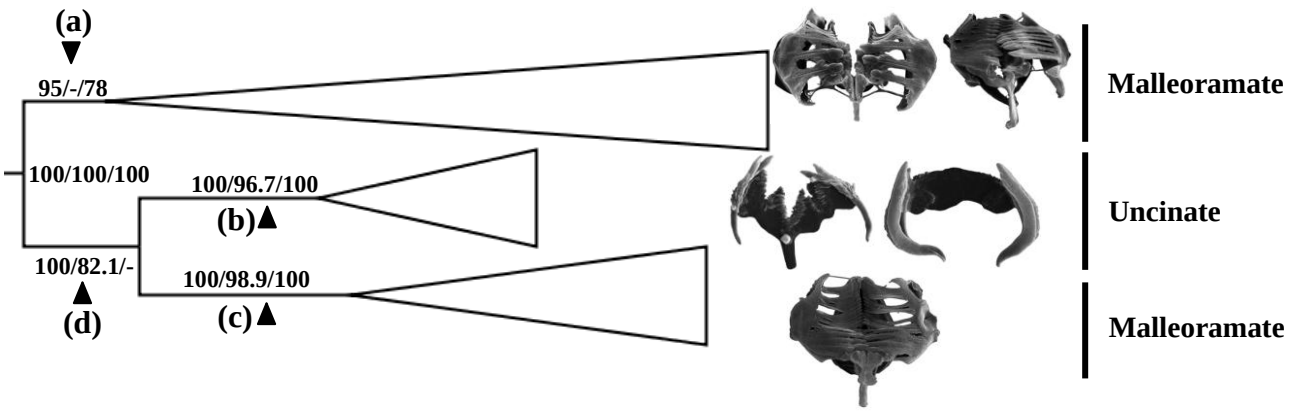


Figure 17 Distribution of trophi types on the tree. The three supporting values – percent of the posterior probability (≥ 90%), bootstrapping (1,000 reps, ≥70%) and jackknifing (1,000 reps, ≥70%), respectively – are indicated on the branches. The letters are referred in the text.

5) Differentiation of unci teeth

There was no pattern regarding differentiation of unci teeth in malleoramate trophi. Neither weakly differentiated nor strongly differentiated (SD) trophi belonged solely to a distinct clade. Rather, each state occurred in different lineages along the tree obtained (Fig. 18).

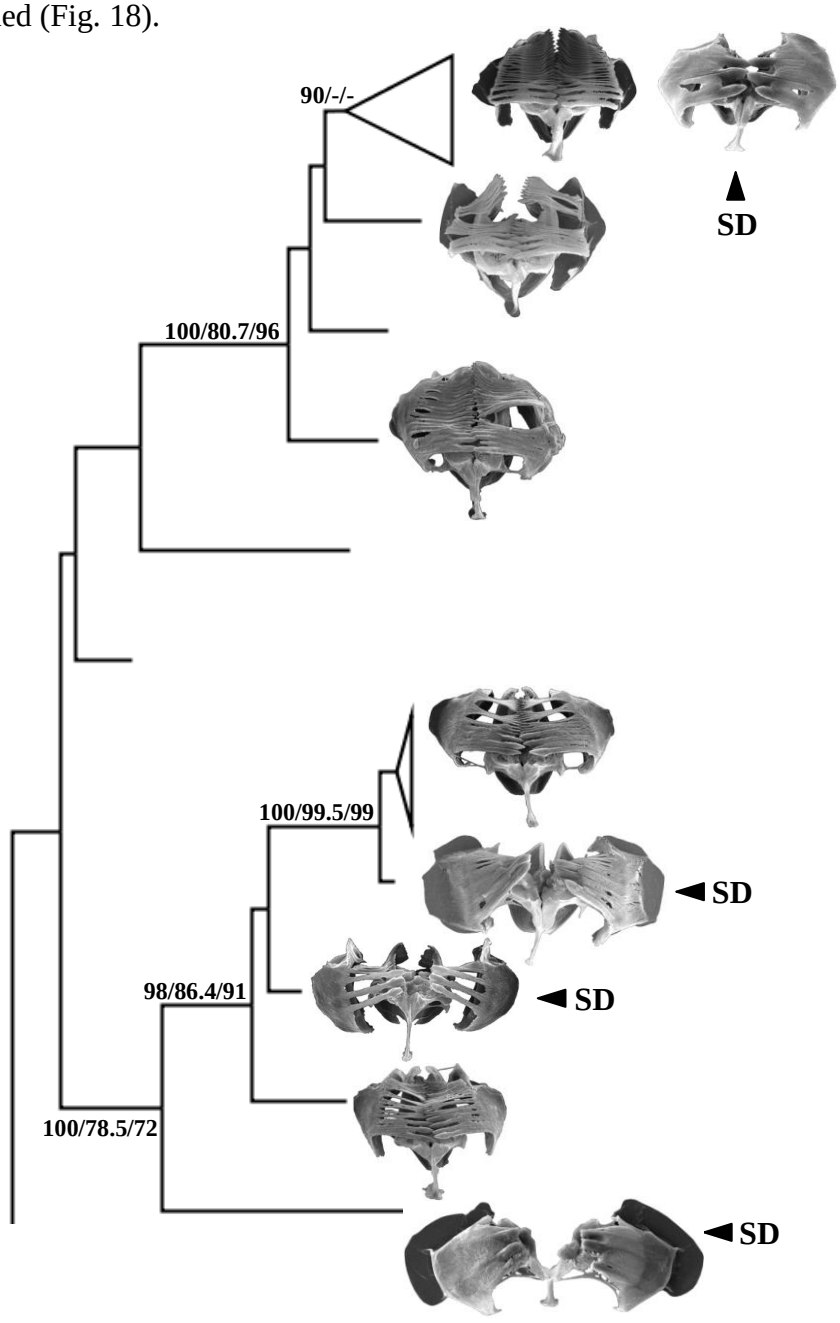


Figure 18 Distribution of differentiation of the unci teeth on the tree. The three supporting values – percent of the posterior probability (≥ 90%), bootstrapping (1,000 reps, ≥70%) and jackknifing (1,000 reps, ≥70%), respectively – are indicated on the branches. The letters are referred in the text. (SDs = strongly differentiated trophi)

6) Symmetry of the trophi

Several representatives carrying asymmetrical trophi formed a clade (Fig. 19a). Although some species possessing asymmetrical state did not belong to this group, their positions contained weak supports (Fig. 19b).

7) Life habits and colony formation

There was no evidence indicating that a certain state of life habits and colony formation corresponded with a single cluster on the tree (Fig. 20). For instance, the character state “sessile solitary” was the most common state and distributed over the entire tree (Fig. 20a). Planktonic colonial species belonged to at least two different lineages (Fig. 20b). Moreover, sessile pseudocolonial lifestyle was also present in distantly related taxa (Fig. 20c).

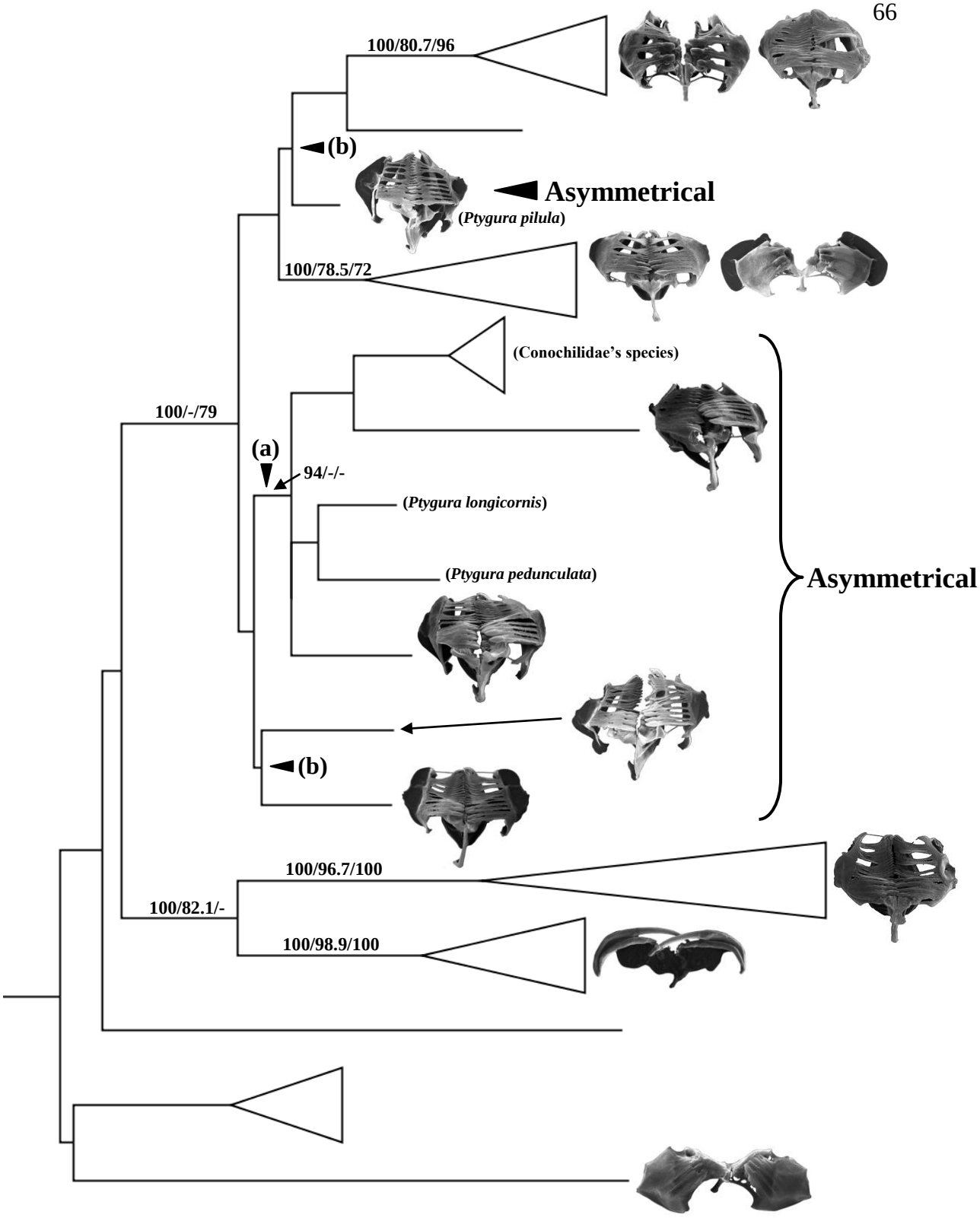


Figure 19 Distribution of symmetry of trophi on the tree. The three supporting values – percent of the posterior probability (≥ 90%), bootstrapping (1,000 reps, ≥70%) and jackknifing (1,000 reps, ≥70%), respectively – are indicated on the branches. The letters are referred in the text.

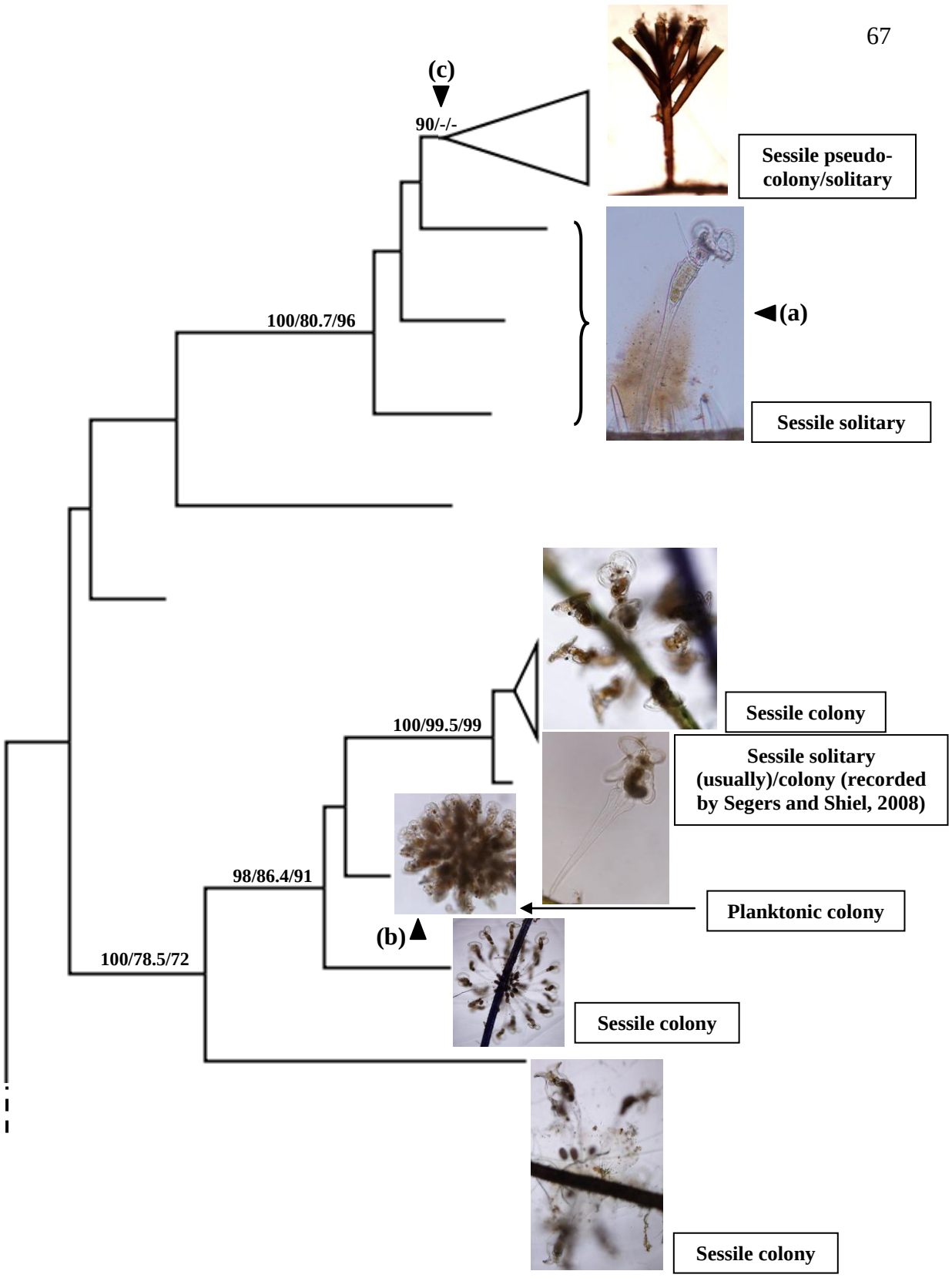


Figure 20 Distribution of life habits and colony formation on the tree. The three supporting values – percent of the posterior probability (≥ 90%), bootstrapping (1,000 reps, ≥70%) and jackknifing (1,000 reps, ≥70%), respectively – are indicated on the branches. The letters are referred in the text.

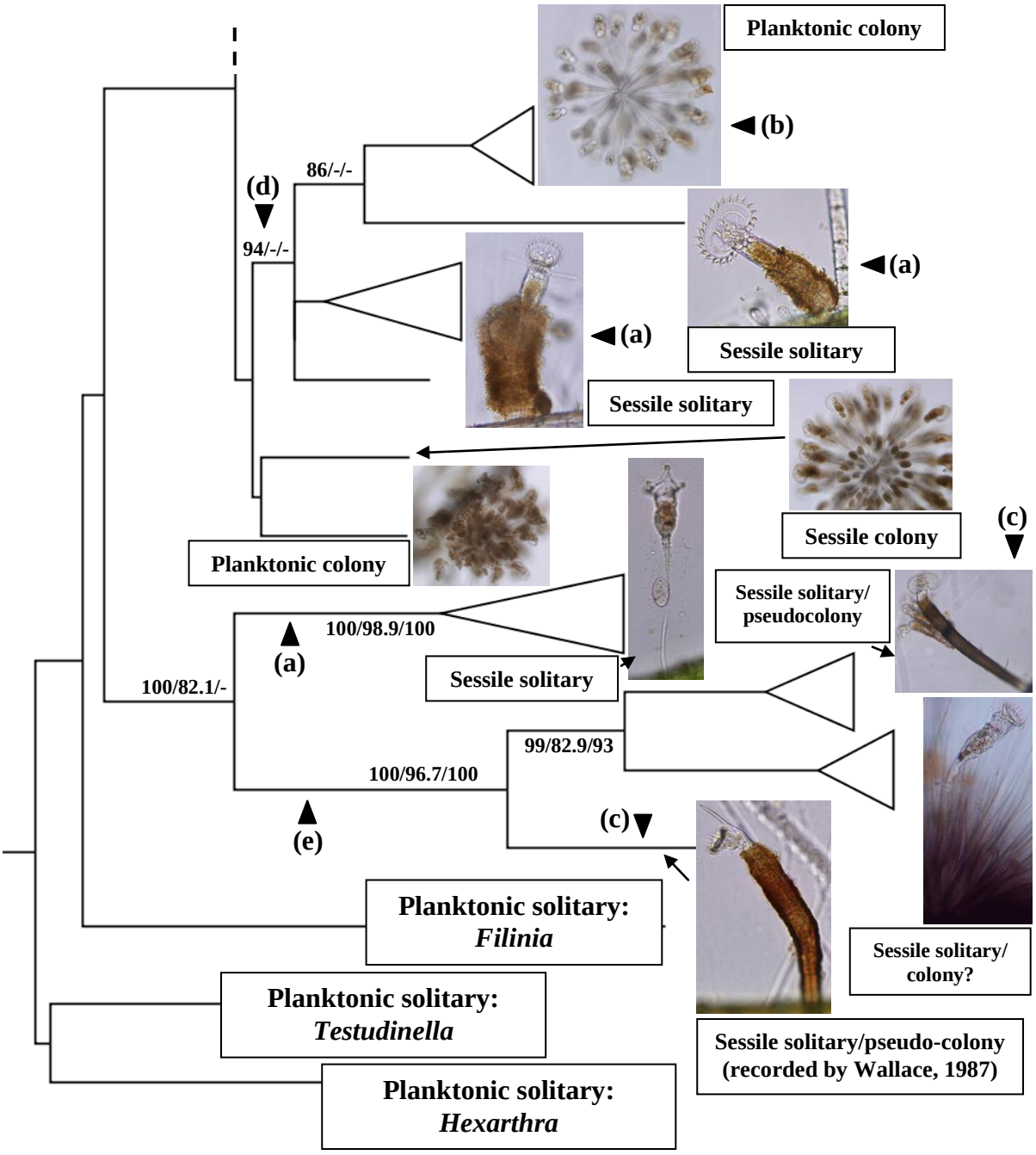


Figure 20 (continued)

CHAPTER 4

DISCUSSION

4.1 Diversity and distribution of sessile rotifers in Thailand observed in the present study

The present study provided two additional species and one infraspecific variant of sessile rotifers known to Thailand. As a result, a total of fifty-six species and three variants of sessile rotifers has been recorded in Thailand (Meksuwan et al., 2013; Sa-ardrit et al., 2013). This number of species observed accounts for about forty-nine percent of species recognized worldwide (Segers, 2007; Segers and Shiel, 2008; Meksuwan et al., 2011, 2013). It is worth noting that this relatively high number of species recorded, including newly described species, is the result of only three studies, which aimed at investigating periphytic and/or sessile rotifer community living around and/or attaching to submerged parts of macrophytes (Koste, 1975; Meksuwan et al., 2011, 2013). Moreover, these works indeed sampled intensively in only two lakes, Bueng-Boraphet lake at central part and Thale-Noi lake at southern part. Although the present study sampled macrophyte materials covering different freshwater habitats in different parts of Thailand (Fig. 3), it was mostly only one-time sampling with relatively low amount of samples, except for the sampling sites in southern part where are near the laboratory, where the present work has been carried out. Accordingly, it seems probable that more extensive and intensive studies would contribute new findings, in particular regarding unrecorded taxa and distribution ranges of sessile rotifers in Thailand. This inference could be illustrated by a case found in the present study that *Ptygura pilula* has just observed in Thale-Noi lake with high abundance after four years of rather intensive observation.

