Phylogeny of Echiura (Annelida, Polychaeta) inferred from morphological and molecular data-implications for character evolution

Dissertation
zur
Erlangung des Doktorgrades (Dr. rer. nat.)
der
Mathematisch-Naturwissenschaftlichen Fakultät
der
Rheinischen Friedrich-Wilhelms-Universität Bonn

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Bonn 2011
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Datum der Disputation: 31.05.2012
Erscheinungsjahr: 2012
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1 General Introduction

1.1 Characterization of Echiura

Echiura is a small taxon of 129 species (Stephen and Edmonds 1972) of soft bodied, sausage shaped, coelomate marine worms with a worldwide distribution. Most species are found in intertidal and shallow waters, but several species live at great depths ranging from 6000 to 10,000 meters (Zenkevitch 1966; Datta-Gupta 1981; Suer 1984; McKenzie and Hughes 1999). Echiurans are hemisessile and can be found in nearly all benthic habitats. Usually they live in burrows in sand and mud; others inhabit rock and coral crevices or live in debris amongst the roots of marine angiosperms (Stephen and Edmonds 1972; Edmonds 1987). Only a few species occur in estuarine brackish waters (Annandale and Kemp 1915).

Echiurans are generally characterized by an unsegmented sac-like trunk and a highly expandable anterior proboscis, which is regarded as a modified prostomium (Baltzer 1931; Korn 1982; Ax 1999; Ruppert et al. 2004; Purschke 2007). The trunk may be from a few cm up to 40 cm long, as observed in *Ikeda taenioides* (Ikeda, 1904). The proboscis is highly mobile and capable of great extension (up to 2 meters in some species), but contrary to the introvert in sipunculans, it cannot be retracted into the trunk (Fig. 1). Externally it is the most distinctive feature of the echiuran body and apomorphic for the group (Ax 1999; Ruppert et al. 2004; Purschke 2007). Primarily it is used to collect sediment from around the burrow as most species are burrowing deposit feeders (exception: filter feeding *Urechis* species, Fisher and MacGinitie 1928). Usually food particles, i.e. epibenthic detritus, are transported on the ciliated ventral surface (Fig. 1B-C). By ciliary action these particles are carried to the mouth located basally (e.g. Nyholm and Bornö 1969; Jaccarini and Schembri 1977a, b, 1979). The proboscis also acts to some extend as a sensory and respiratory organ (Baltzer 1931; Stephen and Edmonds 1972). The common name “spoon worms” is derived from the feeding function of the proboscis and its shape in some species.

In addition to the unique proboscis, echiurans have conspicuous excretory organs, so-called anal sacs (sensu Dawydoff 1959; Harris and Jaccarini 1981; anal vesicles sensu Newby 1940 or posterior nephridia sensu Goodrich 1945), which characterize Echiura as monophyletic taxon (Ax 1999; Harris and Jaccarini 1981). The anal sacs are connected to the hindgut (rectum or cloaca) and may attain nearly half of the length of the echiuran trunk (Pilger 1993). They are generally paired and open into the coelom via numerous ciliated funnels (up to 8500 in female *Bonella viridis* Rolando, 1821 as estimated by Harris and Jaccarini 1981). Anal sacs are assumed to serve their excretory function by
discharging waste products into the cloaca (e.g., Baltzer 1931; Bock 1942; Stephen and Edmonds 1972; Harris and Jaccarini 1981; Ruppert et al. 2004; Schmidt-Rhaesa 2007). In addition, they may function, at least to some extent, in gas exchange and osmoregulation (Brusca and Brusca 2003).

**Figure 1:** Proboscis of *Thalassemata thalassemum* (Thalassematidae). **A:** Specimen in vivo in a “typical” echiuran feeding posture, with the proboscis (pr) extended over the surface of the substratum (bottom of the aquarium). The trunk (tr) is usually hidden under rocks or shells. **B-C:** SEM micrographs showing the tip (B) and the ciliation of the ventral surface (C). Asterisk indicates ventral side.

A further characteristic feature is the arrangement of chaetae in the body wall of most echiurans, i.e. a pair of short hooked chaetae that occurs ventrally on the anterior part of the trunk on each side of the ventral midline just posterior to the mouth (Baltzer 1931). They are used for digging as the animal burrows (e.g. Ruppert et al. 2004). In addition to the anterior ventral chaetae, some echiurans have one or two circles of chaetae around the posterior extremity of the trunk (*Echiurus* and *Urechis* species) (Spengel 1880, 1912; Baltzer 1931). They are assumed to be used for burrow maintenance and anchorage (e.g. Bromley 1999).

While females and males are broadly indistinguishable externally in the majority of echiurans, members of the traditional subgroup Bonelliidae show a pronounced sexual dimorphism (e.g. Ruppert et al. 2004). A ciliated dwarf male, only a few millimeters long, lives usually within the gonoduct of the much larger female and fertilizes the eggs internally (e.g. Baltzer 1931; Edmonds 2000). As the dwarf male retains some larval and juvenile characters, but also contains gametes that mature into ripe cells, this is an example of progenesis in Echiura (Baltzer 1924 for *Bonellia viridis*). Most bonelliid species are also notable in their production of the green toxin bonellin (dermal porphyrin pigment), which probably has an antipredatory role (Baltzer 1924; Giudici 1984; Edmonds 2000; Brusca and Brusca 2003).
In the last decade, several studies substantiated the hypothesis that Echiura are an annelid subtaxon (McHugh 1997, 1999; Hessling and Westheide 2002; Hessling 2002, 2003; Bleidorn et al. 2003a, b; Rousset et al. 2007; Struck et al. 2007; Bourlat et al. 2008; Dunn et al. 2008; Hejnol et al. 2009; Zrzavy et al. 2009; Wu et al. 2009; Struck et al. 2011). Most of the molecular analyses support a sister group relationship with Capitellidae (e.g. Bleidorn et al. 2003a, 2003b, Rousset et al. 2007, Struck et al. 2007; Dunn et al. 2008; Hejnol et al. 2009; Struck et al. 2011), while other analyses yielded inconclusive support for a sister group relationship with Terebellidae (Colgan et al. 2006) or Pectinariidae (Rousset et al. 2007, “restricted” dataset). All these results imply that Echiura have secondarily lost characteristic features of annelids like trunk segmentation, parapodia, and a metamerical nervous system in adults (Purschke et al. 2000; Bleidorn 2007). Based on this hypothesis the sac-like trunk with a the secondary unsegmented coelom, the limitation of chaetae in the anterior and posterior section of the trunk (presence of ventral and anal chaetae) and the secondary loss of parapodia, nuchal organs and cirri on the pygidium are further apomorphies for Echiura (Ax 1999).

