UNIVERSITY OF GOTHENBURG

New Insights into the Evolution of Bryozoa
– An Integrative Approach

Judith Fuchs

Faculty of Science
Department of Zoology
Systematics and Biodiversity
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Judith Fuchs

Department of Zoology, Systematics and Biodiversity
University of Gothenburg
Box 463
SE-40530 Gothenburg
Sweden
Email: judith.fuchs@zool.gu.se

Cover illustration: Life cycle of the gymnolaemate bryozoan *Bugula neritina*.

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Dissertation abstract

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Bryozoa is a group of aquatic, sessile invertebrates with circumglobal distribution and includes about 6000 recent species. Bryozoans have an indirect life cycle with a larval stage that settles and metamorphoses into the adult. Although a bryozoan individual is barely visible with the naked eye, all bryozoans form colonies, which are often macroscopic in size and display a variety of beautiful shapes and forms. Ever since their first scientific description in the 16th century, bryozoan relationships to other animal groups have been enigmatic. Bryozoan morphology and life history show various differences to other invertebrates, so that their closest relatives could not be identified with certainty. Also, a reliable hypothesis about the evolution of the variety of bryozoan larval and adult body forms is greatly in dispute.

In this thesis, questions concerning bryozoan evolution are addressed from diverse angles by exploring different life cycle stages and methodological tools. A new phylogeny of Bryozoa based on molecular data is presented. A similar approach is used to investigate the phylogeny of another animal taxon, Entoprocta, which was long thought to be the sister group of Bryozoa. The results reveal that Bryozoa is a natural group with a single origin (monophyletic clade) and that Bryozoa and Entoprocta are not sister groups. Further, gene expression in the larval stage of the bryozoan *Bugula neritina* was studied and indicates the importance of molecularly pre-patterned blastemic tissues for adult body plan formation. In addition, a new bryozoan species from the West Coast of Sweden is described and a genetic barcode is provided for the new species, which will help to identify this species in the future.

The thesis demonstrates that molecular data combined with high taxon sampling are essential to reveal bryozoan phylogenetic relationships and that gene expression studies of the enigmatic taxon Bryozoa are valuable to get insights into the evolution of their life cycle and to contribute to our general understanding of metazoan body plan evolution.

Keywords: Ectoprocta, moss animal, systematics, phylogeny, barcode, COI, gene expression

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Svensk sammanfattning

Judith Fuchs, 2011

Nya inblickar i mossdjurens (Bryozoa) evolution- ett integrativt tillvägagångssätt

Mossdjur (Bryozoa) är en grupp vattenlevande ryggradslösa djur som finns över hela världen och gruppen innehåller ungefär 6000 arter. Mossdjuren har en indirekt livscykel med en frisimmande larv som, när den landar på en lämplig yta, utvecklas till fastsittande vuxen.

Ett enstaka mossdjur är nästan osynligt för blotta ögat men alla mossdjur bildar kolonier som blir stora nog att synas och som uppvisar en mångfald av vackra former. Det första mossdjuret beskrevs vetenskapligt redan på 1500-talet men deras släktskap med andra ryggradslösa djur har förblivit en omdiskuterad gåta. Både deras utseende och deras livsmönster skiljer sig från andra djur vilket inneburit att det varit svårt att avgöra vilken djurgrupp de står närmast. Det finns inte heller någon helt tillförlitlig hypotes om evolutionen av all variation hos mossdjurens olika former som larver och vuxna. I den här avhandlingen används flera olika metoder, och olika livscykelstadier undersöks, för att försöka besvara några av alla de frågor som rör mossdjurens evolution.

Med hjälp av molekylära data har mossdjurens släktskap undersökts och samma metoder har använts för att undersöka släktskap hos en annan djurgrupp, Entoprocta, som länge ansågs vara den djurgrupp som var närmast besläktad med mossdjuren. Resultaten visar att mossdjur är en naturlig grupp med en gemensam förfader (en monofyletisk klad) och att de inte är närmast släkt med Entoprocta.

Dessutom undersöktes genuttryck av 13 olika gener i larvstadiet hos mossdjuret *Bugula neritina*. Studien visar att de flesta av dessa gener hos larven uttrycks i speciella cell-lager som inte har någon användning i själva larvstadiet, utan har till funktion att bygga upp det adulta djuret.

Slutligen beskrivs en nyupptäckt mossdjursart från svenska västkusten och en genetisk streckkod för den nya arten bifogas i beskrivningen för att underlätta identifiering av denna art i framtiden.

Avhandlingen visar att molekylära data tillsammans med provtagning av många olika arter är nödvändiga redskap för att avslöja mossdjurens släktskap. Vidare är studier av genuttryck hos denna gåtfulla djurgrupp värdefulla för att få en inblick i evolutionen av deras livscykel, något som även bidrar till vår förståelse av evolutionen av de olika livscykel-stadier hos flercelliga djur.

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... from so simple a beginning
endless forms most beautiful and most wonderful
have been, and are being, evolved.

C. Darwin, 1859

Table of Contents

List of Papers

This thesis is based on the following papers:

I. Fuchs J, Obst M, Sundberg P: The first comprehensive molecular phylogeny of Bryozoa (Ectoprocta) based on combined analyses of nuclear and mitochondrial genes. *Molecular Phylogenetics and Evolution* 2009, 52:225-233.
II. Fuchs J, Iseto T, Hirose M, Sundberg P, Obst M: The first internal molecular phylogeny of the animal phylum Entoprocta (Kamptozoa). *Molecular Phylogenetics and Evolution* 2010, 56:370-379.
III. Fuchs J, Martindale MQ, Hejnol A: Gene expression in bryozoan larvae suggest a fundamental importance of pre-patterned blastemic cells in the bryozoan life-cycle. Submitted to *EvoDevo*.
IV. Fuchs J, Sundberg P, Obst M: A new soft-bodied bryozoan (Bryozoa) from the North East Atlantic, and notes on *Arachnoidella dhondti*. Manuscript.

All new taxon names mentioned in this thesis are disclaimed for nomenclatural purposes (ICZN article 8.2.).

Introduction

The name Bryozoa literally means “moss animal” and “mossdjur” in Swedish. Bryozoans are aquatic invertebrates, which have a free-swimming larval stage that develops into a sessile adult through a complex process (metamorphosis). An individual bryozoan is microscopic in size (< 1 mm), but it reproduces asexually and forms a colony, which is usually visible without magnification. Colonies measure in average some cm in size and the coral-like bryozoans *Celleporaria agglutinans* and *Pentapora fascialis* can reach diameters of over 30 cm (Gordon, 2003; Hayward and Ryland, 1999). Bryozoan colonies exhibit a fascinating variety of forms including incrusting, erect, soft-bodied, and calcified types. Bryozoans are distributed in all major oceans and a few species live in freshwater. Colonies are usually permanently attached to all kinds of submerged hard substrates like algae, stones, ropes, garbage, ship hulls, and other aquatic animals.