Comparing number of species observed among different parts of Thailand it was revealed that this seem to be a matter of sampling effort rather than a realistic diversity. As addressed above, sampling effort was highest in southern part. Moreover, this can be seen by the correspondence between species richness and number of sampling sites explored in different parts of Thailand (Table 8). Consequently, it is too early to draw a conclusion about species diversity among different parts of Thailand and geographical distribution of sessile rotifer species recorded in Thailand.

4.2 Diversity of sessile rotifers recorded in Southeast Asia: an overview

Sessile rotifers have been reported from Southeast Asian countries for nearly fifty year. At present, a total of sixty-two species is recorded from Southeast Asia (e.g., Koste, 1968; Koste, 1975; Koste and Tobia, 1990; Meksuwan et al., 2011, 2013). The highest number of the species is recorded in Thailand (56 spp), followed by Cambodia (23 spp) and Singapore (19 spp), whereas no sessile rotifers have been documented in two countries including Brunei and East Timor (Fig. 21).

The difference of numbers of sessile rotifer species among the countries could be explained by two alternative hypotheses. There are 1) these are real numbers of the sessile rotifers living there; 2) an appropriate method to study sessile rotifer diversity has not been applied in some countries. Before one of the alternatives is positioned, there are some points that seem useful considering. Firstly, the method that samples submerged parts of macrophytes in the lake and examines sessile rotifers alive was applied only in the countries in which the highest species numbers have been reported (e.g., Koste and Tobias, 1990; Segers et al., 2010; Meksuwan et al., 2011) (except for Singapore that a study method was not indicated in some observations). On the other hand, other low-diversity records collected plankton-fixed samples (e.g., Fernando and Zankai, 1981; Koste, 1988). Secondly, the number of species recorded in Singapore, where sessile rotifers were reported from four researches, was lower than in Cambodia, where a single study which applied the study method mentioned above was carried out (Fig. 21). Finally, Dr. Hendrik Segers and I had an opportunity to collect small macrophyte materials from a swamp in Central Vietnam with a short observation. We identified about six species that all are new to Vietnam (unpublished data), while one of them is probably an undescribed species that is also recorded from Cambodia as *Ptygura* sp. near *linguata* Edmondson, 1939 (Segers et al., 2010). Accordingly, the second hypothesis seems more probable, being relevant to the information available. Nevertheless, this hypothesis must be tested by a further investigation that samples submerged parts of growing macrophytes from Southeast Asian countries and observes the fresh materials with its local filtrated water under a microscope, to search for living sessile rotifers. Then, knowledge of species diversity, composition and zoogeography of sessile rotifers among Southeast Asian countries could be compared and explained afterwards.

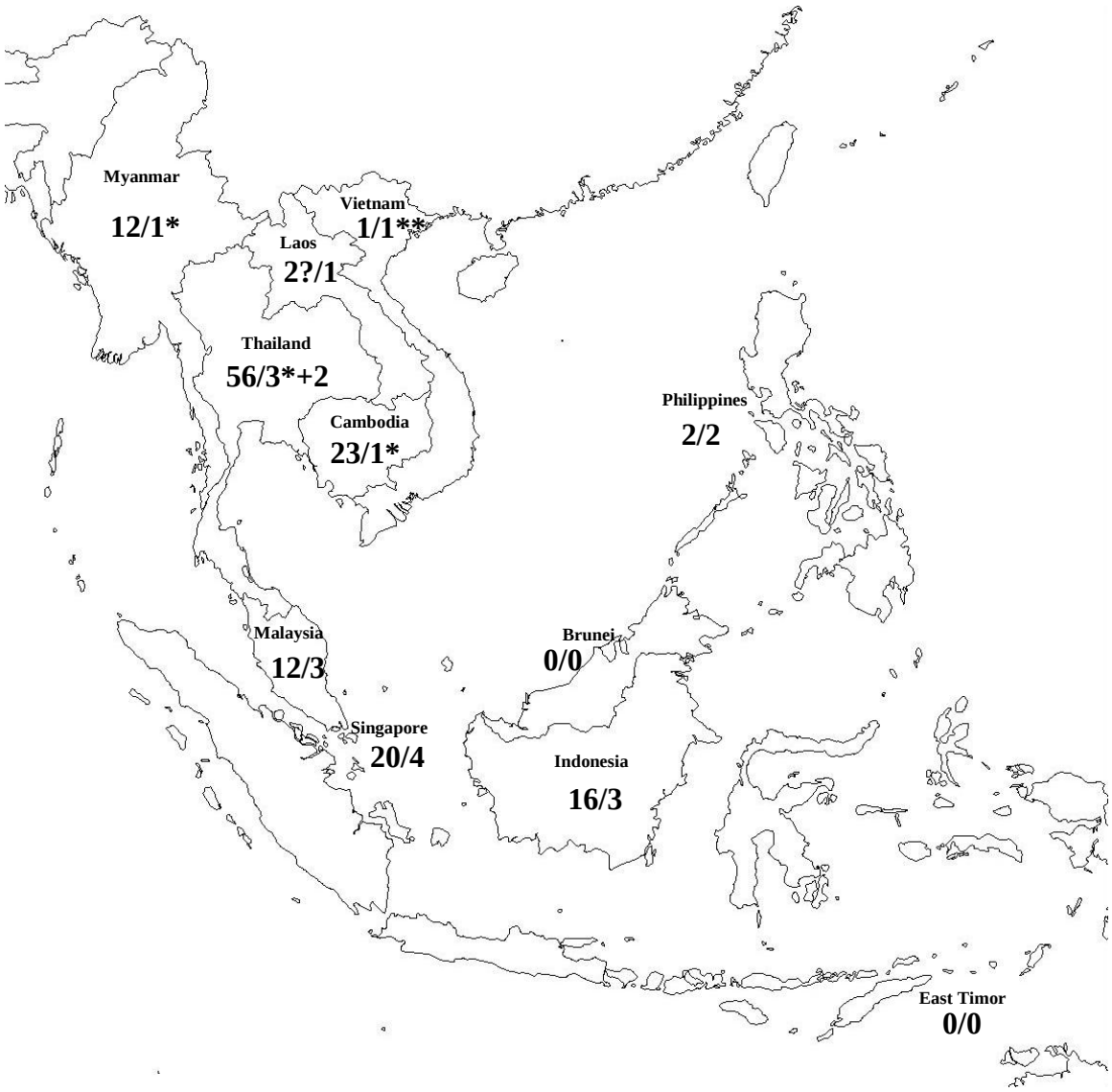


Figure 21 Diversity of sessile rotifer species recorded in Southeast Asian countries. The first number indicated number of species reported; the second indicated number of works that recorded sessile rotifers in the countries; single asterisk indicated the work that applied an appropriate method to study sessile rotifers; two asterisks indicated unpolished data (see Segers et al., 2010); a question mark was for the number that sessile rotifers were not identified into species level. The references for each country are *Cambodia*: Segers et al. (2010); *Indonesia*: Koste (1968), Koste (1988), Sudzuki (1989); *Laos*: Heckman (1974); *Malaysia*: Fernando and Zankai (1981), Koste (1988), Sudzuki (1989); *Myanmar*: Koste and Tobias (1990); *Philippines*: Mamaril and Fernando (1978), Sudzuki (1989); *Singapore*: Karunakaran and Johnson (1978); Fernando and Zankai (1981), Sudzuki (1989, 1991); *Thailand*: Koste (1975), Sanoamuang et al. (1995), Sanoamuang and Savatenalinton (2001), Meksuwan et al. (2011, 2013).

4.3 Phylogenetic trees of 18S rRNA sequences of sessile rotifers

1) Adequacy of the molecular data analyzed

There are major concerns regarding molecular data that determine accuracy of phylogenetic inference which are 1) the sequence quality, 2) correct sequence alignment, 3) few experience of nucleotide substitution saturation, and 4) regularity of the substitution processes along the marker (Lemey et al., 2009).

In the present study, most of the 18S rRNA sequence signals acquired from DNA sequencing analysis (Bio Basic Inc.) contained single, sharp, evenly-spaced peaks, and low noise signals (Fig. 22). This feature indicates rather good quality of the sequences used (https://dnacore.mgh.harvard.edu). However, when a low quality signal was obtained, it was improved by comparing the signal with its complementary-strand signal (can be forward or reverse), and the better one was chosen (Fig. 23). Some regions of the sequences are also double checked by overlapping regions among the three sequence fragments (Fig. 24) obtaining from the three pairs of primers applied (see Table 6).

Since the gene contained rather conserved sites (1,469 bases), the alignment process was done rather straightforwardly and without ambiguous regions (e.g., a large gap) along the gene was present. Thus, the alignment for homologous regions and substitution sites seems straightforward.

The test of nucleotide substitution saturation of the gene sequences revealed that the present data do not reach saturation. It means that the present data still contain information for tracing phylogenetic relationships (Lemey et al., 2009).

However, it is reported that 18S rRNA gene has different nucleotide substitution rates along its length (Mark Welch, 2001). This nature of the gene might less impact on inferring phylogeny than different substitution rates between lineages, making long-branch attraction problem (see following parts). In addition, accuracy (i.e. agreement with other independent characters) of 18S rRNA gene in reconstruction of evolutionary relationships has been evident especially in deep evolutionary time (Hillis and Dixon, 1991; Aguinaldo et al., 1997; Mark Welch, 2001; Giribet et al., 2004).

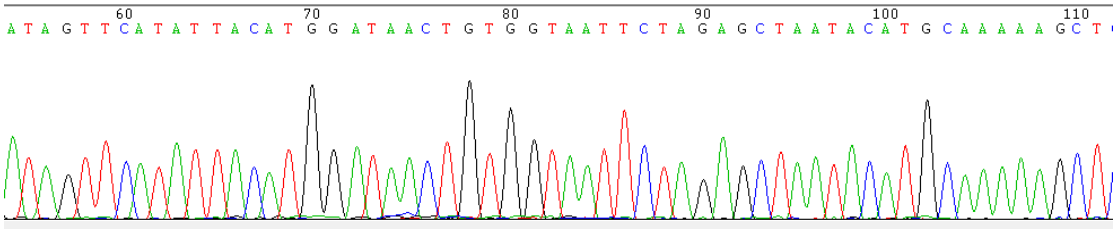


Figure 22 Example of nucleotide signals obtained.

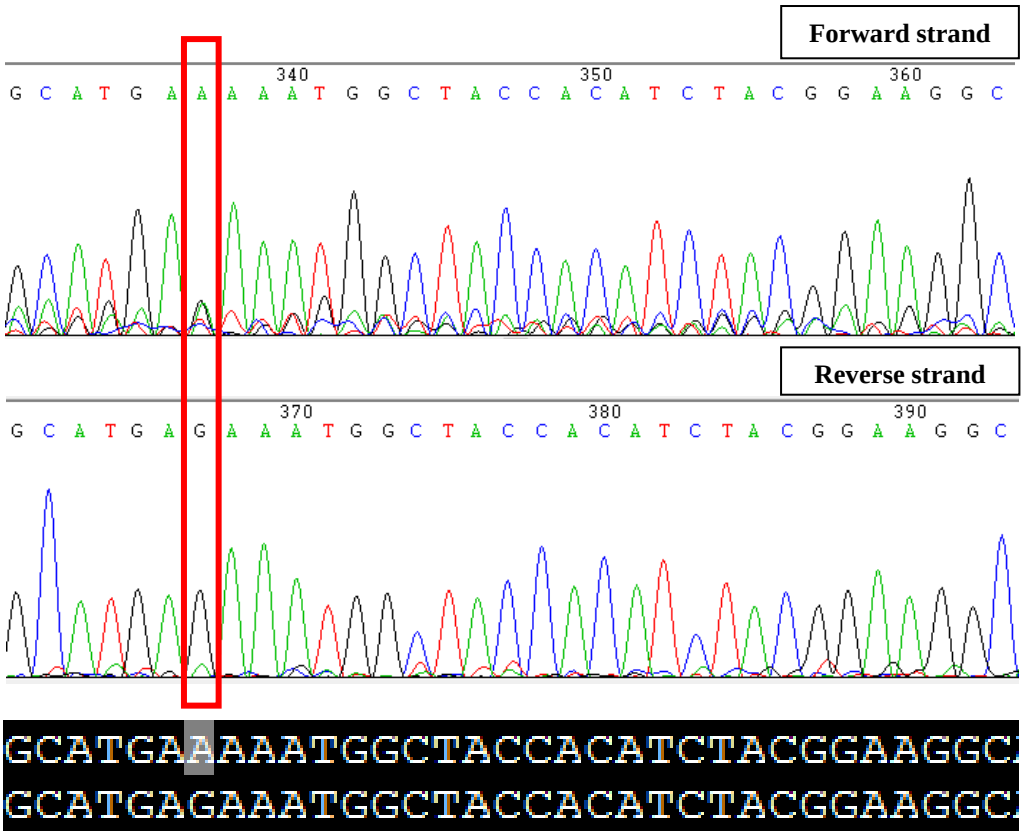


Figure 23 Comparison of DNA sequence signals for sequence improvement. In this sample, reverse strand which contained better quality sequence signals was used to check an ambiguous signal in forward strand (at the site in the red square). Thus, base G was chosen instead of A.

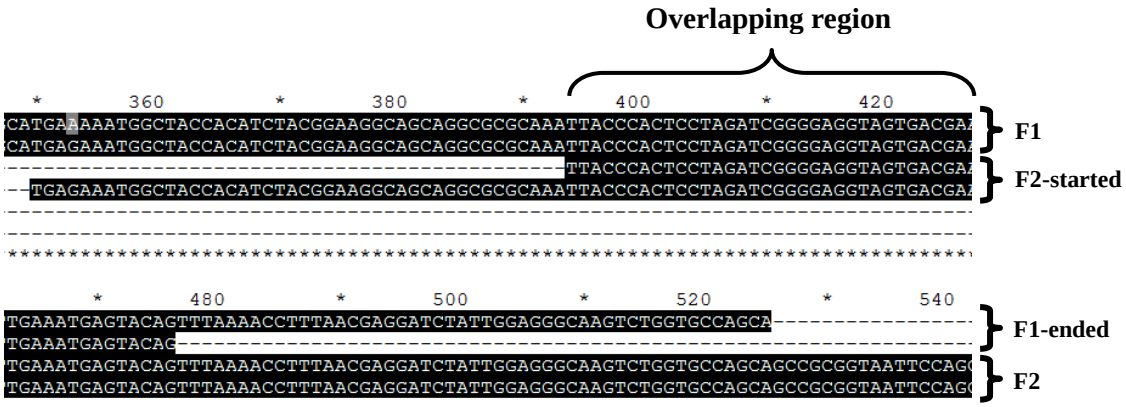


Figure 24 Overlapping region between sequences of the same sample getting from different pairs of primers. **F1**: sequence obtained from the first pair of primer (18SF1-18SR1); **F2**: sequence from the second pair of primer (18SF2-18SR2); **F1-ended**: the most end of the first sequence fragment; **F2-started**: the beginning of the second sequence fragment.

2) Accuracy of the trees reconstructed

Besides careful considering the data used for phylogenetic reconstruction discussed above, other aspects of the tree itself may prove its reliability. Firstly, sufficiency of representative taxa is also one of the factors enhancing resolution of a phylogeny reconstructed (Graybeal, 1998; Mark Welch, 2001). Most taxa in any level examined here were represented by relatively high numbers of the memberships recorded worldwide (100% of the orders, 100% of the families, >80% of the genera, and mostly >50% of the species in each genus; Segers, 2002a, 2007; Segers and Shiel, 2008; Meksuwan et al., 2011), compared with previous studies in which sessile rotifers were included (e.g., Sørensen and Giribet, 2006). This amount of taxa analyzed could result in an accurate phylogeny.

Secondly, several major clades were congruent among the three inference approaches with relatively high branch supports of the three different branch evaluating methods (Posterior probability, Bootstrapping, and Jackknifing) (Figs 11, 12, 13).

Finally, the problem posed by long-branch attraction (LBA) should also be considered in an analysis of phylogenetic relationship. The LBA is a situation of biased grouping between two or more long branches (or short branches), which are distantly related, as sister groups. It is an inherent bias in estimation procedure that can be occurred in any inference method and any level of taxonomic ranks the branches reflect (Bergsten, 2005). In this study, number of the taxon representatives obtained is relatively large (see above). This is one of the conditions that more or less could avoid the LBA (i.e. Adding taxa strategy) (Bergsten, 2005). Moreover, the tree inference methods used, such as, Bayesian inference and Maximum likelihood (ML), evidently are resistant to the LBA especially the ML which is the most resistance among the major methods available (the least is Parsimony) (Anderson and Swofford, 2004; Hordijk and Gascuel, 2005; O'Connor et al., 2010). These conditions could avoid the LBA problem to an effective extent (Graybeal, 1998; Bergsten, 2005).

4.4 Phylogeny and evolution of the major groups of sessile rotifers

1) Evolution and possible ancestral features of the Gnesiotrocha

All analyses demonstrated that all of the gnesiotrochan representatives share a common ancestor (Figs 11b, 12b, 13a). This monophyletic group, as well as being sister group to the pseudotrochans, has been demonstrated by several previous works, using morphological, anatomical and DNA sequence of several genes from both nuclear and mitochondrial genomes (e.g., Wallace and Colburn, 1989; Sørensen and Giribet, 2006). None of these works, however, had a reasonable number of representative taxa (see Sørensen and Giribet, 2006). If this relationship is accepted, the next relevant question would concern the ancestral features of the gnesiotrochan group. Among gnesiotrochans, there are morphological characters that seem useful to discuss these ancestral features, namely, corona modifications, feeding strategies and trophi types, and life habits.