1.2 Phylogeny of Echiura

Traditionally, echiurans are classified after a widely used classification scheme given by Stephen and Edmonds (1972), which is basically that of Bock (1942) and Fisher (1946, 1949). Therein, echiurans are arranged into three traditional orders: i) the Xenopneusta Fisher, 1946, including Urechidae (Urechis species); ii) the Heteromyota Fisher, 1946, including Ikedaidae (Ikeda species), and iii) the Echiuroinea Bock, 1942, comprising the majority of echiuran species (Bonelliidae and Echiuridae). In the taxonomic revision by Nishikawa (2002), however, Heteromyota and Ikedaidae are abolished on the base of previous false information on the arrangement of the body wall musculature, leaving Xenopneusta and Echiuroinea as the only major subgroups within Echiura. According to Nishikawa (2002) the longitudinal musculature of the body wall lies between an outer layer of circular and an inner layer of oblique muscle in all echiurans. He integrated Ikeda species (former Heteromyota, Ikedaidae) into the Echiuroinea, i.e. Echiuridae, without giving arguments. In addition, no further allocation of Ikeda species within the Echiuridae is given, so that its systematic position remains unknown. The Echiuroinea including the traditional families Echiuridae and Bonelliidae is historically based on the presence of a closed vascular system and the absence of a so-called “water lung” (thin-walled, enlarged cloaca, Fisher and MacGinitie 1928; Stephen and Edmonds 1972; Menon and Arp 1992). The Xenopneusta including Urechidae were erected on the presence of a “water lung” as main organ of respiration and the absence of a closed vascular system. Further systematic divisions were made on characters such as the absence or presence of a marked sexual dimorphism (dwarf males), the shape of the proboscis, the number of gonoducts, the absence or presence of anterior ventral or posterior rings of chaetae and the shape of the anal sacs. Thus, species with a pronounced sexual
dimorphism, a proboscis either bifid or tongue-like (not bifid), usually one or two gonoducts, usually branching anal sacs, as well as two ventral chaetae but lacking anal chaetae were arranged into the Bonelliidae (Stephen and Edmonds 1972). In contrast species without sexual dimorphism, without a bifid proboscis but paired nephridia, unbranched anal sacs, two ventral chaetae and usually lacking anal chaetae (except in one genus) were classified as Echiuridae. The Echiuridae in turn were divided into the subfamilies Echiurinae and Thalassematinae on the basis of two rings of anal chaetae and a so-called post-pharyngeal diaphragm (conspicuous septum) in Echiurinae, whereas these characteristics are lacking in Thalassematinae (Stephen and Edmonds 1972).

The only attempt to contribute towards a phylogenetic system was made by Ruppert et al. (2004), who build a phylogeny generally around the hypothesized derived loss of segmental chaetae (Fig. 2). Therein, two sister group relationships are proposed that oppose to the traditional classification of Stephen and Edmonds (1972): monophyletic Thalassematidae (Thalassematinae sensu Stephen and Edmonds 1972) being sister to a clade comprising Bonelliidae and Ikedaidae (Ikedidae sensu Ruppert et al. 2004). This sister group relationship is exclusively based on a loss of anal chaetae in all three subgroups while the sister group relationship of Bonelliidae and Ikedaidae is based on the presence of very long proboscises and unpaired gonoducts. However, the phylogeny of Ruppert et al. (2004) does not provide any information about apomorphic characters for Echiuridae and Thalassematidae. Furthermore, interrelationships of Echiuridae (Echiurinae sensu Stephen and Edmonds 1972) and Urechidae remain unresolved. The assumed basal position of the latter two groups is due to presence of rings of anal chaetae which is a plesiomorphic character which had been developed within the stem species of all echiurans according to Ruppert et al. (2004) (Fig. 2).
Figure 2: Phylogenetic hypothesis on echiuran interrelationships proposed by Ruppert et al. (2004). Apomorphic characters for Echiuridae and Thalassematidae remain unknown. Terminology of characters according to Ruppert et al. (2004), plesiomorphies in brackets. 1, Echiura: [hemal system; outer circular, inner longitudinal muscle layers] elongate, flattened, deposit feeding prostomium; one pair of anterior ventral chaetae; two posterior (anal) rings of chaetae; prostomial and trunk coeloms; segmental metanephridia (gonoducts) with terminal funnels in anterior trunk; excretory funnels and anal sacs open into cloaca; intestine has a ventral siphon. 2, Urechidae: one ring of anal chaetae; reduced prostomium, glandular girdle on anterior trunk; hemal system lost; cloaca is a well developed “water lung”. 3, N.N.: anal rings of chaetae absent. 4, N.N.: very long prostomium; unpaired metanephridium (gonoduct). 5, Bonellidae: forked prostomium; stalked funnels on anal sacs; sexual dimorphism. 6, Ikedaidae: outer longitudinal, inner circular muscle layers, non-segmental multiplication of metanephridia (gonoducts).

1.3 Aims of the present study

Several questions remain unanswered by the classification scheme given in Stephen and Edmonds (1972) and by the phylogeny proposed by Ruppert et al. (2004). These refer basically to the validity of the traditional subgroups (especially Echiuridae and Thalassematidae) and the phylogenetic relationships among all five subgroups (Fig. 2). The attempt of Ruppert et al. (2004) to infer echiuran phylogeny broadly on the basis of one character, the derived absence of rings of anal chaetae, is problematic. It is based on the unconfirmed assumption that the rings of anal chaetae are directly homologous to annelid segmental chaetae, which is controversial because it lacks any supportive comparative studies. In addition, Ruppert et al. (2004) do not provide an explanation for their hypothesis and do not refer to a potential outgroup taxon so that their phylogeny would become more traceable. Furthermore, as there are presently no phylogenetic analyses on echiuran intrarelationships available, it cannot be decided unambiguously whether this negative character (Purschke et al. 2000), namely the absence of anal chaetae, is a primary (plesiomorphic) or a secondary (apomorphic) absence.

Generally, the impression arises that Echiura is a character-poor taxon. Although intensive literature research reveals that there is some detailed information on echiuran morphology available, especially from the older literature. Nevertheless, these cover only very few species with respect to the five echiuran subgroups (e.g. Greef 1879, Spengel 1879, Baltzer 1931, for Echiurus echiurus, Bonellia viridis; Ikeda 1904, 1907 for Ikeda taenioides; Bock 1942 for Maxmuelleria lankesteri; Dawydoff 1959, Stephen and Edmonds 1972 for shorter comprehensive overviews). Thus far, this information has never been compiled systematically, also with respect to comparability and an enlarged taxon sampling considering the current species validity. The latter problem becomes again obvious in Ruppert et al. (2004). The authors refer exclusively to Ikeda taenioides as a valid member of Ikedaidae although a second species Ikeda pirotansis (Menon and Datta-Gupta, 1962) was included by Nishikawa (2002) and was already accepted in the scientific community (compare http://www.marinespecies.org). In conclusion the hypothesis of Ruppert et al. (2004) generally lacks a careful consideration of characters with respect to available information from the literature, the
comprehensible polarization of characters via outgroup comparison and basically the implementation of a cladistic analysis to sustain their hypothesis. Thus the small data set referred to in Ruppert et al. (2004) appears not suitable for the inference of echiuran phylogeny.