The oldest bryozoan fossils are about 460 million years old and major radiations within the taxon have taken place including the extinction of many species (e.g. Ryland, 1970). About 15.000 fossil species and 6000 recent bryozoan species are recognized.

Upon their early scientific discoveries in the 16th century, Bryozoa were regarded as plants or plant-like creatures and their very special body plan and complicated life cycle are so dissimilar to all other animals, that the relationship of Bryozoa with other animals has been enigmatic throughout the centuries. Although it is now well supported scientifically that Bryozoa are protostomes and that they belong to the clade Lophotrochozoa, their affinities within the latter group are unresolved. Furthermore, many questions regarding the evolution of the various larval and adult body forms and their life cycles remain unanswered.

The bryozoan body plan

Bryozoans are microscopic, mostly sessile, and colonial coelomates, which are permanently fastened in exoskeletal cases or gelatinous material of their own secretion. They possess a circular or horseshoe-shaped lophophore (tentacle crown) and a curved digestive tract with the mouth lying inside, and the anus lying outside the tentacle crown. Bryozoans have radial cleavage, lack nephridia and a circulatory system (Hyman, 1959).

An individual bryozoan is called a zooid and consists of the body wall (cystid) as well as the gut and the tentacle crown (polypide) (Fig. 1A, B). The cystid can be soft or calcified. Bryozoans are reported to have the fastest retracting muscles in the animal kingdom (Thorpe et al., 1975). The adult nervous system consists of a cerebral ganglion and nerves in the lophophore and other body parts (e.g. Hyman, 1959; Mukai et al., 1997; Fig. 1C). The ciliated lophophore is extended into the water column to filter small particles from the water. The usual bryozoan zooid feeds, but in many species, some zooids of the colony are non-feeding and instead specialized for e.g. brooding, defence, or cleaning (polymorphism).

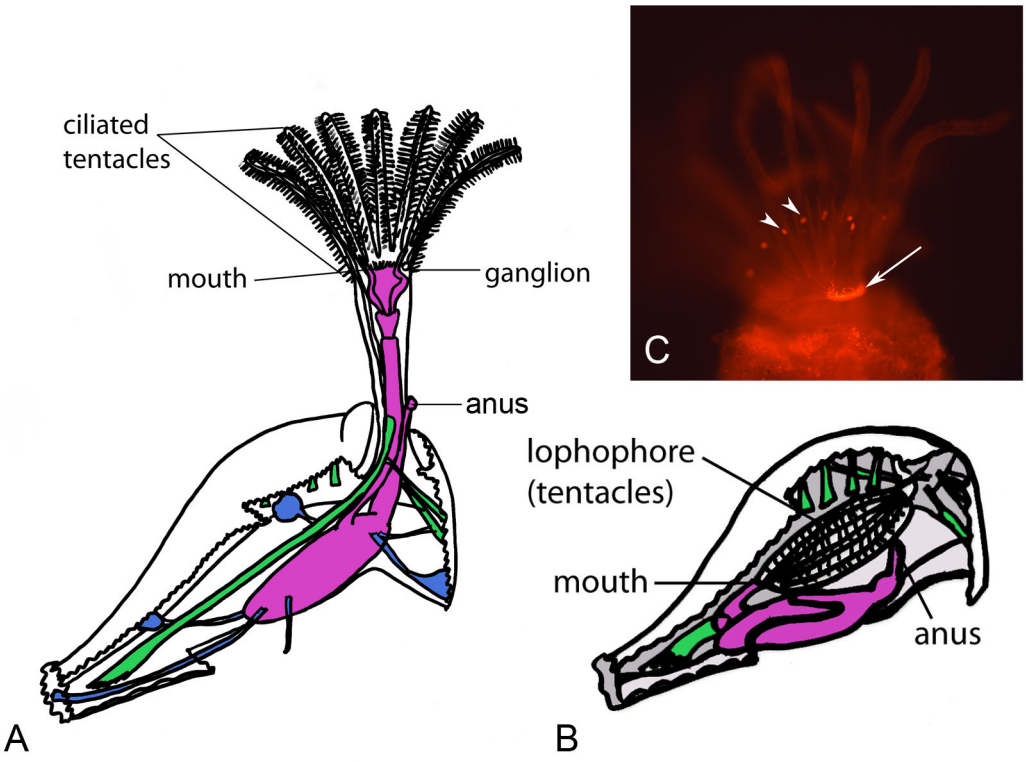

Figure 1. Schematic drawing of a bryozoan individual (zooid) showing the body wall (cystid) and the retractable polypide (digestive tract and lophophore). A. Extended polypide. B. Retracted polypide. pink, digestive tract; blue, tissue connecting to other zooids; green, muscles. Modified after Gordon (2003). C. Part of the nervous system in the lophophore of *Plumatella sp*. (immunocytochemical labeling of serotonin); arrow, cerebral commissure; arrow heads, tentacle nerves.

Bryozoans reproduce both sexually and asexually. Some bryozoans have separate sexes, while most species are probably hermaphrodites. Asexual reproduction is a fundamental part in the life history of bryozoans and includes (1) the production of clones by forming colonies through budding, (2) the cyclic replacement of "old" polypides by new ones, (3) embryonic fission (polyembryony), or (4) regeneration from colony fragments, from damaged colonies, or from spezialised resistant bodies (statoblasts).

The enigmatic position of Bryozoa in the metazoan tree of life

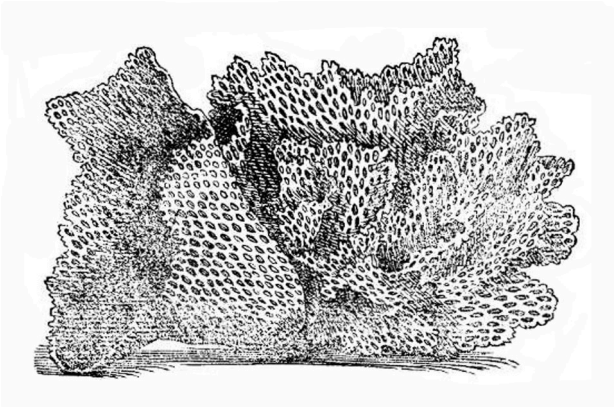

Figure 2. One of the first drawings of a bryozoan colony, *Retepora sp.* (Imperato, 1599).

On their early discoveries, bryozoans were thought to be plants ("marine vegetables" or zoophytes). Probably the first published record of a bryozoan appears in the category "Insects and Zoophytes" (Rondelet, 1555) and then under corals (Imperato, 1599; Fig. 2). Based on their superficial similarity to cnidarians, Jussieu realized the animal nature of bryozoans and named the small individuals "polyps" (1745). Accordingly, bryozoans were treated as Cnidaria in the following years (Lamouroux, 1824; Linnaeus, 1758). Then, bryozoans were erroneously associated with compound ascidians (Tunicata) (Milne Edwards, 1843). When ciliated tentacles and a curved digestive tract with two openings were observed in bryozoans, the group was recognized as unique and named "Polyzoa" (Thompson, 1830). Independently, the name Bryozoa was introduced for the same group of animals (Ehrenberg, 1831), which caused a tedious discussion about which of the two names should be used correctly.