In this study, the results revealed that members of the Flosculariidae and the BLP – the groups that are morphologically most similar (compare Figs 20d and 20e) – are distantly related, and they have split relatively early in the phylogeny inferred (Figs 11: f, L; 13: d, f). Thus, the similarity between them seems to reflect symplesiomorphic character states, retained from their ancestor. Comparing these two clades, it is found that the taxa carrying a complex corona (e.g. five-, eight-lobed corona) are included in the former clade only, the Flosculariidae (Fig. 14d). Hence, it seems probable that an ancestral gnesiotrochan carried a more or less simple corona, and, eventually, the descendants of the lineage of Flosculariidae acquired a much more complicated corona. An alternative hypothesis, that the ancestor possessed a complex corona, and then it was reduced in more recent representatives, of which a majority have a simple corona, seems less likely. This inference might be supported by the fact that newly hatched individuals of all sessile taxa contain less complex corona than the adult stage (e.g., Kutikova, 1995; Fontaneto et al., 2003).

Feeding strategies in the gnesiotrochan group are connected to two major features, the corona structure and the trophi. Filter feeding with malleoramate trophi is present in the two lineages, the Flosculariacea and the BLP, while ambush predation with uncinate trophi and anterior region consisting of an expanded infundibulum with elongate, stiff cilia or without cilia, occurs only in the Collothecacea clade. Based on the outgroup comparison approach (e.g., Watrous and Wheeler, 1981; Bryant, 2001), the former type is plesiomorphic whereas the latter is apomorphic. Accordingly, the parsimonious hypothesis would then be that the gnesiotrochan ancestor was a filter feeder with more or less malleoramate-like trophi. The ambush predation with uncinate trophi evolved later out of this ancestor. The supporting evidence is that the mobile immatures of the ambush predation group contain filtering(?), beating ciliated corona before settling and developing into adult stage that carries ambush predating corona without the filtering corona appearance (Wallace, 1980; personal observation).

To interpret life habit of the gnesiotrochan ancestor, it seems necessary to look at life habits and foot structures of other major groups in the Rotifera – the Bdelloidea, Ploima, and Seisonacea. The bdelloids are mobile but they spend most of the time creeping or immobile, filtering. There are even some that are not able to swim at all. Their foot is terminated by a foot pseudosegment bearing toes or an undifferentiated adhesive surface with openings of numerous pedal glands. This pseudosegment and the toes can be retracted into the foot (Melone and Ricci, 1995; Ricci and Melone, 2000). The ploimids are a group of mobiloe organisms including both periphytic and true planktonic species, in some of which the foot has entirely disappeared. The foot in this group ends with two (or, secondarily, one) toe(s) which does not seem to be retractible into the terminal foot pseudosegment (e.g., Koste, 1978; Nogrady and Pourriot, 1995; Segers, 1995). While almost all members of the two groups mentioned are freshwater rotifers, the seisonids are marine sessile, living permanently attached to a specific taxon of marine crustaceans (*Nebalia* species). This group of rotifers possesses a foot with attachment disc (Ricci et al., 1993). For the phylogenetic relationship among these major groups and gnesiotrochans, I follow the hypothesis that Gnesiotrocha is sister group to the Ploima, these groups together being closely related to the Bdelloidea, and all these together forming a sister group to Seisonacea (Wallace and Colburn, 1989; Segers, 2002a), regardless of the position of

the Acanthocephala that seems to be the closest relative of the Rotifera, and that is a highly specialized and derived group of endoparasites (Wallace et al., 2006).

There are abundant indications that several characters, for example, presence of male, absence of sexual dimorphism, and paired gonads of Seisonacea are primitive features that have been retained from an ancestor of the Rotifera (Epp and Lewis, 1979; Wallace and Colburn, 1989). I accordingly hypothesize that sessile condition of the seisonids also has been retained from the ancestor that, to some extent, may have looked like the seisonids. The evidence that supports this view is that all rotifer taxa, either sessile or planktonic groups, possess pedal glands producing adhesive substance (Wallace et al., 2006). It seems probable that the presence of foot glands in all major rotifer taxa indicated that its ancestors were sessile rather than mobile, and that this ancester gave rise to mobile taxa with an ability to temporarily attach to substrata, such as in both Bdelloidea and Monogononta.

In these two lineages, the ability to be mobile might have developed independently in ploimids and in bdelloids.This implies that the “toes” of ploimids and of bdelloids are not a plesiomorphic feature acquired by a mobile ancestor, but are parallel acquisitions. Such parallel evolution – independent morphological acquisition occurring in the same genotypic basis lineage – appears to have occurred commonly in rotifers (Kutikova, 1983). For the lineage of gnesiotrochans where sessile condition dominates, I propose that the fixo-sessile habit is retained from the group’s ancestor, and not that the sessile condition of seisonids and gensiotrochans are convergent acquisitions from a mobile ancestor. This is at variance from Wallace (1987) who proposed that sessile condition in gnesiotrochans was acquired from a mobile ancestor, and the planktonic life has evolved secondarily in planktonic taxa of Gnesiotrocha.

2) Evolutionary relationship between the Collothecacea and the BLP group, and the switch of feeding modes

A remarkable result of this study is that, the common ancestor of Collothecaceae evolved from a sessile, filter-feeding lineage. Mobile immature individuals of Collothecaceae contain corona with mobile cilia, similar to the young of Flosculariidae and the BLP clade, which might support this inference (Fig. 25).

If this relationship is true, there are two major features that evolved in the Collothecacea lineage – the modified, funnel-shaped infundibulum (including modified corona located along the buccal opening) and the uncinate trophi. These structures serve an ambush-feeding strategy (Fig. 1). One of the possible ways to infer switching of the filter feeding in the ancestor into an ambush predation lineage may be related to food size. It is inferred by the nature of the ambush predation that it usually processes larger food particles than filter feeding (Wallace et al., 2006). Hence, the environmental clue that was precursor for the feeding mode switch might be availability of larger foods. Regarding large foods, two possible selective pressures seem relevant including, 1) domination of the larger foods or, 2) avoidance of food competition. Regardless of the possible selective pressures, the ancestor might have evolved from an organism feeding on small food, to the one processed larger prey. While natural selection favored a larger size of the buccal structure, this may have concurred with acquisition of longer cilia (setae), that confines and/or funnel food to the mouth. The development of long setae from short cilia (of filter feeding) might be illustrated by some species of genus *Collotheca* – one of the genera in this group. Some species (e.g., *C. mutabilis*) possess long, capturing trochus setae while a filtering cingulum band is still present (but completely absence in most species) (see Koste, 1978; personal observation). Although the Atrochidae – one of the other members of this clade – possess no cilia on their corona, the phylogenetic analysis revealed that the group appears to have evolved from the *Collotheca*-stem lineage (Figs 11d, 12e). This interpretation may be also supported by the young individuals of the Atrochidae (e.g., *Acyclus inquietus*) that carry mobile cilia on their corona as in other Collothecaceae (Fig. 25). Since an enlarged buccal structure was gained, I further hypothesize that the uncinate trophi developed after that. Because larger foods

were captured, malleoramate-like form of the ancestor might become to be replaced by a forcepts-like form that has higher capturing ability, in which more or less look like the uncinate form eventually.

In conclusion, I hypothesize that the Collothecacea evolved from sessile, filter feeding ancestors and gained their ambush predation feeding mode with uncinate trophi by exploiting different food source (i.e. larger foods). Meanwhile, the BLP clade has retained several plesiomorphic features including filter feeding with malleoramate trophi.

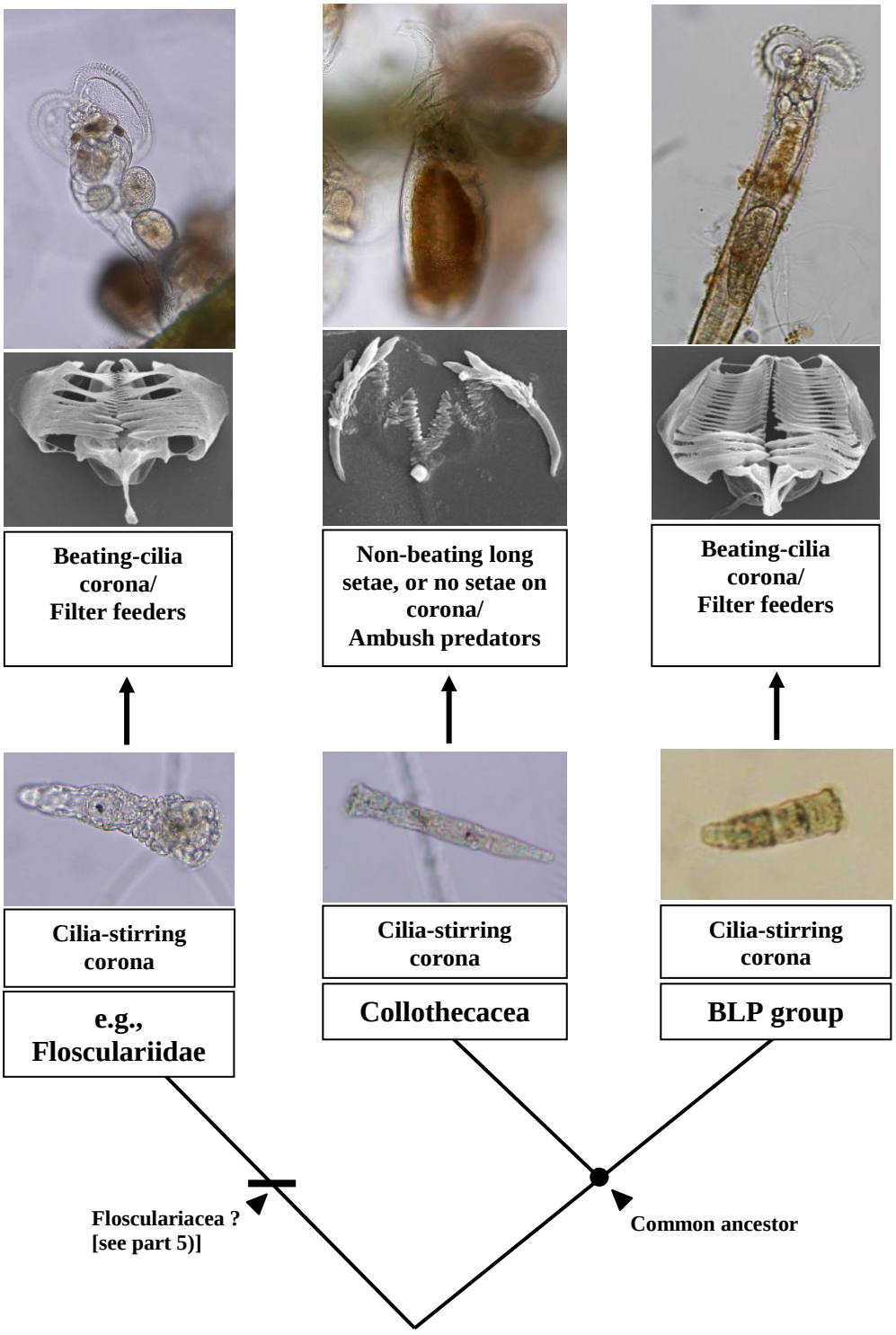


Figure 25 Phylogenetic relationship, corona structure of immature and adult stage, and feeding components of the Collothecacea and the BLP group. The Flosculariidae represents the outgroup of the two lineages.

3) Evolution within the BLP clade

I identified a possible autapomorphic feature to the BLP clade. All members of this group contain a stiff structure (plate) locating beneath the lorica at dorsal-anterior side (Fig. 26). The structure can appear with small to large hooks (Figs 26: A-B), horny processes (Figs 26: C-D), or a long, slender tube (i.e. *Beauchampia crucigera*, see Koste, 1978), all associated with the dorsal antenna. A superficially similar stiff structure can be found outside the BLP clade, such as in genus *Floscularia* and some species of genus *Ptygura* (Fig. 27). In contrary to the BLP clade however, I hypothesize that the stiff structure found outside the BLP clade is formed in a different way. In other words, it may not be homologous to ones found in the BLP group. In those of *Floscularia* and *Ptygura*, the structure seems to consist of a local stiffening of the surface lorica itself, and may be not of a stiff plate, located beneath and separate from the surface lorica. If this would be confirmed by an experiment, the similar appearance of the dorsal stiff structures is likely to be homoplastic convergence. It may be explained by the fact that, when sessile rotifers are disturbed, they contract the corona into its body exposing the dorsal region, where the stiff structure is located. Evolutionary gain of a stiff structure, in particular a horny process, as protection of this exposed body region may enhance defense against predators. Natural selection favors the trait allowing different lineages, such as the BLP clade and the others, to independently gain this structure.

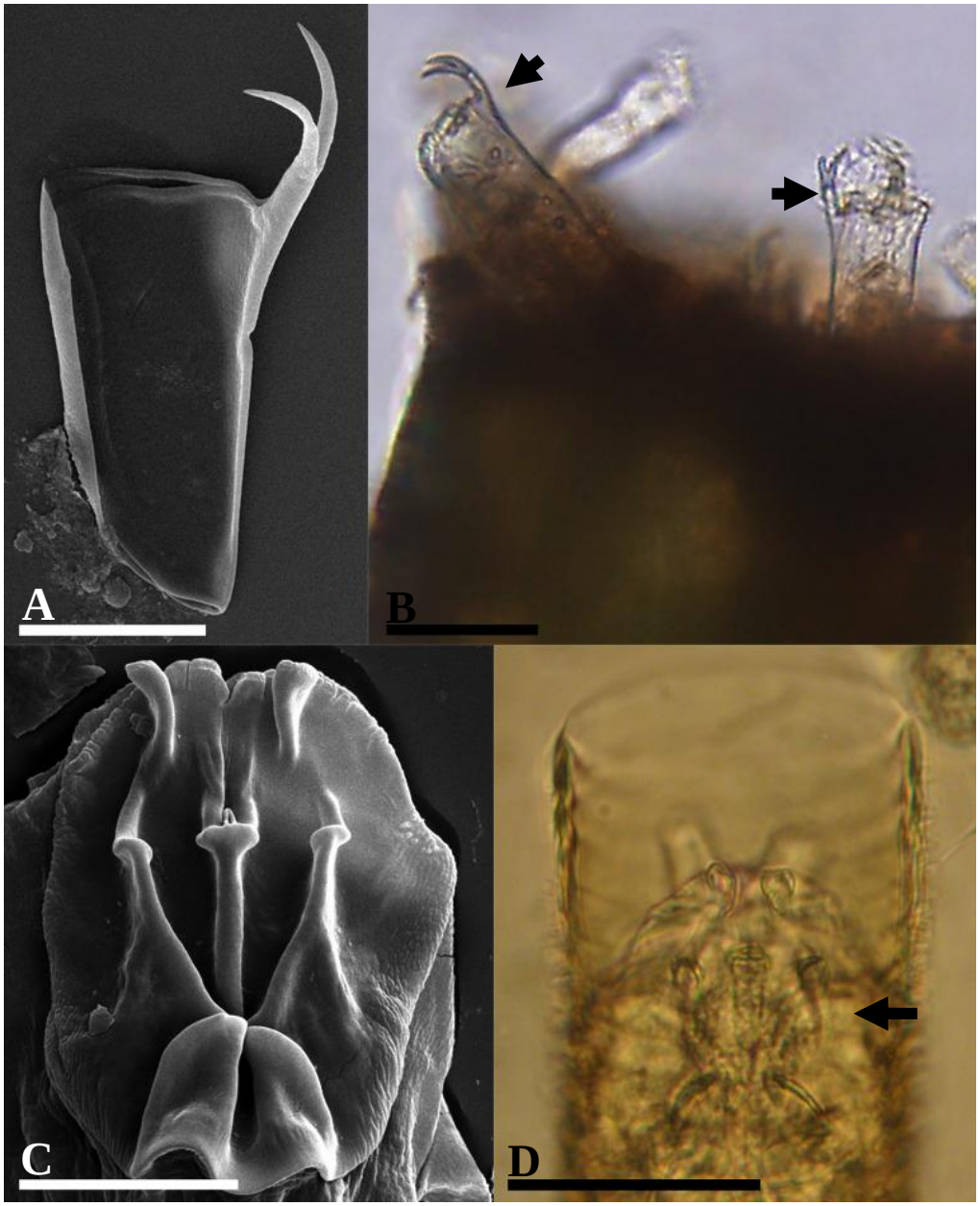


Figure 26 Dorsal stiff plates in the BLP group. A-B (lateral view): *Ptygura furcillata*; C-D (dorsal view): *Limnias melicerta*. Arrows indicate the dorsal stiff plates observed by light microscopes. Scale bars: A, C = 20 µm, B, D = 50 µm.