However, some apomorphic characters have been postulated more or less congruently for certain subgroups (Bonelliidae, Urechidae, Ikedaidae; Stephen and Edmonds 1972; Ruppert et al. 2004), but these permit no conclusions about their interrelationships. In Thalassematidae and Echiuridae there is confusion regarding the absence or presence of apomorphies. Ruppert et al. (2004) did not recognize the presence of a postpharyngeal diaphragm in Echiuridae, contrary to Stephen and Edmonds (1972) who use this character to separate Echiuridae (Echiurinae) from Thalassematidae (Thalassematinae). Furthermore, Thalassematidae is exclusively based on absence characters (negative characters) according to Stephen and Edmonds (1972), which again are difficult to assess without a stable phylogeny. These negative characters hamper their correct interpretation and the decision whether these are primary (plesiomorphic) or secondary (apomorphic) absences in Thalassematidae.

So on the one hand, the difficulties in evaluating the relationships within Echiura are related to the need for a compilation of characters that cover the morphological diversity within all subgroups sufficiently, which is presently not the case, and on the other hand these difficulties are due to the lack of any phylogenetic analyses so far. Due to the persisting uncertainty which characters are comparable respectively homologous among the taxa and phylogenetically significant, morphology based analyses are missing to date. And due to a lack of published echiuran DNA sequences a molecular phylogeny could not be established. Thus, neither a reliable evaluation of the known morphological characters nor an independent test for the hypothesis of Ruppert et al. (2004) was feasible so far. The reason for the lack of sequence data is simultaneously a general problem in echiuran science: the majority of species is hard to obtain, either due to their hidden habitat or their rare occurrence.

The main objectives of the present study are i) assessing the phylogenetic relationships within Echiura on the basis of phylogenetic analyses, ii) testing the appropriateness of the current taxonomic classification schemes (Stephen and Edmond 1972) as well as the hypothesis of Ruppert et al. (2004) and iii) tracing the evolution of the main diagnostic traits (anal sacs, gonoducts, chaetae, probosces) used for taxonomic classification.

To achieve these goals, a molecular phylogeny based on a multigene dataset (MT-CO1 + 16S rRNA + 18S rDNA) comprising all traditional echiuran subgroups was established. New sequence data were analyzed together with sequence data deposited at GenBank (Tab. 1). The evolution of the morphological traits was traced over the molecular phylogeny using the maximum likelihood approach. In addition cladistic analyses were conducted on the basis of an enlarged morphological data matrix using the parsimony approach and a comparable taxon sampling. This was accomplished on the one hand by the comprehensive compilation of potentially phylogenetic informative data from the
literature, which were first critically evaluated and then extracted, on the other hand by the investigation of new morphological character complexes. These new morphological characters were chosen with regard to their potential to suit outgroup comparisons with annelid taxa and to get a broader database across Echiura. Newly studied characters are: the morphology of spermatozoa (chapter 3.1), the morphology of the anal sacs for which particular attention is being paid (chapter 3.2), the structure of the larval protonephridia (chapter 3.3) and the gonostomal lips (chapter 3.4). Except for the latter, all new character complexes are items of the publications on which this thesis is based aside from the unpublished data. In the same manner as done for the diagnostic traits character evolution of these newly investigated characters was traced back on the basis of the molecular phylogeny (chapter 4.6.4). Finally, in this context, questions of convergent evolution and characteristics of the echiuran stem species will be addressed.

The presented recapitulatory data matrix (Appendix 1) may serve as a starting point for future analyses to fill the gaps of our knowledge in echiuran morphology.
2 Material and Methods

For a complete list of first authors regarding all echiuran species referred to in this thesis see Appendix 4.

2.1 Molecular data

2.1.1 Taxon sampling

Sequence data for a total of 13 echiuran taxa comprising all traditional subgroups were drawn from specimens collected or obtained from GenBank (Tab. 1). Specimens for newly sequenced data are deposited in the collection of J. Lehrke (University of Bonn, Germany), except for *Anelassorhynchus porcellus*, which is held in the collection of M. Halt (University of Adelaide, Australia). Additional to the echiuran sequences, sequences of different representatives of the Terebelliformia were considered and serve as outgroups for the reconstruction of the phylogenetic relationships within Echiura, because most molecular studies support a sister-group relationship between terebelliformid taxa and echiurans (Bleidorn et al. 2003a, 2003b, Colgan et al. 2006, Rousset et al. 2007, Struck et al. 2007). Especially the Capitellidae yielded high support by several molecular studies being the potential sister to Echiura (Bleidorn et al. 2003a, 2003b, Rousset et al. 2007, Struck et al. 2007). Therefore and also because of the limited choice of capitellid taxa presently available, one capitellid taxon is included as outgroup, together with two available representatives of the Trichobranchidae and one representative of the Terebellidae. The tree obtained was rooted with the sequences of the errant polychaete *Eunice pennata* (Eunicidae).

Due to limited data availability and sequencing problems with certain genes for certain taxa, it was not possible to obtain an identical taxon sampling for the multigene analysis referring to each single gene (Tab. 1). For this reason so-called “composite taxa” have been used in two cases: the data for the capitellid outgroup taxon were pooled by assembling the available sequences of *Notomastus latericeus* (16S+18S rDNA) and *Dasybranchus* sp. DH1 (CO1). Within echiurans, *Anelassorhynchus* was adopted, which is made up of the newly sequenced species *Anelassorhynchus adelaidensis* (16S+CO1) and *Anelassorhynchus porcellus* (18S).
Table 1: List of echiuran species and polychaete outgroup taxa for which selected sequence data were drawn from specimens collected or were obtained from GenBank (GB) during this thesis. For reasons of clarity the designation of the traditional families follows Dawydoff (1959). Five traditional subgroups are recognized: the Bonelliidae, Echiuridae, Ikedaidae, Thalassematidae and Urechidae + sequences compiled by the author; - sequences presently not available. + a sequence data have been made available by P. Collins (Duke University Marine Laboratory, USA), + b sequence data have been made available by M. Halt (University of Adelaide, Australia).