In 1847 another animal group, Entoprocta, was assigned to "Polyzoa" (Johnston). Although differences between Entoprocta and Bryozoa were realized (Allmann, 1856; Salensky, 1877; Stiasny, 1905), many authors treated the two taxa as one group (Hatschek, 1877; Leidy, 1851; Van Beneden, 1845). In 1869, Entoprocta (meaning "inside-anus" referring to the anus lying inside the tentacle crown) were recognized as a natural group and separated from Ectoprocta (meaning "outside-anus" referring to the anus lying outside the tentacle crown; Bryozoa *sensu stricto*) (Nitsche). Further morphological differences between Entoprocta and Bryozoa are that Entoprocta have spiral cleavage, lack a coelom and possess excretory organs, while Bryozoa have radial cleavage, comprise a coelom and lack excretory organs.

Entoprocta were then erected to "phylum" level and Bryozoa were grouped with Brachiopoda and Phoronida in the clade "Tentaculata" (Molluscoidea) (Hatschek, 1888). The name Tentaculata was later replaced with Lophophorata based on the presence of a lophophore, a tentaculated extension of the mesosome that has a coelomic lumen and embraces the mouth but not the anus (Hyman, 1959). Furthermore, the lophophorates share the presence of a coelom, a U-shaped gut, a simple reproductive system, and they all usually secrete outer casings (i.e. tubes, shells, exoskeletons), and all have radial cleavage. Lophophorates are benthic, mostly marine, and while the wormiform bodies of phoronids can move inside their tubes, bryozoans and brachiopods are entirely anchored inside their casings and usually attached to a substrate. While phoronids and brachiopods share the presence of

circulatory and excretory systems, those are lacking in Bryozoa. It was realized that the lophophorates share morphological similarities with both deuterostomes and protostomes, resulting in ambiguous positions of Bryozoa among Metazoa (Nielsen et al., 1996; Willmer, 1990). However, several authors treated the group as deuterostomes (Brusca and Brusca, 2004; Zimmer, 1973).

Besides the above considerations, from the 1970`s and onwards, a close relationship of Entoprocta and Bryozoa was proposed again based on some similarities of the larval stages, their metamorphosis, and the budding process (Nielsen, 1971, 1977, 2004).

The introduction of molecular methods in biology revolutionized the view of the metazoan tree of life and with it, also the position of Bryozoa. A study based on 18S rDNA data of several bilaterians revealed that Lophophorata are protostomes (Halanych et al., 1995). Further studies supported this view, but subsequently more data became available providing evidence against a close relationship of Bryozoa with Brachiopoda & Phoronida, and thus also against Lophophorata (Paps et al., 2009; Peterson and Eernisse, 2001; Zravý et al., 1998).

The most recent molecular phylogenies of Bilateria based on EST (expressed sequence tag) and nuclear ribosomal data indicate a clade [Bryozoa + (Entoprocta+Cycliophora)], however with low support (Hejnol et al., 2009; Paps et al., 2009; Fig. 3). Thus, besides the assumption that Bryozoa are Lophotrochozoa, their relationship with other animals in this group remains ambiguous.

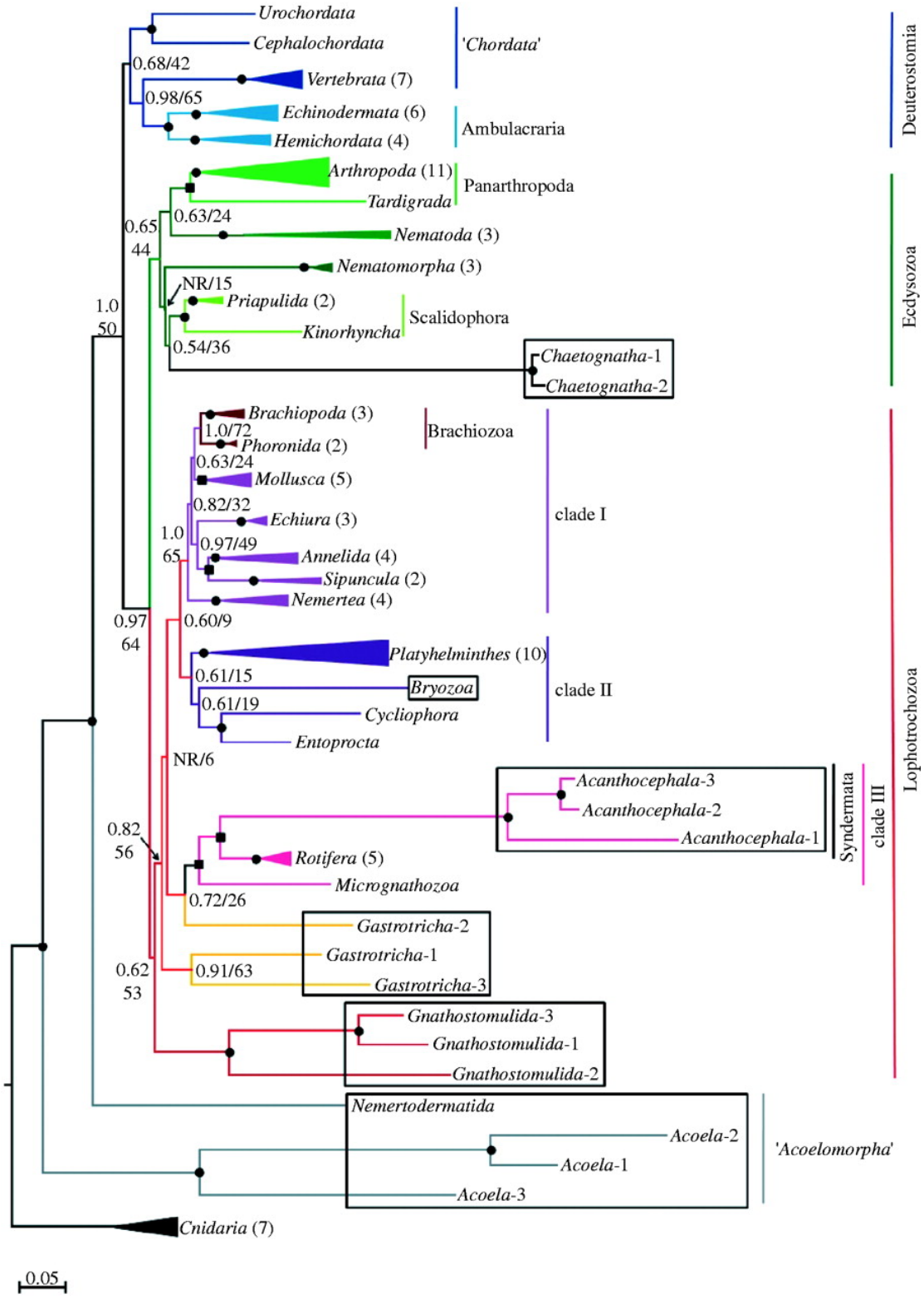

Figure 3. Animal phylogeny based on Bayesian statistics and Maximum likelihood analysis of 18S and 28S rDNA data of 104 taxa. Posterior probabilities (PP) and bootstrap values (BV) are indicated by a circle (PP=1.0; BV>90%) or a square (PP=1.0; BV=75-90%). Bryozoa appear in clade II together with Entoprocta, Cycliophora, and Platyhelminthes. From Paps et al. (2009).