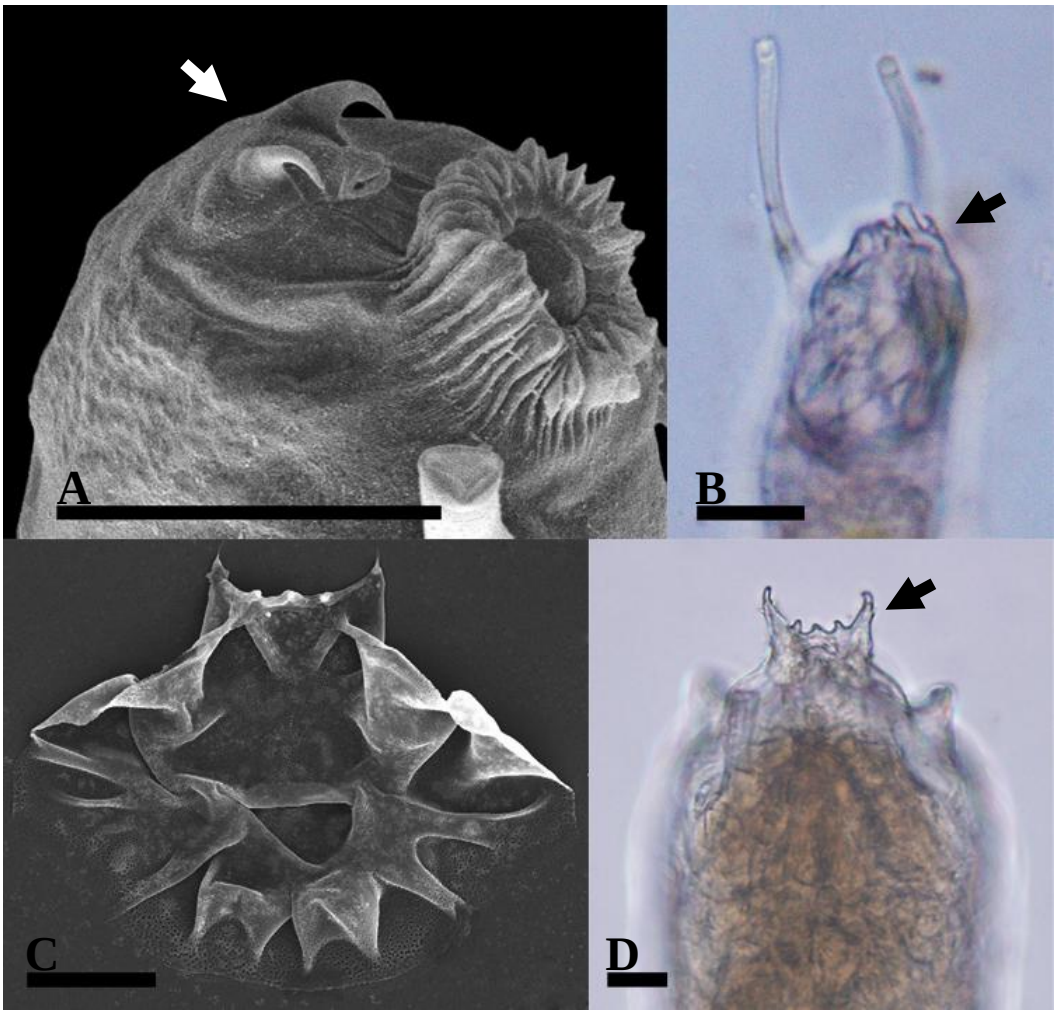


Figure 27 Dorsal stiff lorica in different species. A (ventral-lateral view)-B (dorsal-lateral view): *Ptygura thalenoiensis*; C-D (ventral view): *Floscularia armata*. Arrows indicate the dorsal stiff lorica observed by light microscopes. Scale bars: A-D = 25 μm.

4) Evolutionary relationships among sessile taxa of the Floscullariacea

The analysis revealed three groups in this lineage. Group 1 is composed of three species of genus *Ptygura*, including *P. noodti*, *P. tacita* and *P. thalenoiensis*, and all representatives of genus *Floscularia* (Figs 11m, 12g, 13g). It was noticed that these three *Ptygura* species share a number of morphological features. They possess a dorsal stiff projection similar to that of *Floscularia* species of the *F. ringens* group (Fig. 27). The tube is composed of constructing components including debris and a gelatinous secretion of the species. The tube of *P. tacita* and *P. thalenoiensis* is filamentous, with fine layers, while that of *P. noodti* is constructed of pellets that look similar to some *Floscularia* congeners, in particular *F. janus*, but the pellets of *P. noodti* are longer and are more looser packed (Fig. 28). Moreover, a modulus, which is found in all *Floscularia* species (except for *F. melicerta*, of which no material was found during this study), is present in *P. noodti* (Fig. 5). Hence, both morphological characters as well as the molecular analysis seem to indicate that these three species of *Ptygura* may be closely related to the *Floscularia* of the *F. ringens* group. It could therefore be hypothesized that these *Ptygura* species resemble a possible ancestor that gave rise to the *Floscularia ringens*-group.

Group 2 included *Lacinularia flosculosa*, *Lacinularoides coloniensis*, *Pentatrocha gigantea*, *Sinantherina semibullata* and *S. socialis* (Figs 11o, 12h, 13h). The two sister taxa, *P. gigantea* and *S. socialis*, as well as *S. semibullata* have the oviferon in common. Although the oviferon also appeared in *S. spinosa* – the species locating outside this clade (Figs 11, 12, 13), there are some differences between the oviferon of *S. spinosa* and the three species. In *S. spinosa*, it is present nearly basally at their foot and is formed by a structure which might be separated from the foot and not near the cloaca aperture (Fig. 29B: a). In the letter group in contrast, the oviferon is located much higher up on the body and the organ looks as if it is formed by the trunk part (Figs 29C-E: b). However, a detail investigation, such as an anatomical study, is needed to examine whether the two forms of the oviferon are homologous or not.

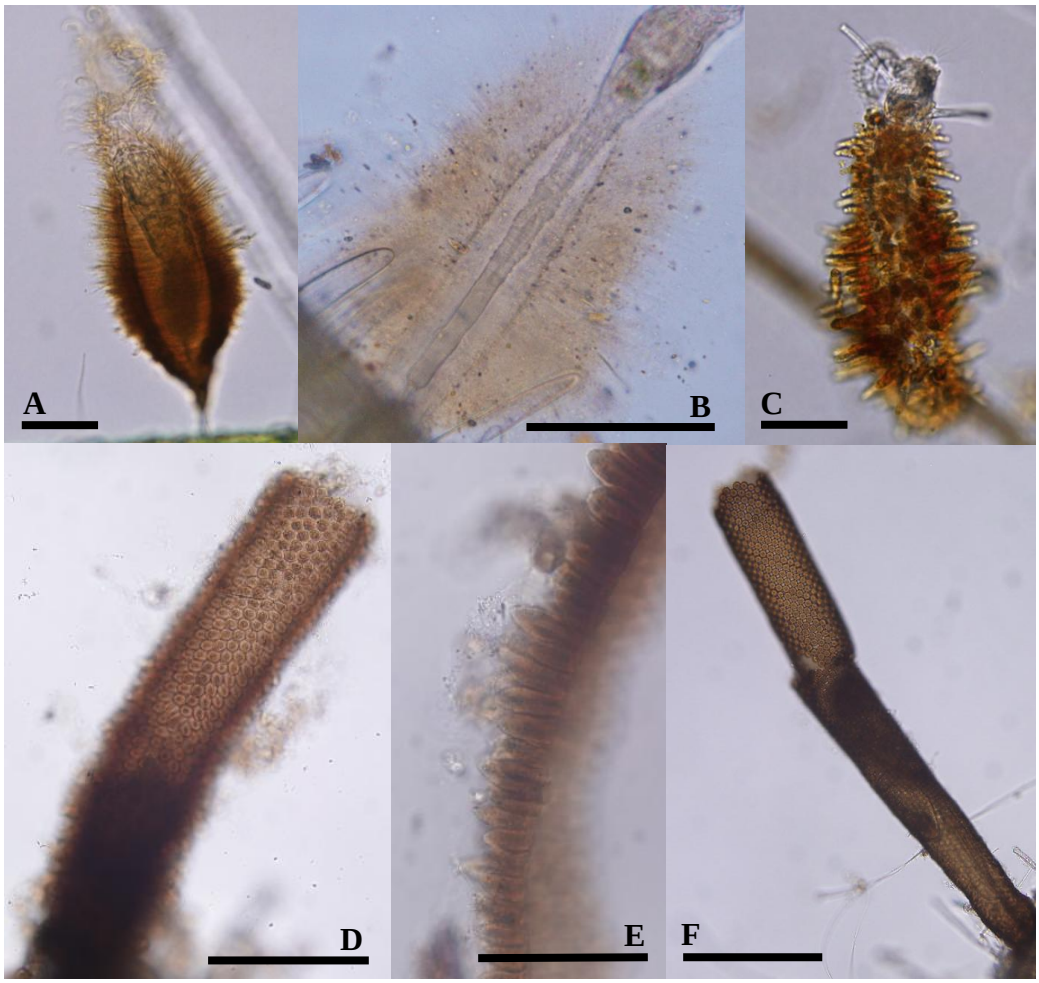


Figure 28 Comparison of case structure in some species. A: *Ptygura tacita*; B: *P. thalenoiensis*; C: *P. noodti*; D-E: *Floscularia conifer*; F: *F. ringens*. Scale bars: A-C, E = 100 µm., D, F = 200 µm.

Figure 29 Comparison of oviferon (egg-carrier organ) in different species (arrows). A-B: *Sinantherina spinosa* (a), C: *S. socialis* (b), D: *S. semibullata* (b), E: *Pentatrocha gigantea* (b). Scale bars: A, E = 250 µm., B-D = 100 µm.

Lacinularia flosculosa and *Lacinularoides coloniensis*, respectively, are closest to the three species carrying the oviferon. To some extent, all of them share some noticeable features. They usually form a relatively large colony and product relatively clear gelatinous case (Fig. 30), although these features can be found in some taxa in different clades. Moreover, there is no an obvious morphological structure, such as spines and horns, that is used for physical protection from predators in these taxa. It appears that colony formation ability – that was also expected to present in their ancestor – might be advantageous to this lineage for its diversification (see Wallace, 1987).

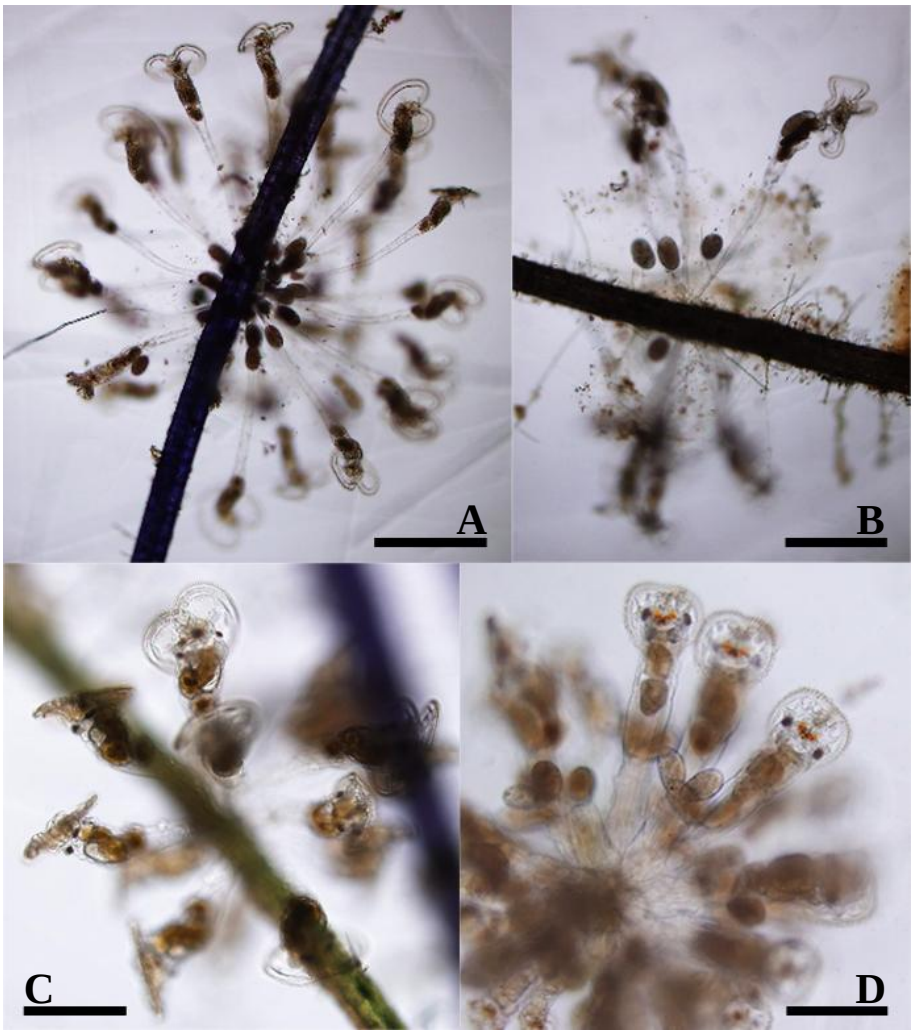


Figure 30 Flosculariidae's species. A: *Lacinularia flosculosa*, B: *Lacinularoides coloniensis*, C: *Sinantherina socialis*, D: *S. spinosa*. Scale bars: A-B = 500 µm., C-D = 250 µm.

Group 3 composed of a single clade of the Conochilidae and a number of *Ptygura* species, although this clade was supported by the BI tree only (Fig. 11s). Since the Conochilidae possesses planktonic habit, interpretation on the evolutionary scenarios of them, as well as of other planktonic groups, are provided in separate section below.

5) Evolutionary relationships between the major planktonic and sessile groups

The representatives of four major planktonic families, including Conochilidae, Hexarthridae, Testudinellidae and Trochosphaeridae (i.e. *Filinia*) (Table 3), were investigated in the present study, while some planktonic species in *Collotheca* and *Ptygura* have not been analyzed yet.

The results revealed that the Conochilidae formed a clade with a majority of the Flosculariidae of which most members are sessile (Figs 11L, 13f). Among the major planktonic groups in the Gnesiotrocha, the Conochilidae is the only taxon that exhibits several features in common with sessile taxa, for example, they have an illoricate body with an elongated foot, relatively large antenna, gelatinous case production, and several features of immature stage of Flosculariidae (Koste, 1978). However, this group also contains several hypothetically derived features, for instance, corona orientation and detail morphology of the trophi (Segers and Wallace, 2001). This could be interpreted as that Conochilidae is a crown group diversified from a Flosculariidae ancestor. Since Conochilidae formed a clade with some of *Ptygura* species (Fig. 11r), it could be inferred that an ancestor of the Conochilidae would more or less have a *Ptygura*-like appearance. However, most species of the *Ptygura* possesses no (true) colonial condition as in most of the conochilids (Koste, 1978; Wallace, 1987; Meksuwan et al., 2011). Accordingly, a more refined analysis is needed to infer an ancestral features of the Conochilidae, for example, obtaining more representatives of either the Conochilidae or the "*Ptygura*", as well as more genes in the analysis. In conclusion nevertheless, membership of the Conochilidae in the Flosculariidae was settled in the present molecular phylogenetic analysis.

In contrary to the Conochilidae, the relationships between the other planktonic groups and sessile groups were not resolved in the present study (Figs 11, 12, 13). Because indeed relatively few representatives of the planktonic groups were obtained, I decided not to interpret any plausible phylogenetic relationship between them and sessile taxa. Nevertheless, there are two (if the planktonic families are monophyletic group) or several (if they are not) possible relationships between the planktonic groups and sessile taxa that could be presented as in Fig. 31.

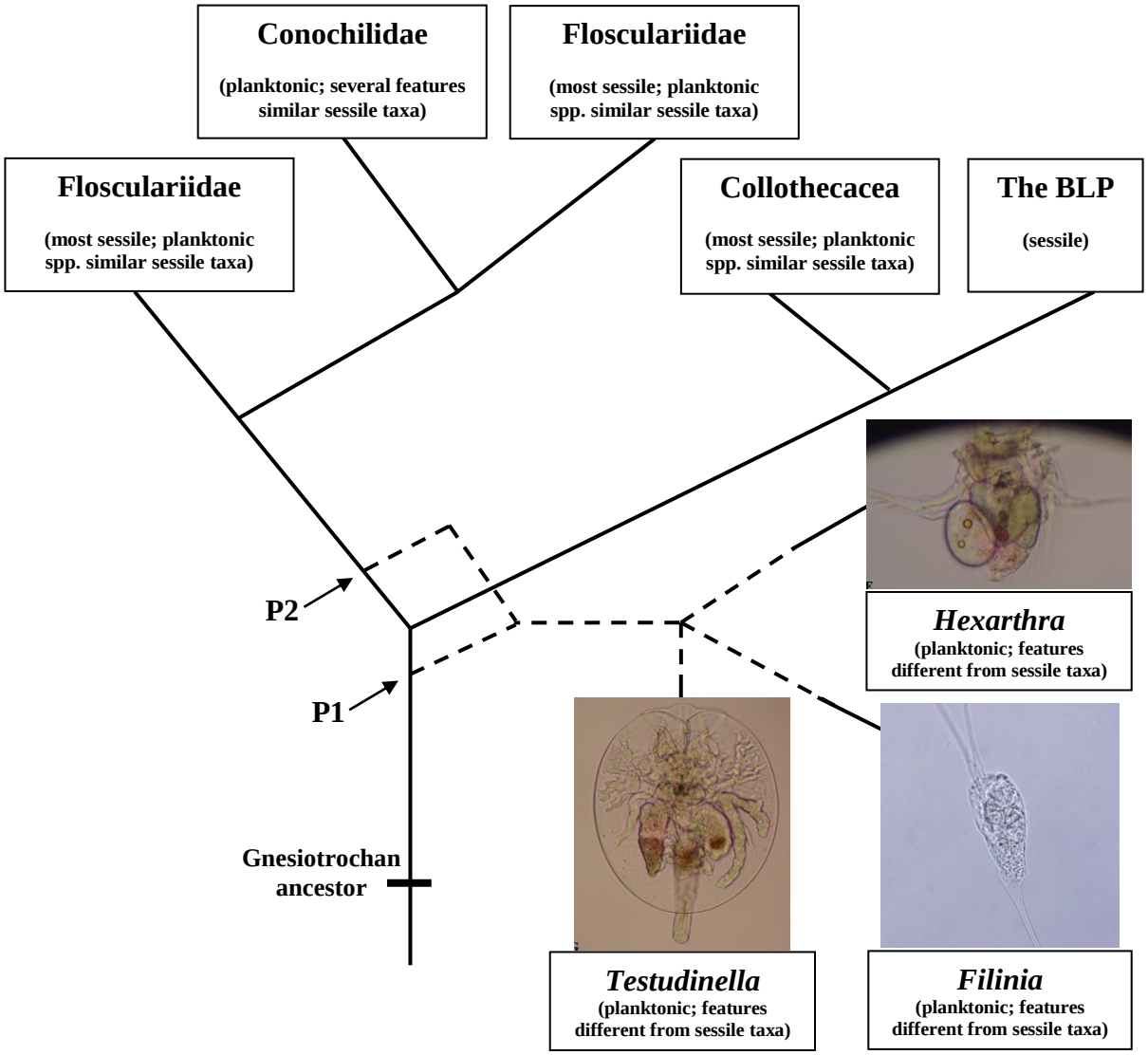


Figure 31 Relationships among the major groups in the Gnesiotrocha uncovered in the present study. The dashed lines indicated the positions that are uncertain in the present analyses and the arrows indicate example of the possible relationships between the planktonic and sessile taxa (P1 and P2) in case the planktonic families are not monophyletic group.

4.5 Phylogeny and evolution of selected morphological characters, and their relation to classification of sessile rotifers

1) Corona (shape and number of lobes)

Among sessile rotifers, similar gain of corona states, e.g., five-lobed corona, appear to have evolved in distantly related lineages (Fig. 14). This could imply that corona structure may be closely related to ecological adaptation involving a selective force that may be related to filtering, hence feeding efficiency of the corona (see e.g., Kutikova, 1983). At present, however, the research area that deals with the connection between certain environmental conditions and corona features has not been explored yet (Wallace et al., 2006).