<table>
<thead>
<tr>
<th>Higher taxon</th>
<th>Species</th>
<th>Source</th>
<th>Studied genes (with accession numbers)</th>
</tr>
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<td>Eunice pennata (O.F. Müller, 1776)</td>
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<td>ECHIURA</td>
<td>Bonelliidae</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Bonellia viridis</td>
<td>Sardinia, Italy (coll. T. Bartolomaeus)</td>
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<td></td>
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<td>Loch Sween, West Scotland (coll. D. Hughes)</td>
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<tr>
<td></td>
<td></td>
<td>Metabonellia haswelli</td>
<td>Edithburgh jetty, South Australia (coll. J. Lehrke)</td>
</tr>
<tr>
<td>Family</td>
<td>Species</td>
<td>Location</td>
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</tr>
<tr>
<td>----------------------</td>
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<td>---------------------------------------------------------------------------</td>
<td>------------------</td>
</tr>
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<td><strong>Protobonellidae</strong></td>
<td>Protobonellia sp. (undescribed species, pers. comm. R. Biseswar)</td>
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<td>Echiurus echiurus (provided by S. Bourlat)</td>
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<tr>
<td><strong>Thalassematidae</strong></td>
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<td>Lizard Island, near Bird Is., Lagoon channel, Queensland, Australia (coll. M. Halt)</td>
<td>-</td>
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<td></td>
<td>Anellassorhynchus adelaidensis</td>
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<td>Arhynchite pugettensis</td>
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<td></td>
<td>GB</td>
</tr>
<tr>
<td></td>
<td>Thalassema thalassenum</td>
<td>Concarneau (Le Cabellou), France (coll. C. Bleidorn)</td>
<td>+</td>
</tr>
<tr>
<td><strong>Ikedidae</strong></td>
<td>Ikeda sp. (undescribed species, pers. comm. G. Rouse)</td>
<td>Victoria, Australia (provided by G. Rouse, 08)</td>
<td>+</td>
</tr>
<tr>
<td><strong>Urechidae</strong></td>
<td>Urechis caupo</td>
<td>GB</td>
<td>NC006379</td>
</tr>
<tr>
<td></td>
<td>Urechis unicinctus</td>
<td>GB</td>
<td>NC012768</td>
</tr>
</tbody>
</table>
2.1.2 PCR amplification, purification and sequencing

Collected specimens were identified, preserved in 100% ethanol and stored at -20°C for later extraction. DNA extraction was performed using Qiagen DNeasy™ Tissue Kit (Qiagen GmbH, 40724 Hilden) according to the manufacturer’s instructions.

PCR amplification of a ~1800bp part of the 18S rRNA gene was performed in 2 overlapping fragments using primer pairs F19 + R993 and F439 + R1843 (Table 2). A ~500bp part of the mitochondrial 16S rRNA gene was amplified using the primer pair 16SarL and 16SbrH (Tab. 2). A ~700bp part of the mitochondrial CO1 gene was amplified using the primer pair LCO1490 and HCO2198 (Tab. 2). The PCR amplifications were carried out in 50 μl reaction volumes, comprising 1 μl dNTP mix (10 mM; Eppendorf, Hamburg, Germany), 0.25 μl Taq DNA polymerase (5 U/μl; 5Prime, Hamburg, Germany), 5 μl 10x Taq buffer advanced (5Prime), 1 μl primer mix (10 μM each,
Metabion), 1 μl DNA template and 41.75 μl sterile distilled water (Eppendorf). All amplifications were carried out on an Eppendorf Mastercycler and Eppendorf Mastercycler gradient. The PCR temperature reaction for the 18S was 94°C for 2 min (initial denaturation); 40 cycles with 94°C for 30 seconds (denaturation), 50°C for 30 seconds (annealing), and 68°C for 1 min 30 seconds (elongation). For the 16S the following file has been used: 94°C for 3 min; 35 cycles with 94°C for 45 seconds, 50°C for 1 min, and 72°C for 1 min; final extension at 72°C for 7 min. The PCR temperature reaction for the CO1 was 94°C for 1 min 30 seconds (initial denaturation); 94°C for 45 seconds (denaturation), 45°C for 45 seconds (annealing), and 68°C for 3 min (elongation).

The quality of PCR products was validated by electrophoresis in 1% TBE ethidium bromide stained agarose gel. PCR products were purified with the NucleoSpin Extract II (Machery-Nagel, Dueren, Germany) kit, as well as the Bluematrix DNA purification kit (EURx, Gdansk, Poland) with comparable results, and finally stored at -20°C.

Some of the PCR products were sequenced in our laboratory on a CEQ 8000 capillary sequencer (Beckman Coulter, software version: 5.0.360, instrument version: 6.0.2), but the great majority of PCR products was outsourced to AGOWA GmbH (Berlin, Germany), which is using a 3730xl DNA Analyzer (ABI). The setup of the CEQ 8000 capillary sequencer as well as the initial cycle sequencing step were executed according to the manufacturer. The CEQ DCTS Quick Start Kit (Beckman Coulter, Krefeld, Germany) was used to set up a single 10 μl reaction volume (1-5 μl of purified PCR product, 4 μl DCTS master mix (Beckman Coulter), and 1 μl primer, 10 mM, 0-4 μl water). Primers and thermocyclers were the same as those used for PCR amplifications, except for the 18S, where primer R1843 was substituted by R1825. Primary sequence analysis was performed with the CEQ software (quality check). If necessary, sequencing reactions were repeated until every part of the sequence was represented by at least two sequences to track down sequencing errors.
Table 2: Amplification and sequence primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence (5´- 3´)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SarL</td>
<td>Forward</td>
<td>CGCCTGTTTAACAAAAACAT</td>
<td>Palumbi 1996</td>
</tr>
<tr>
<td>16SbrH</td>
<td>Reverse</td>
<td>CCGGTCTGAACTCAGATCACGT</td>
<td>Palumbi 1996</td>
</tr>
<tr>
<td>LCO1490</td>
<td>Forward</td>
<td>GGTCAAACAAATCATATAAGATATTGG</td>
<td>Folmer et al. 1994</td>
</tr>
<tr>
<td>HCO2198</td>
<td>Reverse</td>
<td>TAAACTTCAAGGTCAGACCAAAAAATCA</td>
<td>Folmer et al. 1994</td>
</tr>
<tr>
<td>18S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F19</td>
<td>Forward</td>
<td>ACCTGGTTGATCTTGGCAA</td>
<td>Turbeville et al. 1994</td>
</tr>
<tr>
<td>R993</td>
<td>Reverse</td>
<td>CTTGGCAATGCTTTCGC</td>
<td>Giribet et al. 1996</td>
</tr>
<tr>
<td>F439</td>
<td>Forward</td>
<td>GTTCGATTCGGGAGAGGA</td>
<td>Giribet et al. 1996</td>
</tr>
<tr>
<td>R1825</td>
<td>Reverse</td>
<td>CGGAAACCTTGGTACGAC</td>
<td>Bleidorn 2005</td>
</tr>
<tr>
<td>R1843</td>
<td>Reverse</td>
<td>GGATCCAAGCTTGATCTTCTGCAGG</td>
<td>Elwood et al. 1985</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTC ACCTAC</td>
<td></td>
</tr>
</tbody>
</table>