Intrarelationships of Bryozoa

First classifications of Bryozoa date back to the early 19th century (Allmann, 1856; Busk, 1852). Bryozoa were usually divided into the three classes Phylactolaemata, Stenolaemata, and Gymnolaemata from the 20th century and onwards (Woollacott and Zimmer, 1977). Phylactolaemata contain about 80 freshwater species (Fig. 1A), Stenolaemata (Cyclostomata) comprise 700 marine species (Fig. 1B), and Gymnolaemata include approximately 5000 species of which most are marine and a few live in freshwater. Within Gymnolaemata, soft-bodied and calcified species are recognized. The soft-bodied species were named Ctenostomata and calcified species were named Cheilostomata (Fig. 4C-D).

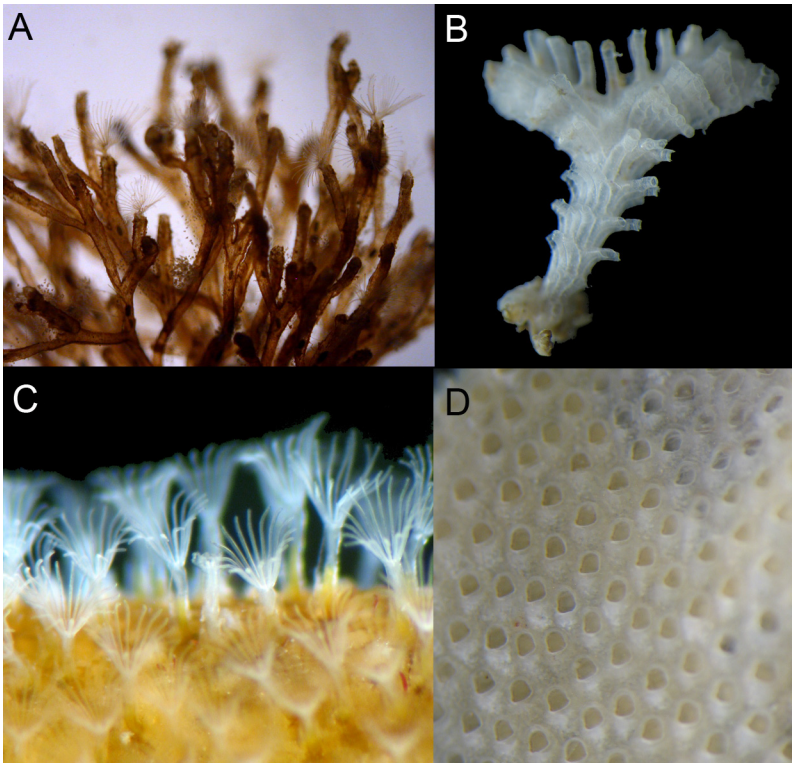

Figure 4. Images of bryozoan colonies. A. Phylactolaemate *Plumatella sp.* B. Stenolaemate *Idmidronea atlantica*. C. Soft-bodied gymnolaemate *Alcyonidium diaphanum* (polypids extended). D. Calcified gymnolaemate *Cryptosula pallasiana* (polypids retracted).

Based on morphology, several hypotheses regarding the interrelationships of the above mentioned bryozoan taxa were proposed during the years. Several authors see the freshwater Phylactolaemata as the basal group, based on characters such as a horseshoe-shaped lophophore and an upper lip organ, the epistome, which is lacking in other bryozoans (Hyman, 1959; Jebram, 1973). A different view was proposed suggesting a closer relationship of Phylactolaemata to Phoronida than to the other Bryozoa (Mundy et al., 1981). Concerning the soft-bodied Ctenostomata, Todd (2000) suggested that the group is paraphyletic with respect to both calcified Stenolaemata and calcified Cheilostomata nesting inside this group. Furthermore, cheilostome polyphyly was suggested by several authors (Gordon, 2000; Voigt, 1991).

The outline above shows that fossil record as well as morphological and life history data are not sufficient to resolve the phylogeny of Bryozoa. Molecular methods to reconstruct the phylogeny of Bryozoa were initiated in the early 21st century. Several molecular studies did not support the monophyly of bryozoans (Helmkampf et al., 2008; Mackey et al., 1996; Passamaneck and Halanych, 2006; Wood and Lore, 2005). A study based on one mitochondrial gene of Gymnolaemata and Stenolaemata gave ambiguous results (Dick et al., 2000). Attempts to clarify the interrelationships of Phylactolaemata using molecular data revealed new insights and did not reflect earlier morphological phylogeny reconstructions (Hirose et al., 2008; Okuyama et al., 2006; Wood and Lore, 2005).

Aims of the thesis

The overall aim of this thesis was to gain new insights into the evolution of the invertebrate animal phylum Bryozoa (Ectoprocta). As outlined above, the evolutionary history and the relationships of Bryozoa to the other animals was not well understood ever since their first discoveries. I used several independent approaches with a focus on molecular methods to tackle specific questions regarding bryozoan phylogeny and evolution.

First, I aimed to reveal some aspects of the phylogeny of the entire phylum Bryozoa using phylogenetic tools (**paper I**). In this work I tested the monophyly of Bryozoa with analyses of molecular data and aimed at getting new insights into the inter- and intrarelationships of the earlier proposed bryozoan groups Phylactolaemata, Stenolaemata, and Gymnolaemata.

In **paper II**, my main approach to learn more about bryozoan evolution, was tackled from a different angle. Over centuries, several scientists argued that the animal taxon Entoprocta was closely related to Bryozoa, based on a few similarities in morphology. I studied the phylogeny of Entoprocta using molecular data and applied similar tools as in the first study. The objective was to reveal important aspects of entoproct evolution and to formulate a hypothesis about the last common ancestor of Entoprocta. In that way, I tried to evaluate a sister group relationship between Entoprocta and Bryozoa.

The idea for **paper III** developed over a longer period. I wanted to gain new insights into the complex bryozoan life cycle by investigating the gene expression of several genes in the larval stage of the bryozoan *Bugula neritina* and by comparing the expression patterns with gene expression of respective gene orthologs in other animals.