Traditionally, corona features, in particular shape and number of its lobes is used for classification at the generic level in family Floscullariidae (Koste, 1978; Segers and Shiel, 2008; Meksuwan et al., 2011). However, the presentstudy reveals that certain character states related to the corona may have evolved several times among sessile rotifers, and may therefore be homoplastic. Therefore, introducing this feature as diagnostic feature for classification of Gnesiotrocha would lead to recognition of para- and/or polyphyletic taxa. In other words, I conclude that corona shape and number of corona lobe should not be used for classification at any higher level in sessile rotifers.

2) Modulus

The result shows that modulus seems to has evolved only once in a lineage of sessile rotifers (Fig. 15). However, modulus is present in species that are presently assigned to different genera and their morphology, in particular corona, is quite distinct. As addressed below, this lineage needs further study to unravel their relationship. Thus, taxonomic consideration on the presence of modulus could be performed thereafter.

3) Oviferon

In this study, an oviferon is present in two different lineages (Fig. 16). As discussed above, the oviferon in the two lineages seems to have developed from different initial structures [part 4.4, 4), page 86]. This indicates that the two groups involved may have acquire it independently. Nevertheless, supports for the phylogenetic position of *Sinantherina spinosa* are weak in all analyses (Fig. 16b). In addition, there are an additional number of species known to carry an oviferon, but they are not included in the present analysis (e.g., *Sinantherina triglandularis*, see Segers et al., 2010). Therefore, more representatives are needed to confirm the evolution of oviferon in sessile rotifers. Accordingly, I suggest not to considered presence of oviferon for classification purposes until the sufficient data isobtained and analysed.

4) Trophi type

Based on outgroup comparison approach, malleoramate trophi type is the plesiomorphic character state, from which the uncinate trophi type is derived as specialized type and therefore apomorphic within Gnesiotrocha. However, the malleoramate type may be considered synapomorphic for Gnesiotrocha as a whole. The uncinate trophi of *Cupelopagis vorax* – the single species of a monotypic genus in Order Collothecacea that is not included in this analysis – are unique. It is made up of a large, single pair of unci teeth with a few smaller, potentially functional teeth (Fig. 3.11 in Wallace et al. (2006)). Whereas uncinate trophi of the remaining species of Collothecacea including *Acyclus inquietus* – a species traditionally located in the same family of *C. vorax* – is composed of two pairs of large unci teeth; in these there are no additional potentially functional teeth (Fig. 32), albeit that a number of subuncinal teeth are present in *A. inquietus* (Fig. 32A: a). Thus, a further study that *C. vorax* in the analysis is needed, testing if the uncinate trophi are indeed a synapomorphic character of Collothecidae or if the trophi of *C. vorax* should be recognized as only superficially resembling collothicid trophi, but autapomorphic for *Cupelopagis*.

According to the present analysis, the malleoramate trophi type should therefore not be used to diagnose higher taxa within Gnesiotrocha (e.g., Floscullariacea). Meanwhile, the uncinate trophi type should be interpreted with caution until additional data is obtained, in particular a phylogenetic analysis that includes additional taxa of Order Collothecacea such as *C. vorax* and other species of Atrochidae.

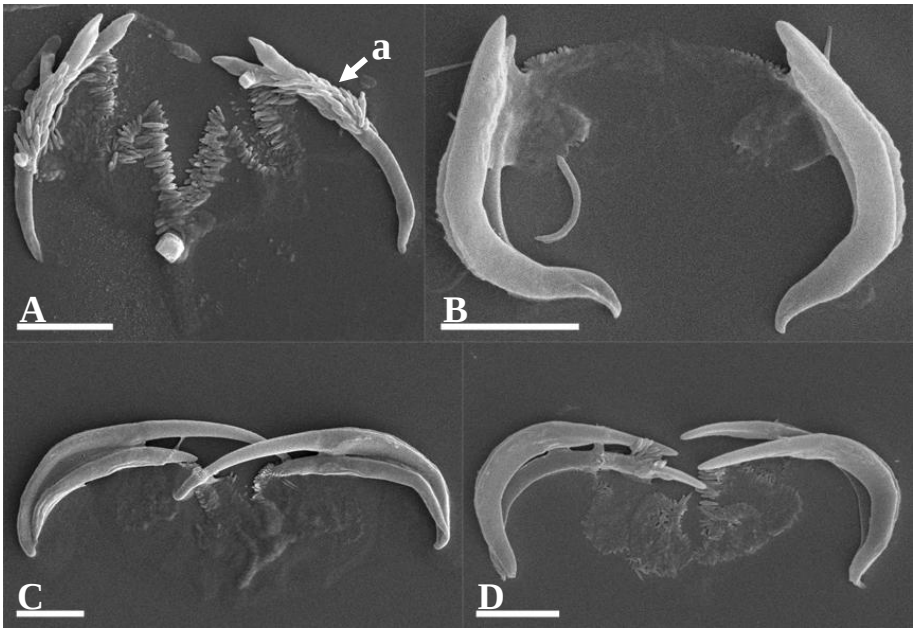


Figure 32 Comparison of uncinate trophi in different species. A: *Acyclus inquietus*, B: *Collotheca campanulata campanulata*, C: *C. trilobata*, D: *Stephanoceros millsii* (a = subordinate, (hypothesized) non-potential functional teeth). Scale bars: A-D = 5 µm.

5) Differentiation of malleoramate unci teeth

The result presented here demonstrate that both character states – either weakly or strongly differentiated teeth - appear to have evolved several times along different lineages of sessile rotifers (Fig. 18). Apart from frequency of transformations (from ancestral to evolved state), Markevich and Kutikova (1989) proposed that simple, separated teeth, usually along with weakly differentiated teeth, is the more

primitive state, which evolvedby consolidation of teeth into more robust, connected teeth plates, a state that usually coincides with strongly differentiated teeth. The interpretation (including that of manubria, rami, and fulcrum) covers transformation toward different types of rotifer trophi, on which the hypothesized pleiomorphic characters (e.g., simple separated teeth) came from comparison with ramate trophi of Subclass Bdelloidea as outgroup. Melone et al. (1998), nevertheless, observed several features in the ramate trophi seem to be autapomorphy of the bdelloids.

Segers (1997) pointed out that differentiation of unci teeth seems connected to food preference. Species carrying weakly differentiated teeth with weak teeth in the proximal group may feed on tiny particles, while those that possess strongly differentiated teeth with strong proximal teeth may feed on studier food particles. He further proposed that the difference of unci teeth in the two groups could explain niche segregation among the congeners. According to the present result, I concur with the hypothesis that the differentiation of unci teeth may have occurred in response to resource partitioning among closely related species rather than being an autapomorphy fixed in a lineage of sessile rotifers.

Taxonomically, classification of higher taxa of sessile rotifers should therefore not rely on degree of differentiation of the malleoramate unci teeth. Nevertheless, at species level, the character states may prove useful in diagnosing morphospecies (Segers, 1997).

6) Symmetry of trophi

The analysis reveals that several species possessing asymmetrical malleoramate trophi are closely related (Fig. 19a), in spite of uncertain position of some of the species that carry asymmetrical trophi. These two species, *Ptygura pedunculata* and *P. longicornis*, and Conochilidae species belong to a clade that appears to be characterised by its asymmetrical trophi (Segers and Wallace, 2001; Meksuwan et al., in preparation) (Fig. 19).

Here, I hypothesize that the structural change affects the efficiency of, for example, mastax muscles for food processing (e.g., crushing), or, ultimately, energy balance of the rotifers. Therefore, I suggest that additional research such as, structural-functional study of the mastax and physiological study in this group would be

promising, and might reveal some aspects of rotifer evolution that have not been discovered.

I refrain from classifying the species carrying asymmetrical malleoramate trophi separately since the calde is in fact supported by only one approach of branch evaluation, and as the position of many of the species has not been adequately resolved.

7) Life habits and colony formation

The present study could not identify characters related to life habits and colony formations that are phylogentically informative (Fig. 20). Regardless, however, of how often each state has evolved, an interesting discussion can be held regarding the evolutionary direction of these features. Wallace (1987) proposed that sessile condition in Gnesiotrocha was the primary requirement for evolution of colonial life. He further suggested that all planktonic species, either solitary or colonial ones, evolved from sessile ancestors. Thus, his hypothesis on the evolutionary direction of the sessile condition and coloniality is, from sessile, solitary ancestors, to sessile colonial, to planktonic colonial and reverting to planktonic solitary taxa. In addition, it is noteworthy that in Collothecacea, colony formation does not seem to occur. In contrast to Flosculariacea, Collothecaceaare ambush predators, not filter feeders. Hence, colony formation may be related to feeding mode of the rotifers, and this is a challenging area for research.

4.6 Phylogeny and classification of sessile rotifers

1) Superorder Gnesiotrocha

The present study reveals that superorder Gnesiotrocha is a monophyletic group, hence a valid taxon in phylum Rotifera. A possible synapomorphic character of the group is not evident, but all ingroup species share absence of (a) toe(s). Furthermore, although the present analysis did not include a representative of subclass Bdelloidea, there are a number of data, especially anatomical and gene sequence, that indicate that order Ploima is sister group to the representative species of sessile rotifers (Wallace and Colburn, 1989; Sørensen, 2002; Giribet et al., 2004; Sørensen and Giribet, 2006). Thus, monophyletic lineage of Gnesiotrochans, which is rooted by representatives of the Ploima, seems acceptable.

2) Order category

At present, two orders – Collothecacea and Flosculariacea – are recognized in superorder Gnesiotrocha (Segers, 2002; Wallace et al., 2006). Based on the present study, Collothecacea is monophyletic group, whereas Flosculariacea is paraphyletic. Flosculariacea is indeed composed of two different lineages, namely the BLP group – which is in a sister-group relation to Collothecacea – and the remaining members of Flosculariacea. As a result, moreover, malleoramate trophi type, which is common to all Flosuclariacea, appears to be symplesiomorphic character status.

As discussed above, molecular data and some features of immature stage indicate that the ancestor of Collothecacea may have evolved from a sessile, filter-feeding lineage (part 4.4, 2, page 80). In addition, Collothecacea forms sister group to the BLP group that has an external morphology that is similar to members of Family Flosculariidae, which belongs to the lineage of Flosculariacea (see Koste, 1978). Hence, I hypothesize that Collothecacea may be a specialized group that evolved from Flosculariidae-BLP-group-like lineage. Taxonomically, if this hypothesis is accepted, there is no ground to recognize Collothecacea and Flosculariacea as two separate taxa at the same level. Accordingly, I propose to include all subtaxa of Collothecacea, as well as the BLP group, in Order Flosculariacea, and the name Collothecacea is a synonym.

As a result, I propose that there is only one order, Floscullariacea, in superorder Gnesiotrocha. The proposed classification at order level of sessile rotifers is below.

Superorder Gnesiotrocha

Order Floscullariacea (syn: Collothecacea)

3) Family category

If the proposal that all of the gnesiotrochans belong to a single Order Floscullariacea is accepted, grouping and ranking of three major clades resolved in this study – former Collothecacea, the BLP group and the Floscullariacea (without the BLP group) – needs to be considered.

At present, Collothecacea is composed of two families, Atrochidae and Collothecidae (Segers, 2002a). According to the view that the taxon should be devalued, I suggest including these two families in Order Floscullariacea. The present phylogenetic analysis revealed that the single representative of Atrochidae is located within Collothecidae clade (Figs 11d, 12e), but not in a sister relation to this clade Moreover, the shared character – absence of cilia on corona in adult stage – of Atrochidae members appear be in contrast with trophi morphology, as the trophy of *Acyclus* appears to be more similar to that of Collothecidae than to that of other Atrochidae such as genus *Cupelopagis* (compare Fig. 32 and Fig. 3.11 in Wallace et al. (2006)). This may indicate that the shared features defining Atrochidae in the current classification may be homoplastic. Whatever the significance of this, the present analysis can only be interpreted as indicating that genera within Collothecidae and Atrochidae are mixed and belong to the same clade, hence the two family-level taxa should be merged. Based on the principle of first reviser (see Art. 24 of the ICZN), I would suggest Collothecidae as the valid name and Atrochidae as the junior synonym. Nonetheless, it should be borne in mind that Atrochidae is represented by a single species in the molecular analysis. Thus, this suggestion remains to be tested by a further study involving more Atrochidae representatives in phylogenetic analysis.

The BLP group appears to be composed of members of genus *Beauchampia*, *Limnias*, and *Ptygura melicerta* group. One distinct character they have in common is the presence of a dorsal stiff plate (Fig. 26). Although the branch of this group is relatively long with remarkably high support (Figs 11f, 12d), their external morphology is similar to members of Family Flosculariidae (Koste, 1978). I therefore hypothesize that these similarities represent ancestral features (plesiomorphic character status). As the BLP group forms sister-group relation to Family Collothecidae, I rank the group at the same level to its sister taxon – family level. As a result, I would propose that the BLP group is to be treated as a new family-level taxon in Order Floscullariacea.

Classically, the Floscullariacea contains five more families including Conochilidae, Flosculariidae, Hexarthridae, Testudinellidae and Trochosphaeridae. Although phylogenetic positions of the latter three planktonic families are not fully resolved in this study, our preliminary results indicate that they seem to be separate taxa, well-separated from Conochilidae and Flosculariidae. Thus, there is no reason to suggest any change to their present taxonomic position (i.e. family-level taxa) (Segers, 2002a, b).

Furthermore, the results reveal that representatives of Conochilidae are indeed an ingroup of Flosculariidae, and they are closely related to some members of genus *Ptygura* (Fig. 11s). The close relationship between conochilids and these *Ptygura* species may be supported by trophi morphology between them. They share flat, arrow-shaped heads of their right proximal unci teeth, and proximal apophysis of right ramus being much longer than the left. These features are absent in other genera of Flosculariidae (Meksuwan et al., in press). Phylogenetically, all of them should be in the same clade. However, *Ptygura* is one of the most diverse genera of Gnesiotrocha, containing about 27 species, but there are only four species included in this study. Thus, these four representatives may not provide sufficient information regarding the relations within the lineage formed by conochilids and *Ptygura* species. Therefore, we refrain from attributing classification significance to both the conochilid clade and/or the total *Ptygura*+conochilids clade as subtaxon within the Flosculariidae, but propose to include the two genera of the former Family Conochilidae, *Conochilopsis* and *Conochilus*, in Flosculariidae.

According to the present results, the proposed classification at family level in the Gnesiotrocha is as below.

Superorder Gnesiotrocha

Order Flosculariacea (syn: Collothecacea)

Family 1 Collothecidae (syn: Atrochidae)

Family 2 Flosculariidae (syn: Conochilidae)

Family 3 Hexarthridae

Family 4 Testudinellidae

Family 5 Trochosphaeridae

Family 6 New family of the BLP group

4) Generic category

The phylogenetic analysis strongly suggested that two genera, *Sinantherina* and *Ptygura*, are not monophyletic groups.

As the result reveals, the single species of *Pentatrocha* – *P. gigantea* – seems to be an ingroup member of genus *Sinantherina* (Fig. 11q). Nevertheless, as all branch evaluating methods indicate low support for a *Sinantherina* (including *Pentatrocha*) clade and as a number of *Sinantherina* species have not been included in the analysis, I prefer not to conclude on the position of *P. gigantea* and await a more inclusive investigation.

Regarding genus *Ptygura*, there are two lineages to be addressed. The first is composed of three species *P. noodti*, *P. tacita* and *P. thalenoiensis* and a group of *Flosuclaria* species. Based on the tree toplogy and some shared features among them, as discussed above, these *Ptygura* species could be considered close relatives to *Floscularia ringens*-group. However, these *Floscularia* species have several unique characters in common such as four-lobed corona, well-developed dorsal projection, special tube constructed of small pellets, as well as pseudocolony formation. To the contrary, colony formation has never been observed in these three *Ptygura* species (e.g., Koste, 1978; Wallace et al., 2006; Meksuwan et al., 2011). In addition, several species of the large genus *Ptygura* are not included in this study, especially *P.*

brachiata, *P. linguata*, which are species that are possibly close to *P. thalenoiensis*. It therefore appears premature to conclude on the classification significance of the *Ptygura+Floscularia* assemblage until this relationship is confirmed by adequate information.

The second is of members of *Ptygura melicerta* group – *P. furcillata* and *P. mucicola* – which are in the BLP group. This group possesses small, simple corona, short lateral antenna, relatively long foot and usually are in a large cluster or colony embedded in gelatinous mass, into which blue-green algae can settle, on bladder-like organ of the aquatic plant *Utricularia*, and in damaged tissues of aquatic plants (Koste, 1978; Wallace, 1980; Meksuwan et al., 2011). Moreover, they have the dorsal stiff plate that is shared by all members of the BLP clade (Fig. 26). If family-level taxon of the BLP clade is accepted as well as existing genera in the clade (*Beauchampia*, *Limnias*), then this group of *Ptygura* would have to be treated as a different genus-level taxon in the new family. Since *P. melicerta* is generotype of genus *Ptygura*, the name "*Ptygura*" would be the correct name for this group. The remaining species of the traditional "*Ptygura"* includes *P. cystallina*, the type species of the genus name *Oecistis,* hence, this name would be available as the genus-level correct name for the taxon uniting those species.

I refrain from assigning any taxonomic treatment to the genera including *Lacinularia* and *Stephanoceros* as their phylogenetic positions are not resolved in this study. Doing so may require including more representative species and/or molecular markers in the analysis.

A proposed classification at generic level of sessile rotifers is below.

Superorder Gnesiotrocha

Order Flosculariacea (syn: Collothecacea)

Family 1 Collothecidae (syn: Atrochidae)

Genus 1 *Acyclus*

Genus 2 *Atrochus*

Genus 3 *Collotheca*

Genus 4 *Cupelopagis*

Genus 5 *Stephanoceros*

Family 2 Flosculariidae (syn: Conochilidae)

- Genus 1 *Conochilopsis*
- Genus 2 *Conochilus*
- Genus 3 *Floscularia*
- Genus 4 *Lacinularia*
- Genus 5 *Lacinularoides*
- Genus 6 *Octotrocha*
- Genus 7 *Oecistes* **stat. nov.**
- Genus 8 *Pentatrocha*
- Genus 9 *Sinantherina*

Family 3 Hexarthridae

Family 4 Testudinellidae

Family 5 Trochosphaeridae

Family 6 New family of the BLP group

- Genus 1 *Beauchampia*
- Genus 2 *Limnias*
- Genus 3 *Ptygura* **(redefined)**

CHAPTER 5

CONCLUSIONS

5.1 Evolution of the major morphological characters and taxonomy of sessile rotifers

Base on analysis of evolutionary pattern of the major morphological characters, two categories relating to taxonomy of sessile rotifers are classified, as in the following.