2.1.3 Phylogenetic Analysis

Protein coding and rRNA genes were identified by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence assembly was done with Bioedit 7.0.1 (Hall 1999). Prior to phylogenetic analysis sequences were aligned with CLUSTAL W (Thompson et al. 1994) as implemented in Bioedit 7.0.1 using default parameters. Two different alignments were used for phylogenetic analysis in order to test the stability of the resulting topologies: (1) An alignment including 100% of the original 3529 positions, i.e. also ambiguously aligned regions were incorporated; (2) An alignment including 76% of the original positions, i.e. poorly aligned positions were excluded from the alignment using Gblocks (version 0.91b) (Castresana 2000). The resulting number of positions analyzed was 2696. The following settings were used for Gblocks: Minimum number of sequences for a conserved position: 9; minimum number of sequences for a flanking position: 9; maximum number of contiguous non-conserved positions: 8; minimum length of a block: 10; allowed gap positions: all.

Maximum likelihood (ML) analyses were done using RAxML version 7.2.8 (Stamatakis 2006) and Treefinder (version of October 2008) (Jobb, von Haeseler, and Strimmer 2004). The analysis was adapted to the given dataset by performing a multigene analysis inferred from 18S rDNA, cytochrome c oxidase I (MT-CO1) and 16S rRNA sequences. Nucleotide substitution was displayed by the GTR +
I+ Γ model and 9 categories of gamma distributed rates across sites; bootstrap support values were determined from 1000 replicates.

2.2 Morphological data

2.2.1 Studied species

A list of studied echiuran species with information on the analyzed character complexes and the methods applied is given in Table 3. Due to reasons of clarity throughout the thesis regarding the naming of the high ranking traditional subgroups, the author follows the simple designation of Dawydoff (1959), which was predominantly adopted by Ruppert et al. (2004). Therein, Echiura simply include five coequal high ranked taxa or subgroups: the Bonelliidae (for members compare Stephen and Edmonds 1972 and “WoRMS, World Register of Marine Species” http://www.marinespecies.org), the Echiuridae (Echiurus species), Ikedaidae (Ikedidae sensu Ruppert et al. 2004, Ikeda species), Thalassematidae (Arhynchite, Anelassorhynchus, Ikedosoma, Listriolobus, Lissomyema, Ochetostoma, and Thalassema) and Urechidae (Urechis species). Anelassorhynchus and Lissomyema were added from the traditional Thalassematinae sensu Stephen and Edmonds (1972). Prashadus (P. pirotansis was excluded from subgroup Thalassematidae, because it was recently assigned to the genus Ikeda (as Ikeda pirotansis see Menon and Datta-Gupta, 1962).

Originally, Dawydoff (1959) and others (e.g. Bock 1942; Fisher 1946) included monotypic Saccosomidae into Echiura, which is based on a single incomplete specimen of Sactosoma vitreum Danielsen and Koren, 1881. Since it also lacks any echiuran apomorphic character, it is regarded as species incertae sedis by Stephen and Edmonds (1972) and is excluded from Echiura in the current classification given by Nishikawa (2002). Thus, it is not recognized in this thesis.

Table 3: List of studied echiuran species with information on the analyzed character complexes and the methods applied in this thesis. For reasons of clarity the designation of the traditional families follows Dawydoff (1959). Five traditional subgroups are recognized: the Bonelliidae, Echiuridae, Ikedaidae, Thalassematidae and Urechidae. Within the scope of this thesis morphological data were collected within all high ranking subgroups, except for the Ikedaidae + character studied by the author; - character not studied by the author. AS Anal sacs, CH chaetae, GO gonoducts, HK head kidneys, SP spermatozoa. cLSM confocal laser scanning microscope, LM light microscopy, histology, SEM scanning electron microscopy, TEM transmission electron microscopy. AWI Alfred-Wegener Institut für Polare Meeresforschung.

<table>
<thead>
<tr>
<th>Higher taxon</th>
<th>Species</th>
<th>Source/Locality</th>
<th>Studied character complexes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AS</td>
</tr>
<tr>
<td>Bonelliidae</td>
<td><em>Bonellia viridis</em></td>
<td>Banyuls sur Mer, France (coll. K. Warnke, 03.06)</td>
<td>SEM</td>
</tr>
<tr>
<td></td>
<td><em>Metabonellia haswelli</em></td>
<td>Edithburgh jetty, South Australia (coll. J. Lehrke, 04.06)</td>
<td>LM, SEM</td>
</tr>
<tr>
<td>Echiuridae</td>
<td><em>Echiurus echiurus</em></td>
<td>Dogger Bank, North Sea, Germany (AWI Helgoland)</td>
<td>-</td>
</tr>
<tr>
<td>Thalassematidae</td>
<td><em>Anelassorhynchus adelaidensis</em></td>
<td>Edithburgh jetty, South Australia (coll. J. Lehrke, 04.06)</td>
<td>LM, SEM</td>
</tr>
<tr>
<td></td>
<td><em>Thalassema thalassemum</em></td>
<td>Le Cabellou, France (coll. J. Lehrke)</td>
<td>cLSM, LM, SEM, TEM</td>
</tr>
<tr>
<td></td>
<td><em>Lissomyema mellita</em></td>
<td>Tampa, Florida, USA (coll. N. Holland)</td>
<td>SEM</td>
</tr>
<tr>
<td>Urechidae</td>
<td><em>Urechis caupo</em></td>
<td>California, USA (coll. G. Rouse, 04.07)</td>
<td>LM, SEM</td>
</tr>
<tr>
<td></td>
<td><em>Urechis unicinctus</em></td>
<td>Suncheon station market, Cheolla Namdo, Republic Korea (Coll. M. Suh, 05.08)</td>
<td>SEM</td>
</tr>
</tbody>
</table>
2.2.2 Rearing of larvae

Reproductive adults of *Thalassema thalassemum* (Thalassematidae) were collected in April and May 2008 in Le Cabellou, Concarneau, France. Animals were taken from rock crevices in the mid-intertidal zone. In the laboratory, adults were kept in small aquaria at the Freie Universität Berlin laboratory with running artificial seawater (13-15°C) until isolation of gametes. Gametes where obtained by dissecting the gonducts of live specimens which contain ripe ova or sperm respectively. Ova where isolated from the gonducts into glass bowls with cooled filtered seawater from Concarneau (9°C; 0.2 µm). Prior to artificial fertilization, the activity of spermatozoa was checked under the light microscope. After 40 minutes, when the ova had rounded up, they were artificially fertilized by adding a few drops of a sperm suspension. The sperm suspension applied comprised diluted filtered seawater and motile spermatozoa in an adequate concentration (slightly cloudy). After 26 h the fertilized eggs were transferred into a large 2 l - beaker containing 1.5 l filtered seawater (20 µm) and a cover of aluminium foil to protect the larvae from dust particles. The water was slowly stirred with a rotating acrylic glass paddle connected to a motor (3V) (Fig. 4). Water was changed three times a week and the water temperature ranged from 15°C to 18°C during the study period. Larvae were fed the alga *Dunaliella* sp. (Chlorophyceae, Chlorophyta) and *Isocrysis galbana* (Prymnesiophyceae, Haptophyta).
2.2.3 Histology and light microscopy