Paper IV "evolved" from my study of the Swedish bryozoan fauna. One part of my PhD was dedicated to generate a Checklist for Swedish Bryozoa. The Swedish Taxonomy Initiative (Miller, 2005) conducted an extensive, marine benthic inventory along the Swedish West Coast in the period 2006 to 2010. As part of the project, over 400 locations were sampled by bottom dredging and we collected the bryozoans at these locations. So far, 45% of the material is determined. The preliminary Checklist for Swedish Bryozoa is added in the additional related material (not a part of the actual thesis). During these studies, we recorded several species new for the Skagerrak and Kattegat (North East Atlantic) and discovered one new species. In **paper IV** (manuscript), the new species is described and notes about another interesting species, which was known only from one location in the West Atlantic, are added.

Methods

Sampling and fixation

Sweden

Papers I, II & **IV** and Checklist for Swedish Bryozoa

Most bryozoans and outgroup taxa were collected along the Swedish West Coast by bottom dredging during the marine benthic inventory of the Swedish Taxonomy Initiative between 2006 and 2010. In addition, several species were sampled by installing settlement plates, snorkeling, and collections in harbors. Most of the collected material was fixed in ethanol (70-96%), while larger stone with colonies were dried. For morphological studies, some species were additionally fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for a few hours and stored in PBS or 0.1% sodium azide in PBS.

Hawaii

Paper III

During my stay in Hawaii, several species of bryozoans were collected in different harbor areas. *Bugula neritina* was collected from three harbors in Honolulu. Colonies were kept in the dark on running seawater tables for some days and then exposed to pointed light sources in the laboratory. Spawned larvae were collected and fixed in RNA*later* for following RNA extraction. Both RNA extraction as well as gene expression studies were most successful with *B. neritina*.

For *in situ* hybridizations, larvae of *B. neritina* were relaxed in 7.14% potassium chloride solution, prefixed in glutaraldehyde fixative (0.3% glutaraldehyde, 3.7% formaldehyde in seawater) for two minutes, fixed in 3.7% formaldehyde for 1h at 4 °C, followed by washes in Phosphatebuffer with 0.1% Tween 20 and in distilled water. The larvae were subsequently stored in 100% methanol at -20 °C.

Other locations

Papers I & **II**

During several occasions and visits to other countries, including Norway, Croatia, and California, additional samples of bryozoans, entoprocts, and outgroups were collected. Few samples were generously provided by co-authors and other collectors (see **papers I** & **II**).

Morphological investigations

Papers I-IV

Species determinations were made with living or alcohol preserved specimens by stereo light microscopy (**papers I - IV** and Checklist for Swedish Bryozoa).

For the species description in **paper IV**, specimens fixed in ethanol or 4% paraformaldehyde were additionally mounted in glycerine on slides or dehydrated in an ascending ethanol series and xylol, and permanently mounted in Canada balsam. Some specimens were stained with aqueous aniline blue or alcoholic paracarmine prior to mounting.

DNA sequencing and phylogeny reconstruction

Papers I-IV

Tissues of ethanol fixed specimens of Bryozoa, Entoprocta, Brachiopoda, and Phoronida were dissected and DNA was extracted. DNA fragments of the mitochondrial genes cytochrome c oxidase subunit I (COI) and 16S rDNA, as well as the nuclear genes 18S rDNA and 28S rDNA were amplified using universal metazoan primer sets (for specifications see **papers I, II & IV)**. The PCR products were separated by gel electrophoresis and positive products were cleaned and sequenced by Macrogen (Korea). The sequences were processed with the software SeqMan (DNAstar) or Geneious (Biomatters) and checked for contaminations by blasting them in the sequence database GenBank. Novel sequences were submitted to the latter (**papers I & II**).

For phylogenetic analyses, the sequences of different species were aligned with Clustal W (Larkin et al., 2007) and corrected by hand (**papers I & II**). Datasets were analyzed separately for each gene. To test the congruence between the genes, the Kishino-Hasegawa and the Shimodaira-Hasegawa tests were used in Paup*4.0b10 (Swofford, 2003). If the datasets of the selected genes were not significantly different, they were analyzed combined. Evolutionary models for the datasets were calculated (a) using Modeltest 3.7 (Posada and Crandall, 1998) for Maximum likelihood analyses in Paup*4.0b10, or (b) with Mr.Modeltest 2.3 (Nylander, 2004) for analyses using Bayesian statistics in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), or (c) using RAxML (Stamatakis, 2006) via the Cipres Portal. Tree support for (a) and (c) was calculated with bootstrapping.

Gene orthology analyses

To show the orthology of the 13 cloned genes of *B. neritina* (**paper III**), respective sequences of the bryozoan were aligned with sequences of other metazoans using MUSCLE (Edgar, 2004). Best-fit evolutionary models for protein evolution were determined with ProtTest (Abascal et al., 2005). Maximum likelihood phylogenetic analyses were performed using PhyML 3.0 (Guindon and Gascuel, 2003). 1000-3000 bootstraps were calculated (see **paper III**, additional data file).

Gene expression studies

Paper III

RNA extraction and probe synthesys

Messenger RNA (mRNA) is the transcribed copy of a gene lacking introns and it serves as template for protein synthesis in eukaryotic cells. Complementary DNA (cDNA) is a transcribed copy of the mRNA. cDNA (lacking introns) is used for cloning eukaryotic DNA in a prokaryotic host (see below), since prokaryotes lack introns and thus the machinery for their removal. Total mRNA of the larvae of *B. neritina* fixed in RNA*later* was extracted using DynaBeads mRNA DIRECT Kit and stored at -80 °C. cDNA was synthesized using the Advantage RT-for-PCR Kit and stored at -20 °C.

An expressed sequence tag (EST) library of *B. neritina* (Dunn et al., 2008) was screened for the genes *Tropomyosin*, *BAMBI*, *Hox4*, *SOXB2*, *SoxE*, *FoxB*, *FoxAB*, and *Wnt1*. Gene specific primers were designed in MacVector (MacVector Inc.) and used to amplify the genes using larval cDNA as template. To yield full-length cDNA fragments, we performed RACE (rapid amplification of cDNA ends) -PCR using the

SMART Race cDNA amplification kit. Fragments of the developmental genes *Cdx*, *FoxA*, *GATA123*, *GATA456*, and *Wnt4* were amplified using degenerate primers (mixture of similar, but not identical primers) with larval cDNA as template.
cDNA fragments were separated by gel electrophoresis and fragments of correct size were cut out of the agarose gel and extracted from the gel with the Quiagen Min Elute Gel Extraction Kit. cDNA fragments were ligated into pGEM-T Easy vectors using the pGEM-T Easy Kit. The vectors were transformed into *E.coli* bacterial cells and the latter were plated on cultivation plates with ampicillin. *E.coli* cells that incorporated the vector were ampicillin resistent and grew on the plates. To confirm that the transformed vector had the correct size, PCR of a fraction of the bacterial clones and subsequent gel electrophoresis were used. Finally, the bacterial clones including the correct cDNA fragments of *B. neritina* were multiplied and the vectors cleaned and sent to Macrogen (Korea) for sequencing. The sequences were blasted on NCBI (BlastX) to check for orthologous sequences (and see gene orthology analyses). Gene sequences were deposited at GenBank. The fragments were used as template to transcribe RNA probes (= riboprobe = single stranded RNA) with the MEGAscript Kit for *in situ* hybridizations.