1) **Characters that need an additional study before being used to unit sessile rotifer taxa**
   - Modulus
   - Oviferon
   - Symmetry of trophi

2) **According to this study, characters that each state was evolved several times and it may not be used to delimit sessile rotifer taxa at higher levels**
   - Corona shape and number of corona lobe
   - Malleoramate trophi
   - Differentiation of unci teeth
   - Life habit
   - Colony formation

5.2 New proposed classification of sessile rotifers and closely related taxa

Current classification (Segers, 2002a; Segers and Shiel, 2008 ; Meksuwanet al., 2011)	**Present proposed classification** (New taxa and positions indicated by an asterisk)
Superorder Gnesiotrocha	Superorder Gnesiotrocha
Order 1 Collothecacea	Order Floscullariacea (syn: *Collothecacea)
Family 1 Atrochidae	Family 1 Collothecidae (syn: *Atrochidae)
Genus 1 *Acyclus*	Genus 1 *Acyclus*
Genus 2 *Atrochus*	Genus 2 *Atrochus*
Genus 3 *Cupelopagis*	Genus 3 *Collotheca*
Family 2 Collothecidae	Genus 4 *Cupelopagis*
Genus 1 *Collotheca*	Genus 5 *Stephanoceros*
Genus 2 *Stephanoceros*	Family 2 Flosculariidae (syn: *Conochilidae)
Order 2 Floscullariacea	Genus 1 *Conochilopsis*
Family 1 Conochilidae	Genus 2 *Conochilus*
Genus 1 *Conochilopsis*	Genus 3 *Floscularia*
Genus 2 *Conochilus*	Genus 4 *Lacinularia*
Subgenus *Conochiloides*	Genus 5 *Lacinularoides*
Subgenus *Conochilus*	Genus 6 *Octotrocha*
Family 2 Flosculariidae	Genus 7 *Oecistes**
Genus 1 *Beauchampia*	Genus 8 *Pentatrocha*
Genus 2 *Floscularia*	Genus 9 *Sinantherina*
Genus 3 *Lacinularia*	*Family 3 New family of the BLP group
Genus 4 *Lacinularoides*	Genus 1 *Beauchampia*
Genus 5 *Limnias*	Genus 2 *Limnias*
Genus 6 *Octotrocha*	Genus 3 *Ptygura*
Genus 7 *Pentatrocha*	Family 4 Hexarthridae
Genus 8 *Ptygura*	Family 5 Testudinellidae
Genus 9 *Sinantherina*	Family 6 Trochosphaeridae
Family 3 Hexarthridae	
Family 4 Testudinellidae	
Family 5 Trochosphaeridae	

5.3 Summary of major findings

1) One new species, *Collotheca orchidacea* Meksuwan, Pholpunthin & Segers, 2013, is described.
2) Causes of low diversity of sessile rotifers in several countries of Southeast Asia seem to be inefficiency of sampling, and specimen investigating methods.
3) Superorder Gnesiotrocha, including all sessile rotifers, is monophyletic taxon.
4) The ancestor of gnesiotrochans seems to be a sessile rotifer population carrying a simple, filtering corona with mastax containing malleoramate-like trophi.
5) Order Collothecacea is synonym of Order Flosculariacea.
6) Family Atrochidae is synonym of Family Collothecidae.
7) Family Conochilidae is synonym of Family Flosculariidae.
8) A new distinct lineage is discovered. It is comprised of genus *Beauchampia* and *Limnias*, and *Ptygura melicerta* group's species. I call it "the BLP" group. Generic name "*Ptygura*" is applied in this group.
9) The generic name "*Oecistes*" is reintroduced for the remaining species of the traditional "*Ptygura"* includes *P. cystallina*, the type species of this generic name.
11) Malleoramate type of trophi is plesiomorphic character.
12) A certain shape (including number of lobes) of corona is not a good character to recognize monophyletic taxa.

BIBLIOGRAPHY

Aguinaldo, A. M. A., Turbeville, J. M., Linford, L. S., Rivera, M. C., Garey, J. R., Raff, R. A. and Lake, J. A. 1997. Evidence for a clade of nematodes, arthropods and other moulting animals. Nature 387: 489-493.

Alfaro, M. E., Zoller, S. and Lutzoni, F. 2003. Bayes or Bootstrap? A simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling and Bootstrapping in assessing phylogenetic confidence. Molecular Biology and Evolution 20(2): 255-266.

Anderson, F. E. and Swofford, D. L. 2004. Should we be worried about long-branch attraction in real data sets? Investigations using metazoan 18S rDNA. Molecular Phylogenetics and Evolution 33: 440-451.

Baum, D. A., DeWitt Smith, S. and Donovan, S. S. S. 2005. The tree-thinking challenge. Science 310: 979-980.

Bergsten, J. 2005. A review of long-branch attraction. Cladistics 21: 163-193.

Bryant, H. N. 2001. Character polarity and the rooting of cladograms. In *The character concept in evolutionary biology* (Wagner, G. P., editor). Academic Press, USA.

Chittapun, S., Pholpunthin, P., and Segers, H. 2007. Diversity of Rotifer Fauna from Five Coastal Peat Swamps on Phuket Island, Southern Thailand. ScienceAsia 33: 383-387.

Crisp, M. D. and Cook, L. G. 2005. Do early branching lineages signify ancestral traits? Trends in Ecology and Evolution 20(3): 122-128.

De Smet, W. H. 1998. Preparation of rotifer trophi for light and scanning electron microscopy. Hydrobiologia 387/388: 117-121.

Dunn, C. W., Hejnol, A., Matus, D. Q., Pang, K., Browne, W. E., Smith, S. A., Seaver, E., Rouse, G. W., Obst, M., Edgecombe, G. D., Sørensen, M. V., Haddock, S. H. D., Schmidt-Rhaesa, A., Okusu, A., Kristensen, R. M., Wheeler, W. C., Martindale, M. Q. and Giribet, G. 2008. Broad phylogenomic sampling improves resolution of the animal tree of life. Nature 452: 745-749. doi:10.1038/nature06614

Edmondson, W. T. 1939. New species of Rotatoria, with notes on heterogonic growth. Transactions of the American Microscopical Society 58(4): 459-472.

Edmondson, W. T. 1940. The sessile Rotatoria of Wisconsin. Transactions of the American Microscopical Society 59(4): 433-459.

Epp, R. W. and Lewis, W. M. 1979. Sexual dimorphism in *Brachionus plicatilus* (Rotifera): evolutionary and adaptive significance. Evolution 33(3): 919-928.

Fernando, C. H. and Zankai, N. P. 1981. The Rotifera of Malaysia and Singapore with remarks on some species. Hydrobiologia 78: 205-219.

Fontaneto, D, Melone, G and Wallace, R. L. 2003. Morphology of *Floscularia ringens* (Rotifera, Monogononta) from egg to adult. Invertebrate Biology 122(3): 231-240.

Futuyma, D. J. 2005. Evolution. Sinauer Associates, Inc., USA.

Garey, J. R., Near, T. J., Nonnemacher, M. R. and Nadler, S. A. 1996. Molecular evidence for Acanthocephala as a subtaxon of Rotifera. Journal of Molecular Evolution 43: 287-292.

Gilbert, J. J. 1977. Mictic-female production in monogonont rotifers. Archiv für Hydrobiologie–BeiheftErgebnisse der Limnologie 8: 142-155.

Giribet, G., Carranza, S., Baguñà, J., Riutort, M. and Ribera, C. 1996. First molecular evidence for the existence of a Tardigrada+Arthropoda clade. Molecular Biology and Evolution 13(1): 76-84.

Giribet, G., Sørensen, M. V., Funch, P., Kristensen, R. M. and Sterrer, W. 2004. Investigations into the phylogenetic position of Micrognathozoa using four molecular loci. Cladistics 20: 1-13.

Graybeal, A. 1998. Is it better to add taxa or characters to a difficult phylogenetic problem? Systematic Biology 47(1): 9-17.

Gregory, T. R. 2008. Understanding evolutionary trees. Evolution: Education and Outreach 1: 121-137.

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M. Hordijk, W. and Gascuel, O. 2010. New algorithms and methods to estimate Maximum-likelihood phylogenies: assessing the performance of PhyML3.0. Systematic Biology 59(3): 307-321.

Halanych, K. M., Bacheller, J. D., Aguinaldo, A. M. A., Liva, S. M., Hillis, D. M. and Lake, J. A. 1995. Evidence from 18S ribosomal DNA that lophophorates are protostome animals. Science 267: 1641-1643.

Hall, B. K. and Hallgrimsson, B. 2008. Strickberger's evolution. Jones and Bartlett Publishers, Inc., USA.

Harring, H. K. 1913. Synopsis of the Rotatoria. Smithsonian Institution. United States National Museum. Bulletin 81: 1-226.

Heckman, C. W. 1974. The seasonal succession of species in a Rice Paddy in Vientiane, Laos. Internat. Rev. ges. Hydrobiol. 59: 489-507.

Herlyn, H., Piskurek, O., Schmitz, J., Ehlers, U. and Zischler, H. 2003. The syndermatan phylogeny and the evolution of acanthocephalan endoparasitism as inferred from 18S rDNA sequences. Molecular Phylogenetics and Evolution 26: 155-164.

Hillis, D. M. and Dixon, M. T. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. The Quarterly Review of Biology 66(4): 411-453.

Hochberg, R. and Lilley, G. 2010. Neuromuscular organization of the freshwater colonial rotifer, *Sinantherina socialis*, and its implications for understanding the evolution of coloniality in Rotifera. Zoomorphology 129: 153-162.

Hochberg, R., O'Brien, S. and Puleo, A. 2010. Behavior, metamorphosis, and muscular organization of the predatory rotifer Acyclus inquietus (Rotifera, Monogononta). Invertebrate Biology 129(3): 210-219.

Hordijk, W. and Gascuel, O. 2005. Improving the efficiency of SPR moves in phylogenetic tree search methods based on maximum likelihood. Bioinformatics 21(24): 4338-4347. doi:10.1093/bioinformatics/bti713

Hudson, C. T. and Gosse, P. H. 1886. The Rotifera or Wheel-animalcules. London, Longmans, Green & Co.

International Commission on Zoological Nomenclature. 1999. International Code of Zoological Nomenclature, 4th ed. The International Trust for Zoological Nomenclature. The Natural History Museum, Cromwell road, London.

Karunakaran, L. and Johnson, A. 1978. A contribution to the rotifer fauna of Singapore and Malaysia. Malayan Nat. Jnl. 32: 173-208.

Katoh, K. and Standley, D. M. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30(4): 772-780.

Koste, W. 1968. Ueber moosbewohnende Rädertiere von Strohdächern alter Gebäude in Hahlen und Kl. Bokern. Mitt. n° 15 Kreisheimatb. Bersenbrück: 123-128.

Koste, W. 1972. Über ein sessiles Rädertier aus Amazonien, *Ptygura elsteri* n. sp., mit Bemerkungen zur Taxonomie des Artkomplexes *Ptygura melicerta* (Ehrenberg, 1832). Int. Revue ges. Hydrobiol. 57(6): 875-882.

Koste, W. 1975. Über den Rotatorienbestand einer Mikrobiozönose in einem tropischen aquatischen Saumbiotop, der *Eichhornia-crassipes*-Zone im Litoral des Bung-Borapet, einem Stausee in Zentralthailand. Gewässer und Abwässer 57/58: 43-58.

Koste, W. 1978. Rotatoria. Die Rädertiere Mitteleuropas. Borntraeger, Berlin, 2 vols, 673 pp, 234 plates.

Koste, W. 1988. Rotatorien aus Gewässern am Mittleren Sungai Makaham, einem Ueberschwimmungsgebiet in E Kalimantan, Indonesian Borneo. Osnabr. Naturwiss. Mitt. 14: 91-136

Koste, W. and Tobias, W. 1990. Zur Kenntnis der Rädertierfauna des Kinda-Stausees in Zentral-Birma (Aschelminthes: Rotatoria). Osnabr. Naturwiss. Mitt. 16: 83-110.

Kutikova, L. A. 1983. Parallelism in the evolution of rotifers. Hydrobiologia 104: 3-7.

Kutikova, L. A. 1995. Larval metamorphosis in sessile rotifers. Hydrobiologia 313/314: 133-138.

Kutikova, L. A. and Markevich, G. I. 1993. Principal direction of the evolution of Monimotrochida. Hydrobiologia 255/256: 545-549.

Lemey, P., Salemi, M. and Vandamme, A-M. 2009. The phylogenetic handbook: a practical approach to phylogenetic analysis and hypothesis testing (second edition). Cambridge University Press, UK.

Lipscomb, D. 1998. Basics of Cladistic analysis. George Washington University, Washington D.C., USA.

Mamaril, A. C. Sr. and Fernando, C. H. 1978. Freshwater zooplankton of the Philippines (Rotifera, Cladocera and Copepoda). Natural and Applied Science Bulletin 30: 109-221.

Mark Welch, D. B. 2001. Early contributions of molecular phylogenetics to understanding the evolution of Rotifera. Hydrobiologia 446/447: 315-322.

Markevich, G. I. and Kutikova, L. A. 1989. Mastax morphology under SEM and its usefulness in reconstructing rotifer phylogeny and systematics. Hydrobiologia 186/187: 285-289.

Mayr, E. 1999. Systematics and the origin of species, from the viewpoint of a zoologist, with a new introduction by the author. Harvard University Press, USA.

Mayr, E. and Ashlock, P. D. 1991. Principles of systematic zoology (second edition). McGraw-Hill, Inc., Singapore.

Meksuwan, P., Pholpunthin, P. and Segers, H. 2011. Diversity of sessile rotifers (Gnesiotrocha, Monogononta, Rotifera) in Thale Noi Lake, Thailand. Zootaxa 2997: 1-18.

Meksuwan, P., Pholpunthin, P. and Segers, H. 2013. The Collothecidae (Rotifera, Collothecacea) of Thailand, with the description of a new species and an illustrated key to the Southeast Asian fauna. ZooKeys 315: 1-16. doi: 10.3897/zookeys.313.5330

Meksuwan, P., Pholpunthin, P. and Segers, H. Molecular phylogeny confirms Conochilidae as ingroup of Flosculariidae (Rotifera, Gnesiotrocha) (in press).

Melone, G. and Ricci, C. 1995. Rotatory apparatus in bdelloids. Hydrobiologia 313/314: 91-98.

Melone, G. and Fontaneto, D. 2005. Trophi structure in bdelloid rotifers. Hydrobiologia 546: 197-202.

Melone, G., Ricci, C. and Segers, H. 1998. The trophi of Bdelloidea (Rotifera): a comparative study across the class. Canadian Journal of Zoology 76: 1755-1765.

Miller, M. A., Pfeiffer, W. and Schwartz, T. 2010. "Creating the CIPRES Science Gateway for inference of large phylogenetic trees" in Proceeding of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA pp 1-8.

Minelli, A. 1993. Biological systematics: the state of the art. Chapman & Hall, London, UK.

Nicholas, K. B., Nicholas Jr., H. B. and Deerfield II, D. W. 1997. GeneDoc: analysis and visualization of genetic variation. EMBnet.news 4: 1-4.

Nogrady, T. and Pourriot, R. 1995. Rotifera. Volume 3: the Notommatidae. *In Guides to the Identification of the Microinvertebrates of the Continental Waters of the World, Volume 8.* (Dumont, H.J.F., ed). SPB Publishing bv. Amsterdam. The Netherlands.: 1-299.

O'Connor, T., Sundberg, K., Carroll, H., Clement, M. and Snell, Q. 2010. Analysis of long branch extraction and long branch shortening. BMC Genomics 11(supplement 2): 2-9.

Page, R. D. M. and Holmes, E. C. 1998. Molecular evolution: a phylogenetic approach. Blackwell Science Ltd, Oxford, UK.

Posada, D. 2008. jModelTest: phylogenetic model averaging. Molecular Biology and Evolution 25(7): 1253-1256. doi:10.1093/molbev/msn083

Ricci, C. and Melone, G. 1998. Dwarf males in monogonont rotifers. Aquatic Ecology 32: 361-365.

Ricci, C. and Melone, G. 2000. Key to the identification of the genera of bdelloid rotifers. Hydrobiologia 418: 73-80.

Ricci, C., Melone, G. and Sotgia, C. 1993. Old and new data on Seisonidea (Rotifera). Hydrobiologia 255/256: 495-511.

Ridley, M. 1993. Evolution. Blackwell Scientific Publications, Inc., New York, USA.

Ronquist, F., Teslenko, van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. and Huelsenbeck, J. P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539-542.

Sa-ardrit, P., Pholpunthin, P. and Segers, H. 2013. A checklist of the freshwater rotifer fauna of Thailand (Rotifera, Monogononta, Bdelloidea). Journal of Limnology 72(s2): 361-375. doi: 10.4081/jlimnol.2013.s2.e18

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4(4): 406-425.

Salt, G. W. 1987. The components of feeding behavior in rotifers. Hydrobiologia 147: 271-281.

Sanoamuang, L. 2002. Rotifera. Volume 6: *Filinia*. In *Guides to the Identification of the Microinvertebrates of the Continental Waters of the World, volume 18*. (Nogrady, T. & H. Segers, eds.). Backhuys Publishers Leiden, The Netherlands.

Sanoamuang, L. and Savatenalinton, S. 2001. The rotifer fauna of Lake Kud-Thing, a shallow lake in Nong Khai Province, northeast Thailand. Hydrobiologia 446/447:297-304.

Sanoamuang, L., Segers, H. and Dumont, H. J. 1995. Additions to the rotifer fauna of south-east Asia: new and rare species from north-east Thailand. Hydrobiologia 313/314: 35-45.

Santo, N., Fontaneto, D., Fascio, U., Melone, G. and Caprioli, M. 2005. External morphology and muscle arrangement of *Brachionus urceolaris*, *Floscularia ringens*, *Hexarthra mira* and *Notommata glyphura* (Rotifera, Monogononta). Hydrobiologia 546: 223-229.

Santos-Medrano, G. E., Rico-Martinez, R. and Velázquez-Rojas, C. A. 2001. Swimming speed and Reynolds numbers of eleven freshwater rotifer populations. Hydrobiologia 446/447: 35-38.

Segers, H. 1993. Rotifera of some lakes in the floodplain of the River Niger (Imo State, Nigeria): I. New species and other taxonomic considerations. Hydrobiologia 250: 39-61.

Segers, H. 1995. Rotifera. Volume 2: The Lecanidae (Monogononta). In *Guides to the Identification of the Microinvertebrates of the Continental Waters of the World, Volume 6*. (Dumont, H. J. F., ed.). SPB Academic Publishing bv, The Hague, The Netherlands. 1-226.