For histological studies the anal sacs of *T. thalassemum* (Thalassematidae) were isolated from the dissected, relaxed specimen (90 minutes, 7% MgCl₂) and were fixed with Bouin’s fluid at room temperature for 24 h. Anal sacs of *Metabonellia haswelli* (Bonelliidae), *Anelassorhynchus adelaidensis* (Thalassematidae) and *Urechis caupo* (Urechidae) were first fixed with 7-10% formalin at room temperature for 24 h. Prior to fixation, complete specimens of *A. adelaidensis, M. haswelli* and *U. caupo* were relaxed for 30 minutes in a 7% MgCl₂ solution at +4°C. After fixation, these specimens were then rinsed in double distilled water for 1 h, transferred into 70 % ethanol (EtOH) for 30 minutes and subsequently transferred into Bouin’s solution overnight. Bouin’s fluid was washed out with 70 % EtOH. All isolated sacs were then dehydrated in a graded ethanol series, transferred in butanol and embedded in paraplast. Sections of 5 µm thickness were stained according to the Azan method. The sections were examined with an OLYMPUS BX61 light microscope equipped with colour digital camera (Colour view, SIS) for documentation purposes. The same microscope and camera was used to test the motility of the spermatozoa of *T. thalassemum* and to document the different larval stages of *T. thalassemum* in vivo.

The gonoducts of *T. thalassemum* and the chaetae of *T. thalassemum* as well as *E. echiurus* were fixed with Bouin’s fluid and have been treated in the same way as the anal sacs. All images and plates were edited with Adobe Photoshop CS and Adobe Illustrator CS software.

2.2.4 Transmission electron microscopy (TEM)

For ultrastructural studies, mature spermatozoa of *T. thalassemum* (Thalassematidae) and *E. echiurus* (Echiuridae) were fixed, embedded and sectioned as described in Lehrke and Bartolomaeus (2009).

For ultrastructural studies of the anal sacs of *T. thalassemum* (Thalassematidae), entire specimens were first relaxed for 90 minutes in a 7% MgCl₂ solution and subsequently fixed with 1.25% glutaraldehyde buffered in 0.05 M sodium phosphate (0.3 M NaCl, pH 7.2) with traces of ruthenium red at room temperature for 2 h. Anal sacs were then isolated and fixed again for additional 2 h at room temperature. Details of further treatment can be seen in Lehrke and Bartolomaeus (2011).

98-h old larvae of *T. thalassemum* (Thalassematidae) were fixed with 1.25% glutaraldehyde buffered in filtered 0.05 M sodium phosphate (0.3 M NaCl, 0.2 µm, pH 7.2, 4°C) with traces of ruthenium red for 1 h. They were washed in the same buffer and postfixed in 1% OsO₄ buffered in 0.05M phosphate
with 0.3M sodium-chloride (4°C) for 1 h. Details of further treatment can be seen in Kato et al. (2011).

2.2.5 Scanning electron microscopy (SEM)

All material studied was dehydrated in a graded ethanol or acetone series with slight variations referring to the different specimens (compare following paragraphs). Subsequently, all specimens were dried with the critical point drying method (CPD 030, Bal-Tec), except for the spermatozoa (see next paragraph). Samples were then mounted on stubs and sputtered with gold (SCD 040, Balzers Union), except for the larvae of T. thalassemum (see last paragraph). After all, samples were examined with a Quanta 200 Scanning Electron Microscope (Fei), and the resulting images and plates were edited with Adobe Photoshop CS and Adobe Illustrator CS software. Fixation of material and further protocols of treatment are described for each single character complex in the following paragraphs:

The spermatozoa of T. thalassemum (Thalassematidae) were treated in the same manner as the sectioned spermatozoa (TEM), but instead of embedding them in araldite, they were resuspended in 100% ethanol, mounted and dried under normal conditions (no critical point drying device was used) (see also Lehrke and Bartolomaeus, 2009).

For scanning electron microscope studies of the anal sacs the following species were examined: Bonellia viridis (Bonelliidae), Metabonellia haswelli (Bonelliidae), T. thalassemum (Thalassematidae), Anellassorhynchus adelaidensis (Thalassematidae), Lissomyema mellita (Thalassematidae), Urechis caupo (Urechidae) and Urechis unicinctus (Urechidae). For the studies of the anal sacs of T. thalassemum, entire specimens were first relaxed for 90 minutes in a 7% MgCl₂ solution and subsequently fixed with 1.25% glutaraldehyde buffered in 0.05 M sodium phosphate (0.3 M NaCl, pH 7.2) with traces of ruthenium red at room temperature. Anal sacs were then isolated within the fixative, so that the entire fixation process persists 2 h. Afterwards they were washed three times (after 10 minutes, 30 minutes and 60 minutes) in the same buffer, and were then postfixed in 1% OsO₄ buffered in 0.05M phosphate with 0.3M sodium-chloride (4°C) for 60 min. Dehydration in an graded acetone series followed (starting with 30 % acetone at 4°C). The anal sacs of the remaining studied specimens were fixed in 7-10% formalin, except for L. mellita which was fixed in 100% EtOH. Prior to fixation, complete specimens of A. adelaidensis, M. haswelli and U. caupo were relaxed for 30 minutes in a 7% MgCl₂ solution at + 4°C. U. unicinctus was not relaxed prior to fixation. After fixation, the anal sacs of all formalin-specimens were rinsed in a graded ethanol series.
starting with 70 % EtOH. At the 70 % EtOH step 1.2 % phosphotungstic acid (PWS) was added. Any further treatment of the anal sacs follows the protocol used for the anal sacs of *T. thalassemum*.