In situ hybridization

In situ hybridizations were performed with fixed *Bugula neritina* larvae stored in 100% methanol. The procedure takes between two and three days and includes pretreatment of the larvae, a hybridization step, and visualization of the riboprobe. In the first step, the larvae were permeabilized for uptake of the riboprobe (RNA). The riboprobe was labeled with a dioxigenin (DIG) antigen and added to the hybe-solution containing the larvae. The riboprobe hybridized with the according mRNA in the larvae. An alkaline-phosphatase-conjugated anti-DIG antibody was added to the solution and attached to the antigen on the riboprobe. The chemical compound NBT/BCIP was added to the solution, which reacted with the alkaline phosphatase and visualized the probe by dyeing it dark blue. The color development was monitored and stopped by changing the pH. The larvae were stored in 70% glycerol and mounted on glass slides for observations of the expression patterns of the 13 cloned genes. The entire *in situ* hybridization protocol can be obtained from Hejnol and Martindale (2008).

Histology

The precise localization of the gene expressions could not satisfactorily be observed in the whole mounted, *in situ* hybridized larvae. Therefore, the larvae were sectioned to localize the expression patterns in the larval tissues in greater detail. Larvae stored in 70% glycerol were washed in phosphate buffered saline and dehydrated in an ascending ethanol series with a final step in 100% propylene oxide. The larvae were embedded in Low Viscosity Resin LVR and semithin serial sectioned (2 µm) on a Leica RM2255 microtome with a Diatome Histo Jumbo Diamond Knife (Blumer et al., 2002). Sections were stained with an alcoholic solution of 1% basic fuchsine (p-Rosanilin) or toluidine blue and embedded in LVR on slides.

Image processing

Overview pictures of bryozoan colonies were taken with a Canon Powershot S3IS digital camera mounted on a stereo microscope. Light microscopical observations were mainly conducted on a Nikon Eclipse E1000 microscope and images were taken with a Nikon DXM1200 digital camera mounted on the former. Adobe Illustrator and Photoshop CS3-CS5 were used for drawings and image presentation.

Main results and discussion

In **paper I**, a hypothesis of the phylogeny of Bryozoa is presented. The study included datasets of one mitochondrial gene (COI) and two nuclear genes (28S and 18S) of 32 bryozoan species and four outgroup species. We showed that Bryozoa is a natural grouping (monophyletic clade). The result corroborates morphology and challenges some molecular studies, which showed Bryozoa polyphyletic (Helmkampf et al., 2008; Mackey et al., 1996; Passamaneck and Halanych, 2006; Tsyganov-Bodounov et al., 2009; Wood and Lore, 2005).

Our combined dataset further showed support for the previous defined bryozoan "classes" Phylactolaemata, Stenolaemata, and Gymnolaemata. We reveal that Gymnolaemata is a natural group containing both soft-bodied and calcified species and propose that calcification has evolved several times independently in this clade.

The interrelationship of the three main clades, however, is not entirely resolved. We have, however, indications that Bryozoa have evolved from a common ancestor, into the freshwater Phylactolaemata on one hand and a group of marine bryozoans (Stenolaemata+Gymnolaemata) on the other hand. These two groupings might thus have evolved parallel to each other over a long time. The latter claim is to some degree supported by e.g., the different larval forms that we see in (1) Phylactolaemata and (2) Stenolaemata & Gymnolaemata. However, this consideration needs further evaluation by cladistic and phylogenetic approaches.

In **paper II**, the phylogeny of Entoprocta was reconstructed based on analyses of one mitochondrial gene (COI) and two nuclear genes (28S, 18S) of 18 entoproct species and 10 outgroup species. Many outgroups were included, since there was no certainty about the entoproct sister group (an outgroup is an important reference group in phylogenetic reconstructions and it should be closely related to the ingroup as it is used to root the phylogenetic tree).

The study revealed that (1) Entoprocta is a monophyletic clade, (2) there was an early split leading to colonial species on one hand and solitary species on the other hand, (3) the solitary *Loxosomella* are probably paraphyletic, (4) there is high support for Cycliophora being the sister group of Entoprocta. The latter result provides evidence against a sister group relationship of Entoprocta and Bryozoa, an earlier proposed hypothesis based on some morphological details or phylogeny reconstructions with insufficient taxon sampling (Hausdorf et al., 2007; Nielsen, 2004).

In **paper III,** the expression patterns of 13 metazoan developmental genes in the larval stage of the gymnoalemate bryozoan *Bugula neritina* were investigated. The study reveals that most genes are expressed in certain blastemic tissues in the larvae, which are crucial to build tissues of the adult bryozoan during metamorphosis. This result suggests, that the cells in the blastemic tissues are molecularly pre-patterned according to their future fate in the adult. Only two of the 13 genes were exclusively expressed in truly larval tissues, which are discarded at metamorphosis. Comparison of the gene expression patterns in the bryozoan larva with corresponding data of other metazoans show that some of the bryozoan larval tissues correspond to adult tissues of other animals. Overall, this paper gives insight into bryozoan life history and raises important questions regarding the evolution of metazoan larval stages in general.

In **paper IV**, a new soft-bodied gymnolaemate bryozoan from the Skagerrak (North East Atlantic) based on morphological data is described. The species seems to grow preferentially on Hydrozoa. Further, new data of another soft-bodied bryozoan species are presented. Previous to our study, the latter species was found growing epizoically on hermit crabs (Crustacea) at the West coast of the USA (West Atlantic), and we discovered the bryozoan growing on squat lobsters (Crustacea) in the North East Atlantic. Barcodes for the bryozoans and the crustacean host from the North East Atlantic are provided.

Conclusions and future perspectives

This thesis demonstrates that phylogeny reconstructions based on molecular data are important to reveal the phylogeny of Bryozoa (and Entoprocta). In addition, non-molecular characters, e.g. morphological, ecological, life-history, or palaeontological, can be mapped on the molecular trees serving as backbones. Thus, molecular and morphological data complement each other and are both equally needed to understand the evolution of these taxa.

Our studies prove that main questions concerning bryozoan evolution can be answered by an integrative approach implementing systematics tools (taxonomy, phylogeny) and developmental biology (i.e. gene expression analyses of the larval stage). The larval stage especially, is an important life-cycle stage, not only in Bryozoa, but in several aquatic metazoan taxa. The evolution of indirect life cycles within Metazoa, however, is not clear and the homology of metazoan larvae remains enigmatic. Investigating the clade of indirect developing Bryozoa can provide insights into larval evolution and thus metazoan evolution in general.

The results of this thesis give way to many challenging questions regarding the taxon Bryozoa. I will list a few ideas for potential future studies here.