Segers, H. 1997. Contribution to a revision of *Floscularia* Cuvier, 1798 (Rotifera: Monogononta): notes on some Neotropical taxa. Hydrobiologia 354: 165-175.

Segers, H. 2002a. The nomenclature of the Rotifera: annotated checklist of valid family- and genus-group names. Journal of Natural History 36: 631-640.

Segers, H. 2002b. Rotifera. Volume 6: Trochosphaeridae. In *Guides to the Identification of the Microinvertebrates of the Continental Waters of the World, volume 18* (Nogrady, T. and Segers, H. eds.). Backhuys Publishers, Leiden, The Netherlands: 212-223.

Segers, H. 2007. Annotated checklist of the rotifers (Phylum Rotifera), with notes on nomenclature, taxonomy and distribution. Zootaxa 1564: 1-104.

Segers, H. and Chittapun, S. 2001. The interstitial Rotifera of a tropical freshwater peat swamp on Phuket Island, Thailand. Belgian Journal of Zoology 131: 65-71.

Segers, H. and Shiel, R. J. 2008. Diversity of cryptic Metazoa in Australian freshwaters: a new genus and two new species of sessile rotifer (Rotifera, Monogononta, Gnesiotrocha, Flosculariidae). Zootaxa 1750: 19-31.

Segers, H. and Wallace, R. L. 2001. Phylogeny and classification of the Conochilidae (Rotifera, Monogononta, Flosculariacea). Zoologica Scripta 30: 37-48.

Segers, H., Kotethip, W. and Sanoamuang, L. 2004. Biodiversity of freshwater microfauna in the floodplain of the River Mun, Northeast Thailand: the Rotifera Monogononta. Hydrobiologia 515:1-9.

Segers, H., Meksuwan, P. and Sanoamuang, L. 2010. New records of sessile rotifers (Phylum Rotifera: Flosculariacea, Collothecacea) from Southeast Asia. Belgian Journal of Zoology 140(2): 235-240.

Sørensen, M. V. 2002. On the evolution and morphology of the rotiferan trophi, with a cladistics analysis of Rotifera. Journal of Zoological Systematics and Evolutionary Research 40: 129-154.

Sørensen, M. V. and Giribet, G. 2006. A modern approach to rotiferan phylogeny: combining morphological and molecular data. Molecular Phylogenetics and Evolution 40: 585-608.

Stearns, S. C. and Hoekstra, R. F. 2005. Evolution: an introduction (second edition). Oxford University Press Inc., New York, USA.

Stevens, P. F. 1980. Evolutionary polarity of character states. Annual Review of Ecology, Evolution, and Systematics 11: 333-358.

Sudzuki, M. 1989. Rotifera from the Oriental region and their characteristics. *Special issue* celebrating the Centennial Aniversary of the Foundation of "Nihon Daigaku" University Tokyo 3: 325-366

Sudzuki, M. 1991. The rotifera from Singapore and Taiwan. Proceedings of the Japanese Society of Systematic Zoology 43: 1-34

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24(8): 1596-1599.

Thorpe, V. G. 1893. The Rotifera of China. Transactions of the Society: 145-152.

Vidrine, M. E., McLaughlin, R. E. and Willis, O. R. 1985. Free-swimming colonial rotifers (Monogononta: Flosculariacea: Flosculariidae) in Southwestern Louisiana rice fields. Freshwater Invertebrate Biology 4: 187-193.

Wallace, R. L. 1978. Substrate selection by larvae of the sessile rotifer *Ptygura beauchampi*. Ecology 59: 221-227.

Wallace, R. L. 1980. Ecology of sessile rotifers. Hydrobiologia 73: 181-193.

Wallace, R. L. 1987. Coloniality in the phylum Rotifera. Hydrobiologia 147: 141-155.

Wallace, R. L. 2002. Rotifers: exquisite metazoans. Integrative and Comparative Biology 42: 660-667.

Wallace, R. L. and Colburn, R. A. 1989. Phylogenetic relationships within phylum Rotifera: orders and genus *Notholca*. Hydrobiologia 186/187: 311-318.

Wallace, R. L. and Edmondson, W. T. 1986. Mechanism and adaptive significance of substrate selection by a sessile rotifer. Ecology 67: 314-323.

Wallace, R. L., Snell, T. W., Ricci, C. and Nogrady, T. 2006. Rotifera. Volume 1: Biology, Ecology and Systematics. In *Guides to the Identification of the Microinvertebrates of the Continental Waters of the World, volume 23*. (Segers, H. and Dumont, H. J. F., ed). Backhuys Publishers, Leiden, The Netherlands.

Watrous, L. E. and Wheeler, Q. D. 1981. The out-group comparison method of character analysis. Systematic Zoology 30(1): 1-11.

Whiting, M F., Carpenter, J. C., Wheeler, Q. D. and Wheeler, W. C. 1997. The Strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. Systematic Biology 46(1): 1-68.

Winston, J. E. 1999. Describing species: practical taxonomic procedure for biologists. Columbia University Press, New York, USA.

Xia, X. 2013. DAMBE5: A comprehensive software package for data analysis in molecular biology and evolution. Molecular Biology and Evolution 30: 1720-1728.

Zander, R. H. 2004. Minimal values for reliability of Bootstrap and Jackknife proportions, Decay index, and Bayesian posterior probability. PhyloInformatics 2: 1-13.

APPENDICES

Appendix A Photographs of unidentified species. A-B: *Lacinularia* sp.; C: *Limnias ceratophylli* group sp.1; D: *Limnias ceratophylli* group sp.2. Scale bars: A = 1 mm., B-D = 200 µm.

Appendix B DNA extraction protocol applied in the present study.

DNA extraction kit: AGL-R-200600 (Agilent technologies, USA)

Cell lysis

1. A female/single-cloned egg(s) in absolute ethanol in 1.5-ml microcentrifuge tube
2. Expel the ethanol in the 1.5-ml tube as much as possible
3. Add the SOLUTION 2: 300 µl
4. Add ProtenaseK: 0.2 µl
5. Centrifuge at 13,000 rpm for 20 min
6. Incubate at 55^{o}C for at least 2 hr within a water bath

Protein precipitation

7. After incubation, cool the tube on ice for 10 min
8. Add SOLUTION 3: 100 µl
9. Mix the solution on ice for 5 min
10. Centrifuge at 10,000 rpm for 5 min
11. Transfer supernatant into a new 1.5-ml tube (about 350 µl)
12. Centrifuge at 10,000 rpm for 5 min
13. Transfer supernatant into a new 1.5-ml tube (about 350 µl)
14. Add RNase: 0.2 µl
15. Incubate at 37^{o}C for 15 min

DNA precipitation

16. Add 3M NaAc: 35 µl
17. Add cooled absolute ethanol: 700-850 µl
18. Invert gently 20 times
19. Store overnight at -20^{o}C

Appendix B (continued)

DNA dehydration

20. After storing overnight, centrifuge at 13,000 rpm (4^{o}C if any) for 20 min
21. Pour all solution
22. Add cooled 80% ethanol: 500 µl
23. Centrifuge at 13,000 rpm for 10 min
24. Pour all ethanol
25. Air-dry the tube (with DNA precipitate) for 3-5 hr (or until well-dried)
26. Add DI water: 20 µl
27. Incubate in a water bath at 50^{o}C for 1 hr and mix intermittently during the incubation
28. DNA solution is ready for utilization

Appendix C Purification protocol of PCR product.

Purification kit: DF100 (Geneaid, Taiwan)

1. Transfer PCR product (≥50 µl) into a 1.5-ml microtube

2. Add DF buffer 5-folds of the PCR product volume (e.g., 300 µl)

3. Mix well (vortex) and spin down

4. Transfer the solution (PCR product and DF buffer) into a DF column (with its collection tube)

5. Centrifuge at 12,000 rpm for 1 min

6. Remove the filtered solution (at the bottom of the collection tube) (pick up the DF column and put it back)

7. Add Wash buffer 600 µl, wait for 1 min, and centrifuge at 12,000 rpm for 1 min

8. Remove the filtered solution

9. Centrifuge at 12,000 rpm for 1 min (dry column)

10. Place the DF column in a 1.5-ml microtube (used as collection tube)

10. Add Elution buffer 30 µl, wait for 1 min, and centrifuge at 13,000 rpm for 1 min

11. This purified PCR product (30 µl) is ready for DNA sequencing

Appendix D Distribution of sessile rotifers in different parts of Thailand observed during the present study

Sampling sites / Species	Southern									Central	Northern			Northeastern				
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**C1**	**N1**	**N2**	**N3**	**NE1**	**NE2**	**NE3**	**NE4**	**NE5**
Acyclus inquietus							+											
Collotheca algicola				+	+		+											
C. ambigua		+					+											
C. campanulata f. *campanulata*	+			+			+										+	+
C. campanulata f. *longicaudata*							+									+	+	
C. ferox				+														
C. heptabrachiata							+											
C. orchidacea							+											
C. ornata f. *ornata*	+	+					+											
C. ornata f. *cornuta*					+													
C. stephanochaeta							+										+	
C. tenuilobata		+				+	+								+		+	+
C. trilobata							+											+
Collotheca sp.					+													

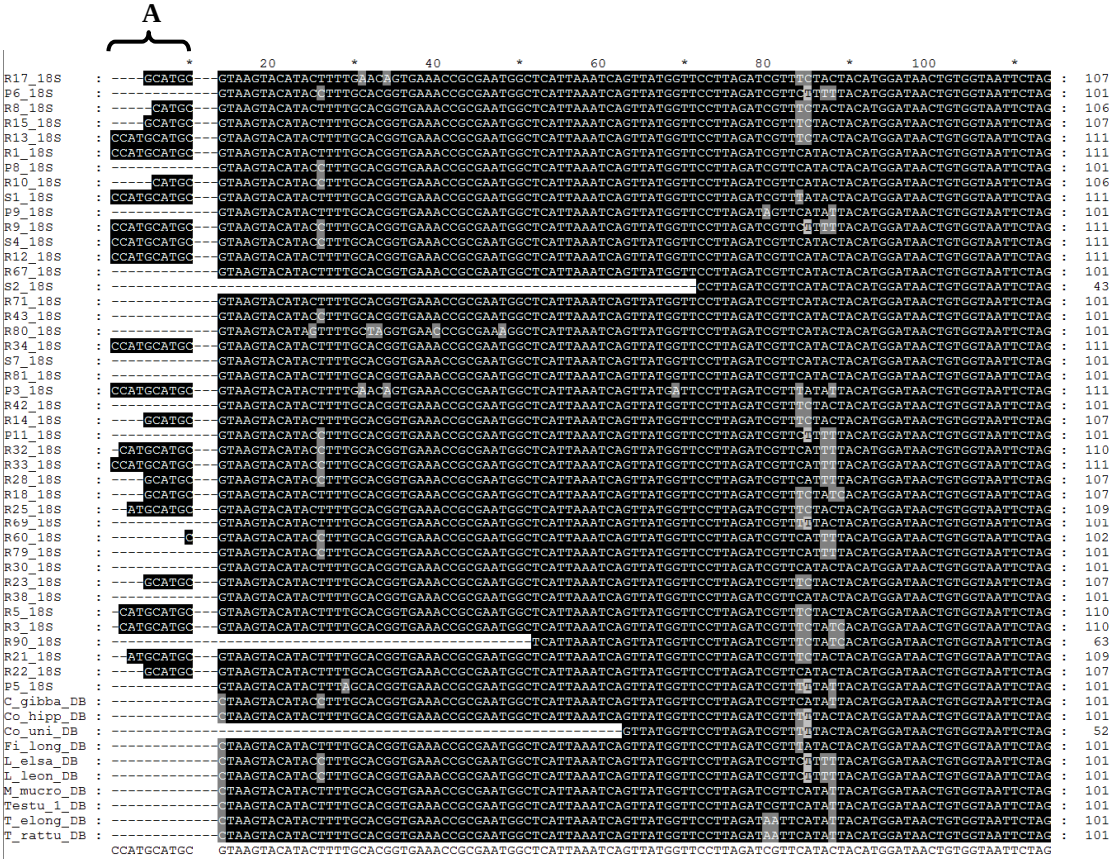
Appendix D (continued)

	Southern									Central	Northern			Northeastern				
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**C1**	**N1**	**N2**	**N3**	**NE1**	**NE2**	**NE3**	**NE4**	**NE5**
Stephanoceros fimbriatus							+											
S. millsii	+						+										+	
Beauchampia crucigera	+	+		+			+				+	+			+	+		
Floscularia armata							+											
F. bifida							+											
F. conifera							+		+						+			
F. pedunculata							+											
Lacinularia flosculosa		+					+				+	+				+	+	
Lacinularia cf. *pedunculata*							+						+			+		+
Lacinularoides coloniensis							+				+				+	+	+	
Limnias ceratophylli group sp.1							+			+	+							
L. ceratophylli group sp.2	+						+	+			+	+			+		+	+
L. melicerta	+	+		+			+			+								
Octotrocha speciosa							+					+			+	+	+	+

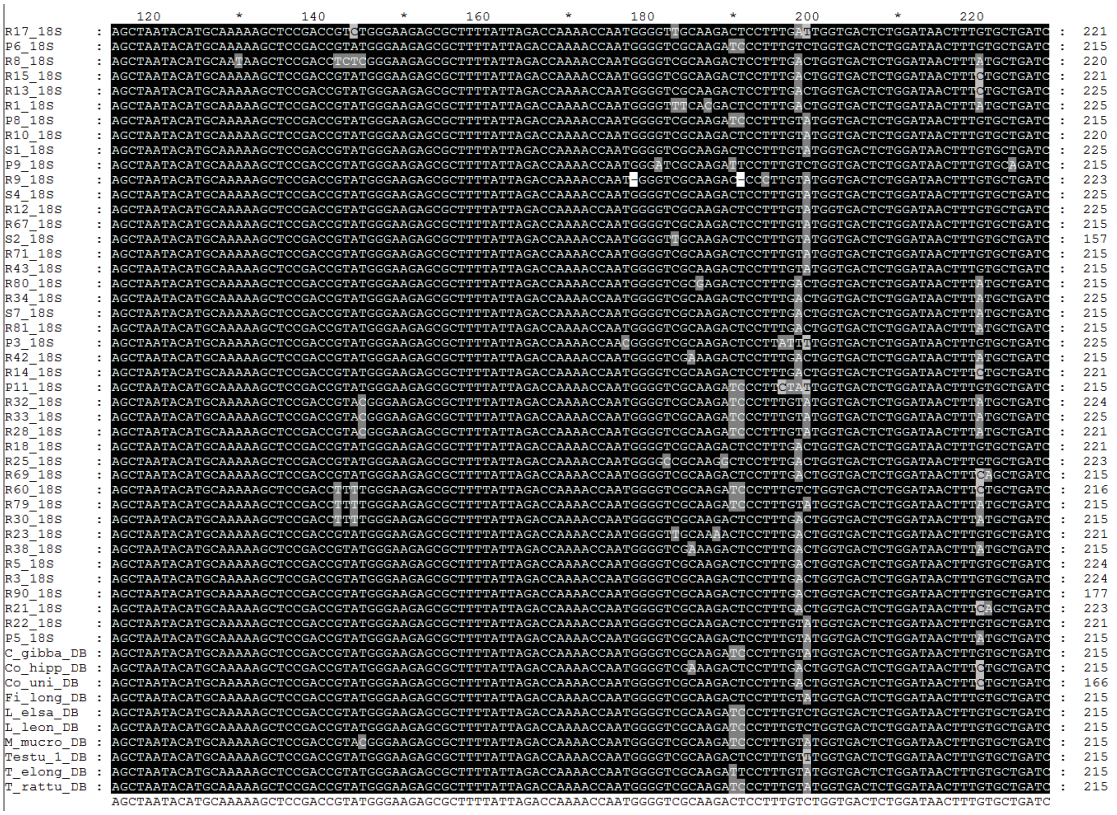
Appendix D (continued)

	Southern									Central	Northern			Northeastern				
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**C1**	**N1**	**N2**	**N3**	**NE1**	**NE2**	**NE3**	**NE4**	**NE5**
Pentatrocha gigantea	+	+					+								+			
Ptygura barbata		+					+		+	+								
P. beauchampi		+					+							+	+	+	+	+
P. crystallina	+	+			+		+					+			+	+	+	+
P. furcillata							+											
P. longicornis	+						+			+					+			
P. mucicola		+					+											
P. noodti							+					+			+			
P. pedunculata							+											
P. pilula							+		+							+	+	+
P. tacita					+		+										+	+
P. thalenoiensis					+		+		+									+
Sinantherina semibullata			+				+											
S. socialis						+	+				+	+				+	+	
S. spinosa			+				+											

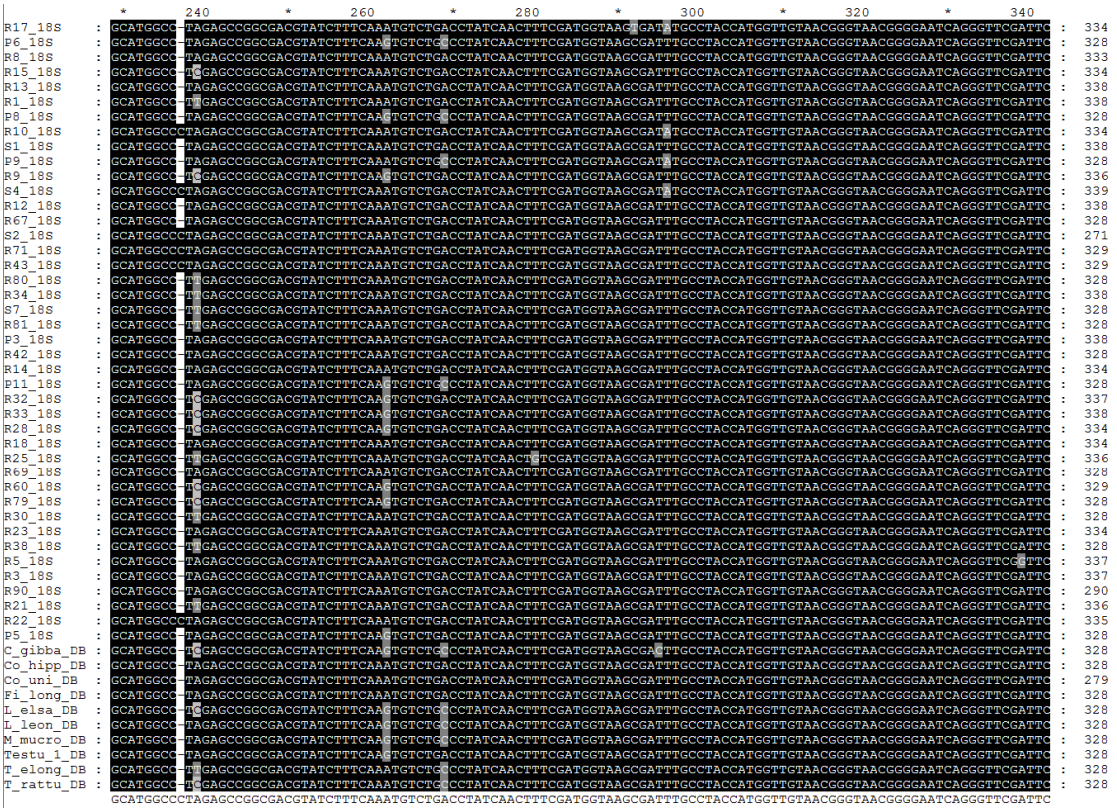
Appendix E Nucleotide sequence alignment of 18S rRNA gene of all taxa analyzed. The sequences acquired (newly sequenced by the present study) ranging from 1,655-1,749 bases. The bases at beginning and end of the alignment which were separated by three gaps (A and B, respectively) were removed before analyzing phylogenetic relationship to maximize the sameness of the data among the taxa studied.