For scanning electron microscope studies of the gonoducts the following species were examined: *T. thalassemum* (Thalassematidae), *A. adelaidensis* (Thalassematidae), *U. caupo* (Urechidae) and *U. unicinctus* (Urechidae). Investigated specimens were partly fixed in different fixatives. Entire specimens of *T. thalassemum* were fixed in 1.25% glutaraldehyde buffered in 0.05 M sodium phosphate (0.3 M NaCl, pH 7.2) at room temperature. Gonoducts were then isolated within the fixative, so that the entire fixation process persists 2 h. A specimen of *A. adelaidensis* was fixed in 10% formalin, and *U. caupo* was fixed in 7 % formalin. After fixation, the gonoducts of the formalin-specimens were rinsed for 1 h in double distilled water and afterwards they were rinsed in a graded ethanol series starting with 20 % EtOH. At the 70 % EtOH step 1.2 % phosphotungstic acid (PWS) was added. *U. unicinctus* was fixed in 70 % EtOH and passes subsequently a graded ethanol series starting with 70 % EtOH.

The protocol for studying the anterior ventral chaetae of *T. thalassemum* (Thalassematidae) with a scanning electron microscope follows the protocol applied for the anal sacs of *T. thalassemum*.

The 98-122-h-old larvae of *T. thalassemum* (Thalassematidae) were fixed and treated in the same manner as the sectioned larvae (TEM), but deviating from the protocol they were postfixes for 20 minutes in 1% OsO₄ buffered in 0.05M phosphate with 0.3M sodium-chloride (4° C). Fixation was stopped by adding 30% acetone (4°C), and the specimens were accordingly held for 15 minutes at room temperature. Subsequently they were dehydrated in an acetone series (30%, 50%, 70%, 80%, 90%, 95%). Larvae were further dehydrated in two changes of 100% ethanol and one additional change of 100% ethanol (p.A.). Then they were critical point dried and mounting on stubs, they were sputtered with a gold-palladium mixture in a Voltage Cool Sputter Coater (EMITECH K 550).

### 2.2.6 Confocal laserscan microscopy (cLSM)

Anal sacs of *T. thalassemum* were fixed for 30 min at room temperature in 4% paraformaldehyde and filtered sea water. Afterwards, anal sacs were transferred in 0.01 M PB in Na-cacodylate (NaN₃). Following fixation, samples were permeabilized in four 15 min changes of 0.01 M PB + NaN₃/0.25 % BSA/1% Triton (Triton buffer). For confocal microscopy anal sacs were stained with 5 µl Phalloidin conjugated with Alexa Flour® 568 (1 U/1 µl DMSO) (Molecular Probes, Cat. No. A12380) in 100 µl Triton buffer for 1 h at room temperature. Staining was stopped by rinsing samples three times in fresh
Triton buffer (three 10min changes). Clearing of samples was done in three 5min changes of glycerol. Details of further treatment can be seen in Lehrke and Bartolomaeus (2011). To examine their internal morphology, the anal sacs were mounted on hollow ground microscope slides and examined in a Leica confocal laser scanning microscope (TCS SPE) using the 532-nm excitation laser line. Resulting stacks were projected with ImageJ (ImageJ 1.38w).

2.2.7 Cladistic analysis of morphological data

Phylogenetic analyses were based on a data set that includes potentially informative characters selected from the studied character complexes (Table 3, chapters 4.1-4.4), as well as characters compiled from the literature (Table 4; chapter 4.5). A survey of all species first considered for cladistic analysis is given in Appendix 1. Since this first dataset includes more than 50% uncertain states, the cladistic analysis did not succeed regarding a higher resolution among echiurans. Nevertheless, it is presented here for reasons of clarity and in order to visualize the knowledge gaps. Finally, the morphological data set comprises 47 characters (all unordered) and 15 echiuran terminal taxa that can be assigned to all high-ranking traditional sub-taxa (Appendix 2).

Compared to the taxon sampling of the molecular analysis a slightly different sampling was applied for the morphological data set (compare Table 1 and Table 3). On the hand this is due to the difficulties to obtain the relevant specimens. On the other hand this was also due to preliminary results from the cladistic analyses indicating an unresolved morphological tree in case the same species from the molecular analysis were included into the morphological analysis.

Deviating additional taxa within the morphological taxon sampling are: Pseudoikedella achaeta and Hamingia arctica (both Bonelliidae), Ikeda pirotansis (Ikedaidae) as well as Ochetostoma caudex (presumably synonym to Ochetostoma erythrogrammon used in the molecular analysis (Stephen and Edmonds 1972), Listriolobus pelodes, Ikedosoma gogoshimense (all Thalassematidae). These taxa were also sorted with the intention to better cover the variety of morphological characters among Echiura.

Capitella teleta Blake, Grassle and Eckelbarger 2009 (Annelida, Capitellidae) was chosen as outgroup taxon, because the Capitellidae yielded high support by several molecular studies being the potential sister to Echiura (Bleidorn et al. 2003a, 2003b, Rousset et al. 2007, Struck et al. 2007). Data for the outgroup taxon was compiled from Franzén (1982), Eckelbarger and Grassle (1987) and Kato et al. (2011).
Phylogenetic analyses were computed with TNT (Goloboff et al. 2003) using both equally weighted characters and implied weights (Goloboff 1993) under variable weighting strengths (concavity function k=1-6). All Maximum Parsimony (MP) analyses were performed with exact search (implicit enumeration). Optimizations of characters and character evolution were analyzed using the software WinClada 10.00.08 (© Nixon 2002).

Table 4: Complete list of echiuran species (in alphabetical order) for which literature data were included into the character matrices (Appendix 1, 2). Species from the molecular analysis are also considered with corresponding references. Data for the outgroup taxon Capitella teleta (Capitellidae, Annelida) compiled from Franzén (1982), Eckelbarger and Grassle (1987) and Kato et al. (2011).