First, Gymnolaemata is the most species-rich bryozoan clade with over 5000 species. We have shown in **paper I**, that the soft-bodied gymnolaemate "Ctenostomata" and the calcified gymnolaemate "Cheilostomata" are probably polyphyetic, which was also indicated in some other studies (Todd, 2000; Tsyganov-Bodounov et al., 2009). More taxon intense phylogenetic reconstructions of the species-rich clade Gymnolaemata should be accomplished and future studies should also include important "key taxa". Such work will ultimately help resolving important questions such as wether a planktotrophic (feeding) larva or a lecitotrophic (non-feeding) larva was ancestral in Gymnolaemata and how the diverse polymorphisms evolved in this group.

To pinpoint the position of Bryozoa in Lophotrochozoa, phylogenomic studies should be conducted, which include high taxonomic sampling of Bryozoa, as well as representatives of all other lophotrochozoan taxa.

Another interesting question concerns the homology of bryozoan larvae with other lophotrochozoan larvae. This could be tackled by investigating the expression of certain genes involved in patterning the apical organs (larval character) among lophotrochozoan larvae.

A future study could focus on comparing the socalled "set-aside-cells" among metazoan larvae to adress the question how often an indirect life cycle evolved among metazoans.

Gene expression in bryozoan developmental stages and especially in the adult stage should be conducted for investigating the development of the nervous system

and the digestive tract. As **paper III** indicates, study of gene expression of the adult bryozoan stage could potentially provide additional important insight into the homology of bryozoan adult organs with that of other lophotrochozoans, and can help to reveal the oral-aboral axis polarity in bryozoans compared to other lophotrochozoans.

Further, a comprehensive cell lineage study of Bryozoa is urgently needed and modern methods such as 4D microscopy and cell labeling could be applied. Cell fate maps of Bryozoa have the potential to answer important questions, amongst others, if the "corona", the bryozoan larval ciliary band, is homologous to the "prototroch", a ciliary band of other lophotrochozoan larvae.

It would be interesting to know if the blastemic cells in bryozoan larvae show molecular similarities to cells involved in regeneration in other animals. Gene expression studies could be used to test this question and expression of e.g. *vasa*, a gene expressed in the germ line of mollusks and in stem cells in deuterostomes, could be investigated in bryozoans.

Additional related material

Remarks on the taxon Entoprocta

Today, approximately 180 entoproct species are recognized and the taxon comprises solitary as well as colonial species. Two species live in fresh water, while the rest is marine. Entoprocts live attached to submerged hard substrates, but several species are able to move transitorily (e.g. Assheton, 1912; pers. obs.). Most solitary species live in ectosymbiosis with other invertebrates (cf. Iseto, 2005; Nielsen, 1964).

The entoproct body plan

Entoprocts are microscopic, aquatic acoelomates. Their body consists of (1) a *calyx* with most inner organs and a circular tentacle crown, (2) a *stalk*, and (3) a *basal attachment* of the stalk (Fig. 1). The mouth and the anus of the curved digestive tract lie inside the tentacle crown. Entoprocts have spiral cleavage and possess excretory organs, and colonial species comprise a unique circulatory system (see Schwaha et al., 2010). Entoprocts are indirect developers with a swimming-type or a creeping-type larva (Fuchs and Wanninger, 2008; Wanninger et al., 2007). Despite the sexual production of larvae, they also propagate by asexual budding.

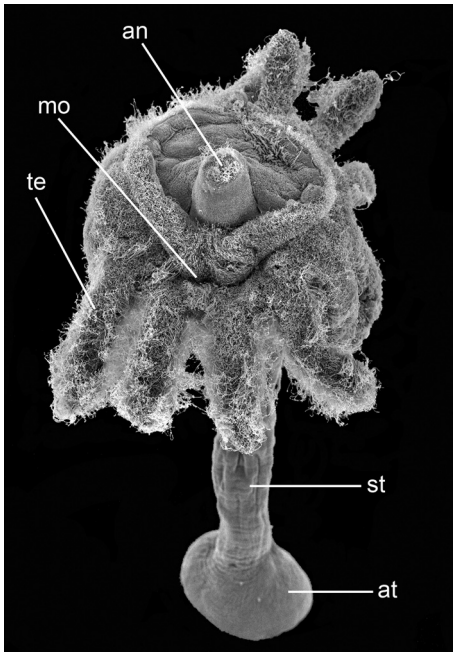

Figure 1. The solitary entoproct *Loxosoma pectinaricola* showing the calyx with tentacles (te), the stalk (st), and the basal attachment disc (at). Mouth (mo) and anus (an) on the anal cone lie inside the tentacle crown. Total length of the specimen about 350 *μ*m.

Entoproct phylogenetic relationships

The history of entoproct research encompasses a period of more than 250 years. Like bryozoans, entoprocts were considered plant-animals (zoophytes) and the latter were confused with rotifers, cnidarians, and bryozoans (Bosc, 1830; Johnston, 1838; Pallas, 1774). However, their uniqueness was probably realized by all investigators (e.g. Deshayes and Milne Edwards, 1836; Emschermann, 1985; Nielsen, 2001; Sars, 1835). Entoprocta were elevated to "phylum" level in 1888 (Hatschek). Due to morphological characteristics, entoprocts were usually considered spiralian

protostomes. In the 20th century, molecular studies confirmed the position of Entoprocta in Protostomia and Lophotrochozoa (e.g. Dunn et al., 2008; Mackey et al., 1996; Paps et al., 2009). Only few zoologists focused on studying the phylogeny of Entoprocta. Prior to our study (**paper II**), no more than two entoproct species were included in molecular studies. Our study implies the usefulness of molecular data in entoproct phylogeny reconstruction and reveals high support for a sister group relationship of Entoprocta with Cycliophora. Additional studies are needed to pinpoint the position of these sister taxa in Lophotrochozoa.

A few words about evolutionary biology and systematics

In the following I outline some concepts of evolutionary biology and systematics, which have a major impact on this thesis.

It is apparent that certain animals share an identical body plan with some other animals, as certain plants do with certain other plants. Think of a group such as "birds". One can usually identify within seconds, that the animal that just crossed the sky was "a bird". In that case, we realize that the individual belongs to a group of animals, which share several characteristics, i.e. they have a common body plan.

Such similarities entailed people, to intuitively sort living creatures with similar body forms into groups and give them a common name. An early classification scheme, the *scala naturae*, listed all entities of our world in a ladder-like system beginning with "the elements" and subsequently the human as the highest being (Bonnet, 1745). The ladder reflected the belief in a constant world shaped by god. In the early 18th century, amongst others, Linnaeus, the influential Swedish naturalist categorized plants and animals in his well-known *Systema Naturae* (1758). He invented several hierarchical categories in this system, while trying to include and classify the growing number of described organisms. The smallest entity in his system was the species, which should ideally be recognized by unique morphological characteristics.

In the mid 19th century, a major revolution regarding the perception of the natural world took place, initiated by the English naturalist Charles Darwin. He presented to the scientific community the idea of the common ancestry of all species, with the explanation that all species evolved through a process of natural selection (Darwin, 1859). This major concept regarding all species on this planet being related to each other and having evolved from a common ancestor, is the essence of all present biological work.