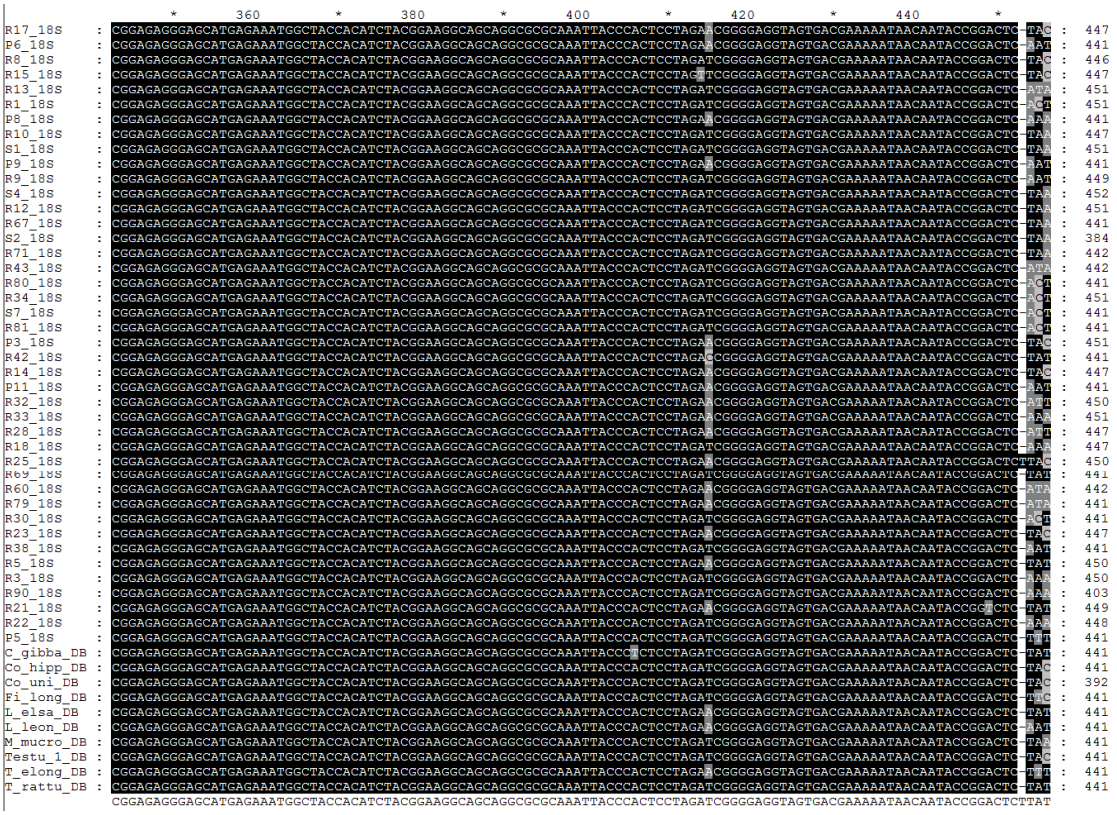
Appendix E (continued)



Appendix E (continued)



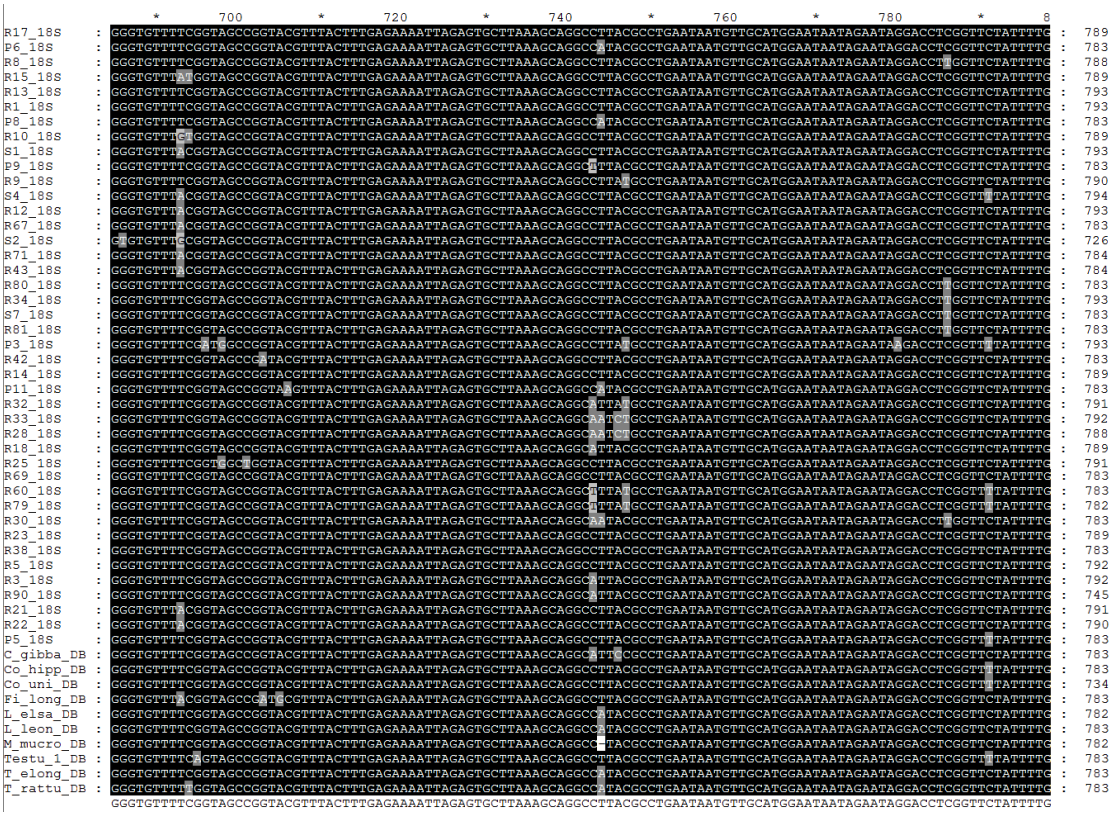
Appendix E (continued)



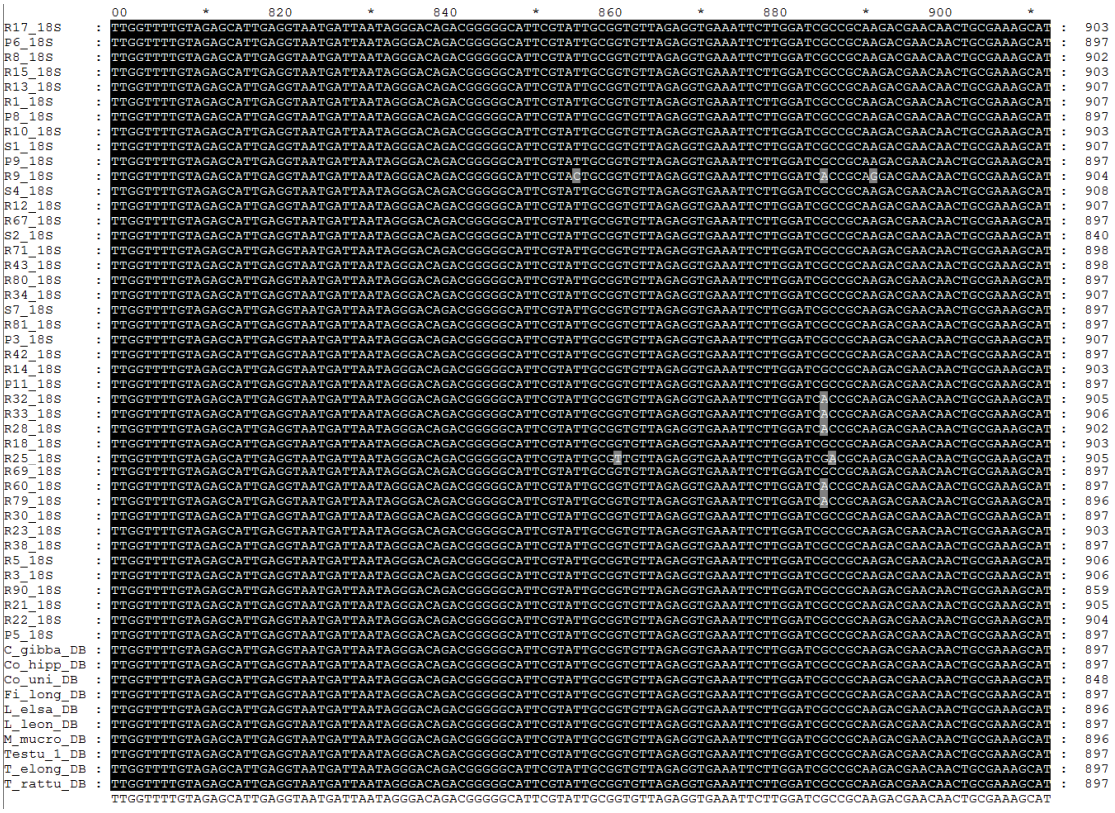
Appendix E (continued)

Appendix E (continued)

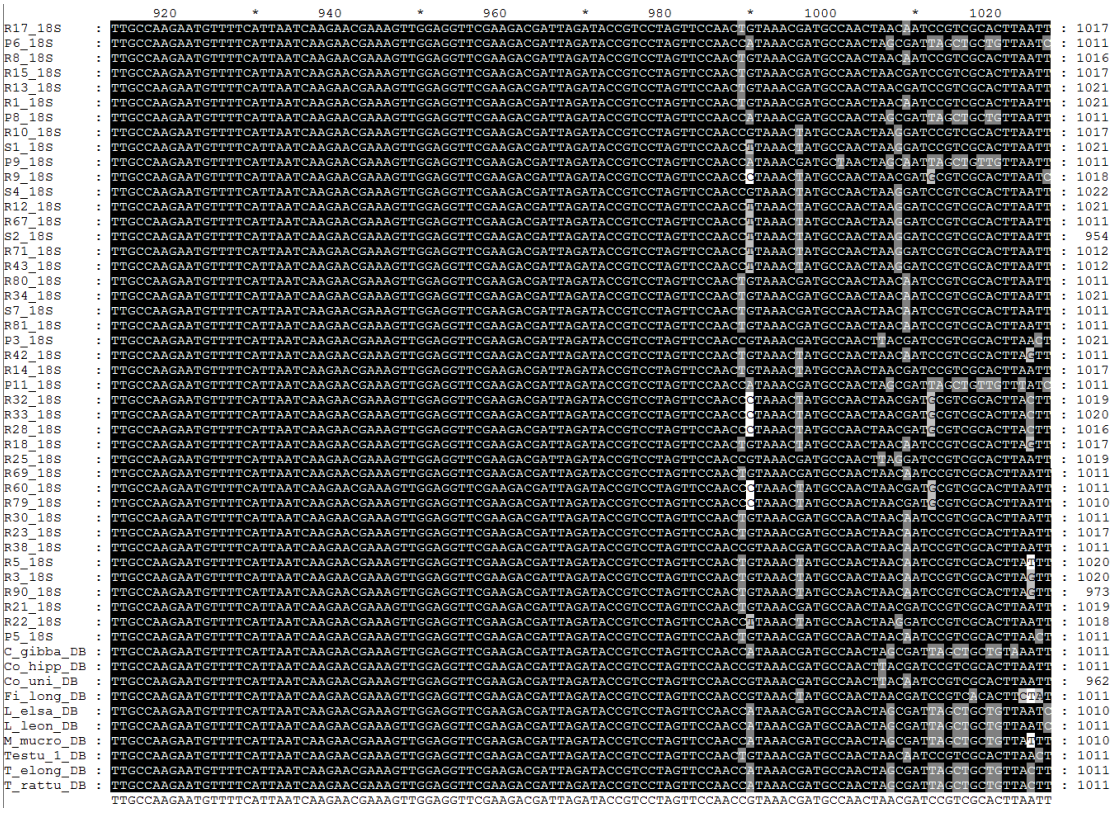
Appendix E (continued)



Appendix E (continued)



Appendix E (continued)



Appendix E (continued)

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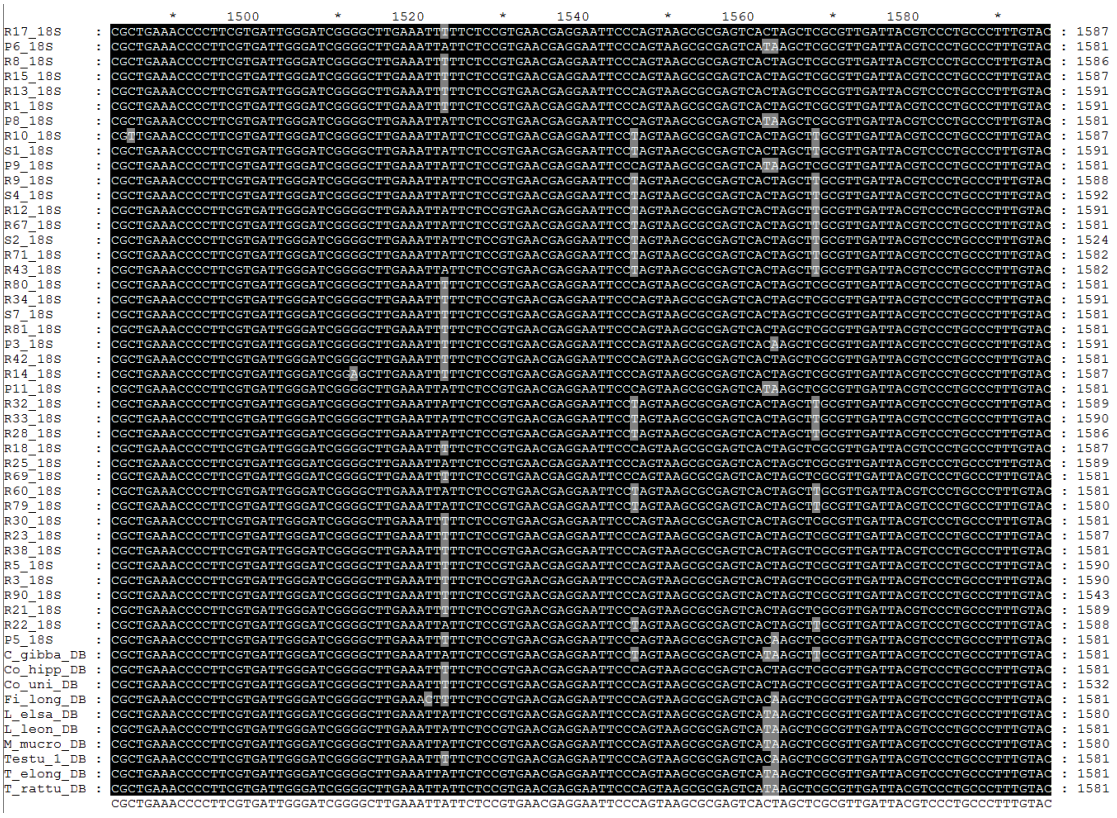
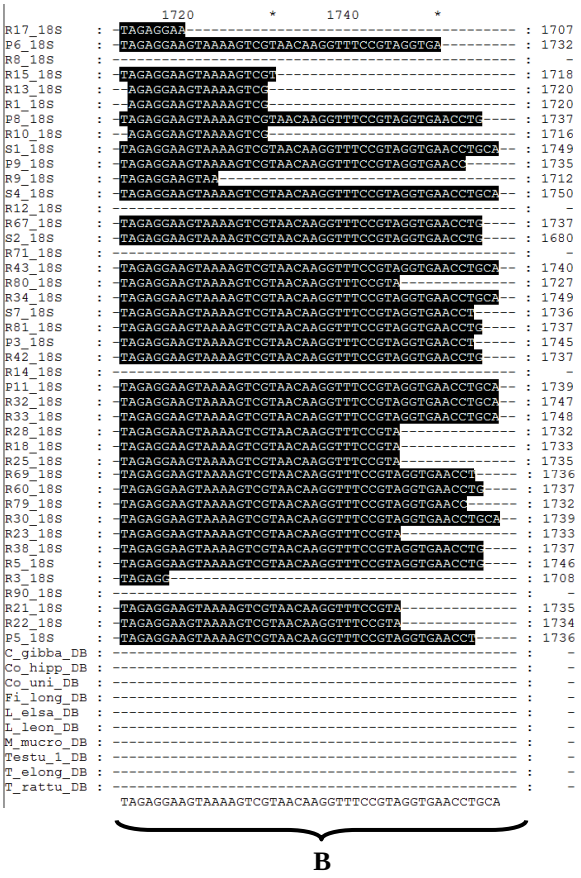


Appendix E (continued)

Appendix E (continued)



PUBLICATIONS

1. Meksuwan, P., Pholpunthin, P. and Segers, H. 2013. The Collothecidae (Rotifera, Collothecacea) of Thailand, with the description of a new species and an illustrated key to the Southeast Asian fauna. ZooKeys 315: 1-16. doi: 10.3897/zookeys.313.5330
2. Meksuwan, P., Pholpunthin, P. and Segers, H. Molecular phylogeny confirms Conochilidae as ingroup of Floscullariidae (Rotifera, Gnesiotrocha) (in press).

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Meksuwan, P., Pholpunthin, P. and Segers, H. 2013. The Collothecidae (Rotifera, Collothecacea) of Thailand, with the description of a new species and an illustrated key to the Southeast Asian fauna. ZooKeys 315: 1-16. doi: 10.3897/zookeys.313.5330

Meksuwan, P., Pholpunthin, P. and Segers, H. Molecular phylogeny confirms Conochilidae as ingroup of Floscullariidae (Rotifera, Gnesiotrocha) (in press).