Darwin´s theory had, and still has, major impact on the superficial system of classifying animals, since the classification (taxonomy) should make sense in the way that it refers to "natural groupings", meaning that a name should be given to a group of animals, which have evolved from a last common ancestor. Darwin`s tree-like view of evolution, in which lineages split up and become increasingly different in time, was much different from the earlier view of the diversity of life.

Darwin`s causal explanations for evolution met enormous resistance, however, his concepts were confirmed, when the basis of heredity was bit by bit discovered by the studies of the researchers Gregor Mendel, William Bateson, Frederick Griffith, Oswald Theodore Avery, Colin McLeod, Maclyn McCarty, James Watson, and Francis Crick. It was found that animals diverge from a common ancestor by changes in their DNA.

Underlying Darwin`s theory is the concept of homology, referring to characters which share a common descent. The concept of homology was amongst

others revisited by the German biologist Willi Hennig, who introduced the concept of primitive (plesiomorphic) and derived (apomorphic) characters in evolutionary theory. The main idea was to search for shared derived characters (synapomorphies) among taxa to reconstruct their evolutionary history (Hennig, 1950).

The above outlined concepts are the essence of systematics. Systematics is the study of biological diversity and its origins and aims at understanding the evolutionary relatedness of biological entities, their phylogeny. This means in practice, that matrices are filled with (often molecular) characters, which are then analyzed with suitable phylogenetic programs. The outcome is a phylogenetic tree, which is a hypothesis of the relationships among the included entities. In recent years, phylogenetics has gone molecular for inferring relationships of living organisms, because molecular characters have often turned out to be less controversial when compared to morphological data. However, the main aim of a systematics researcher should ideally be to try to collect informative data of an animal group or another biological entity of interest, and use this information critically and unbiased to try to reveal their evolutionary history.

Checklist for Swedish Bryozoa

Group/ Number	Species	New record or new species
GYMNOLAEMATA		
1	*Aetea anguina*	
2	*Aetea sica*	
3	*Aetea truncata*	
4	*Alcyonidium albidum*	
5	*Alcyonidium diaphanum*	
6	*Alcyonidium gelatinosum*	
7	*Alcyonidium hirsutum*	
8	*Alcyonidium mamillatum*	
9	*Alcyonidium parasiticum*	
10	*Alderina imbellis*	
11	*Amphiblestrum aurita*	x
12	*Amphiblestrum flemingii*	
13	*Arachnidium hippothoides*	x
14	*Arachnidium simplex*	x
15	*Arachnoidella dhondti*	x
16	*Beania mirabilis*	
17	*Bicellariella ciliata*	
18	*Bowerbankia gracilis*	
19	*Bugula avicularia*	
20	*Bugula purpurotincta*	
21	*Buskea quincuncialis*	x
22	*Callopora craticula*	
23	*Callopora dumerilii*	
24	*Callopora lineata*	
25	*Callopora rylandi*	
26	*Cauloramphus spiniferum*	x
27	*Cellaria fistulosa*	
28	*Cellaria sinuosa*	x
29	*Celleporella hyalina*	
30	*Celleporina decipiens*	x
31	*Celleporina sp.*	
32	*Chartella barlei*	
33	*Chorizopora brogniartii*	
34	*Conopeum seurati*	
35	*Cribrilina annulata*	x
36	*Cribrilina cryptoecium*	x
37	*Cribrilina punctata*	x
38	*Cribrilina sp. nov.?*	?
39	*Cryptosula pallasiana*	
40	*Electra crustulenta*	
41	*Electra pilosa*	

75	*Escharella immersa*	
42	*Escharella klugei*	x
43	*Escharella laqueata*	
44	*Escharella ventricosa*	
45	*Escharina vulgaris*	x
46	*Eucratea loricata*	
47	*Fenestrulina malusii*	
48	*Flustra foliacea*	
49	*Flustrellidra hispida*	
50	*Hemicyclopora microstoma*	x
51	*Hippothoa divaricata*	x
52	*Hippothoa flagellum*	x
53	*Hypophorella expansa*	
54	*Kinetoskias smitti*	
55	*Membranipora membranacea*	
56	*Microporella ciliata*	
57	*Nolella dilatata*	x
58	*Nolella loveni sp. nov.*	x
59	*Notoplites harmeri*	
60	*Notoplites jeffreysii*	
61	*Notoplites loricata*	
62	*Palmiskenea skenei*	
63	*Panolicella nutans*	
64	*Parasmittina trispinosa*	
65	*Penetrantia concharum*	
66	*Phaeostachys spinifera*	
67	*Porella compressa*	
68	*Porella concinna*	
69	*Porella laevis*	x
70	*Porella patula*	
71	*Pyripora catenularia*	x
72	*Ragionula rosacea*	x
73	*Ramphonotus minax*	
74	*Reteporella beaniana*	
76	*Schizomavella cf. hastata*	x
77	*Schizomavella linearis*	
78	*Schizoporella alderi/ hexagona*	x
79	*Schizoporella unicornis*	x
80	*Scruparia ambigua*	
81	*Scruparia chelata*	
82	*Scrupocellaria reptans*	
83	*Scrupocellaria scabra*	
84	*Scrupocellaria scruposa*	
85	*Scupocellaria scrupea*	x
86	*Securiflustra securifrons*	
87	*Smittina bella*	
88	*Smittoidea reticulata*	

89	*Stomachetosella cruenta*	x
90	*Stomachetosella sinuosa*	
91	*Tegella sp.*	?
92	*Tegella unicornis*	
93	*Tessaradoma boreale*	
94	*Tricellaria peachii*	x
95	*Triticella flava*	
96	*Triticella pedicellata*	
97	*Turbicellepora avicularis*	
98	*Victorella sp.*	?
99	*Walkeria ulva*	
CYCLOSTOMATA		
100	*Crisia aculeata*	
101	*Crisia calyptosoma*	x
102	*Crisia cornuta*	
103	*Crisia eburnea*	
104	*Crisia klugei*	
105	*Crisiella producta*	
106	*Diplosolen obelia*	
107	*Disporella hispida*	
108	*Entalophoroecia deflexa*	x
109	*Filicrisia geniculata*	x
110	*Hornera lichenoides*	
111	*Idmidronea atlantica*	
112	*Lichenopora verrucaria*	
113	*Oncousoecia dilatans*	
114	*Plagioecia patina*	
115	*Tubulipora aperta*	
116	*Tubulipora liliacea*	
117	*Tubulipora lobifera*	
118	*Tubulipora penicillata*	
119	*Tubulipora phalangea*	
120	*Tubulipora plumosa*	
PHYLACTOLAEMATA		
121	*Cristatella mucedo*	
122	*Fredericella sultana*	
123	*Gelatinella toanensis*	
124	*Plumatella repens*	
125	*Plumatella fruticosa*	

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Nothing in biology makes sense except in the light of evolution.

(Dobzhansky, 1973)