<table>
<thead>
<tr>
<th>Species</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthobonellia miyajimai (Ikeda, 1904)</td>
<td>Menon et al. (1964)</td>
</tr>
<tr>
<td>Acanthobonellia pirotanensis José, 1964</td>
<td>Menon et al. (1964); José (1964)</td>
</tr>
<tr>
<td>Acanthobonellia rollandae Menon, Datta-Gupta and Johnson, 1964</td>
<td>Menon et al. (1964); Stephen and Edmonds (1972), Fig. 43G(</td>
</tr>
<tr>
<td>Alomasoma belyaevi Zenkevitch, 1964</td>
<td>Saiz-Salinas (1996); Biseswar (2010); Biseswar pers. comm.</td>
</tr>
<tr>
<td>Alomasoma nordpacificum Zenkevitch, 1958</td>
<td>Stephen and Edmonds (1972), Fig. 44D-F; Biseswar (2010)</td>
</tr>
<tr>
<td>Amalosoma eddystonense Stephen, 1956</td>
<td>Stephen (1956)</td>
</tr>
<tr>
<td>Amalosoma paradolum (Fisher, 1946)</td>
<td>Fisher (1946), Pl. 31, Fig. 6; Stephen and Edmonds (1972)</td>
</tr>
<tr>
<td>Anelassorhynchus adelaidensis Edmonds, 1960</td>
<td>Edmonds (1987); this study</td>
</tr>
<tr>
<td>Anelassorhynchus branchiorhynchus (Annandale and Kemp, 1915)</td>
<td>Stephen and Edmonds (1972)</td>
</tr>
<tr>
<td>Anelassorhynchus dendrorhynchus (Annandale and Kemp, 1915)</td>
<td>Stephen and Edmonds (1972)</td>
</tr>
<tr>
<td>Anelassorhynchus microrhynchus (Prashad, 1919)</td>
<td>Stephen and Edmonds (1972)</td>
</tr>
<tr>
<td>Anelassorhynchus mucosus (Ikeda, 1904)</td>
<td>Stephen and Edmonds (1972)</td>
</tr>
<tr>
<td>Arhynchite arhynchite (Ikeda, 1924)</td>
<td>Stephen and Edmonds (1972)</td>
</tr>
<tr>
<td>Arhynchite californicus Fisher, 1949</td>
<td>Stephen and Edmonds (1972)</td>
</tr>
<tr>
<td>Arhynchite hiscocki Edmonds, 1960</td>
<td>Edmonds 1987</td>
</tr>
<tr>
<td>Arhynchite inamoenus Fisher, 1946</td>
<td>Stephen and Edmonds (1972)</td>
</tr>
<tr>
<td>Arhynchite pugettensis Fisher, 1949</td>
<td>Stephen and Edmonds (1972)</td>
</tr>
</tbody>
</table>
Bengalus longiductus Biseswar, 2006
Bonellia viridis Rolando, 1821
Bonelliopsis alaskana Fisher, 1946
Bruunellia bandae Zenkevitch, 1966
Charcotus charcotus Datta-Gupta, 1981
Choanostomellia bruuni
Echiurus echiurus (Pallas, 1767)
Eubonellia valida Fisher, 1946
Hamingia arctica Danielssen and Koren, 1881
Ikeda pirotansis (Menon and Datta-Gupta, 1962)
Ikeda taenioides (Ikeda, 1904)
Ikeda sp.
Ikedella bogorovi Zenkevitch, 1964
Ikedella misakiensis (Ikeda, 1904)
Ikedosoma gogoshimense (Ikeda, 1904)
Jakobia densopapillata Biseswar, 2006
Listriolobus pelodes Fisher, 1946
Lissomyema mellita (Conn, 1886)
Maxmuelleria lankesteri (Herdmann, 1898)
Metabonellia haswelli (Johnston and Tieg, 1920)
Ochetostoma australiense Edmonds, 1960
Ochetostoma baronii (Greef, 1879)
Ochetostoma bombayense (Prashad and Awati, 1929)
Ochetostoma capense Jones and Stephen, 1955
Ochetostoma caudex (Lampert, 1883)
Ochetostoma erythrogrammon Leuckart and Rüppel, 1828
<table>
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<th>Scientific Name</th>
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<td><em>Ochetostoma hornelli</em> (Prashad, 1921)</td>
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<td><em>Ochetostoma septemyotum</em> Datta-Gupta, Menon and Johnson, 1963</td>
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<td><em>Thalassema fuscum</em> Ikeda 1904</td>
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3 Results

3.1 Spermatozoa

In order to reveal phylogenetically significant data, the ultrastructure of the spermatozoa in *T. thalassemum* (Thalassematidae) and *E. echiurus* (Echiuridae) was investigated by means of SEM and TEM.

3.1.1 *Thalassema thalassemum* (Thalassematidae)

Spermatozoa are approximately 54 μm long and consist of three externally distinguishable parts: a head with a long conical acrosome and a spherical nucleus, a midpiece with a variable number of mitochondria and a tail (Fig. 5A-B). The tail is more than five times longer than the head-midpiece-complex, which measures 3.6 ± 0.41 μm (n=8) in length. The acrosomal complex is tapering towards the anterior tip of the spermatozoon. The acrosomal vesicle is differentiated into a thin apex (50 nm wide) filled with electron-grey material, and an electron-dense stained basal ring component with lateral electron-grey margins (Fig. 5C). This basal ring component faces the nucleus and measures 1037 ± 93.2 nm (n=8) at its broadest diameter. The subacrosomal space contains non-homogenous, electron-grey material. The subacrosomal space measures 1.9 ± 0.35 μm (n=7) in longitudinal extension and 130 ± 19.8 nm (n=7) in diameter on a level of the basal margin of the acrosome. At the apex of the acrosome the subacrosomal space measures 455 ± 62.6 nm (n=7) in diameter. The subacrosomal space is not membrane bound (Fig. 5B-C). The nucleus is approximately circular in cross section and the chromatin is homogeneously electron-dense. A nuclear membrane surrounds the entire nucleus. In the midpiece, the mitochondria posteriorly surround the centrioles at the base of the ciliary axoneme and anteriorly extend to one third of the nucleus, so that the nucleus is basally encircled by a variable number (1-4) of mitochondria with well developed cristae (Fig. 5A-B). Electron-dense granules resembling glycogen are located in ridges between the mitochondria. The proximal centriole is perpendicular to the distal centriole that represents the basal body of the ciliary axoneme of the tail shaft (Fig. 5A-B). Associated with the distal centriole are some satellite structures which seem to anchor the ciliary axoneme to the plasma membrane covering the mid-piece (compare Lehrke and Bartolomaeus 2009). The axoneme shows a 9 x 2 + 2 microtubular pattern and measures up to 48 μm in length. The tail is provided with lateral fin-like extensions of the plasma membrane that can reach a maximum of approximately 440 nm in length. These extensions dispose an angle of approximately 90°.
to an analogical longitudinal axis through the cross section of the flagellum (compare Lehrke and Bartolomaeus 2009).

Figure 5: Thalassema thalassum (Thalassematidae), mature spermatozoon. A: Schematic 3D-view. B: Reconstruction (longitudinal section) based on a series of complete ultrathin sections. C: Details of the acrosome (acrosomal vesicle + subacrosomal material), longitudinal section (TEM). Basal ring component encloses approximately two-thirds of subacrosomal space filled with subacrosomal material. The subacrosomal space is not membrane bound. Arrow indicates acrosomal membrane. av acrosomal vesicle, bb basal body, br basal ring component, ci cilium, mt mitochondrion, n nucleus, pc proximal centriole, sm subacrosomal material, ss subacrosomal space, v vesicle (modified from Lehrke and Bartolomaeus 2009).

3.1.2 Echiurus echiurus (Echiuridae)

Without the tail, the spermatozoon is approximately 2.3 ± 0.19µm (n=6) long and consists of three parts (Fig. 6A-B): a head with a complanate conical acrosome (Fig. 6A-C) and a spherical nucleus, a midpiece with a c-shaped mitochondrion encircling the basal body (Fig. 6A) and a tail. The acrosomal vesicle is more or less saucer-shaped. It is differentiated into a thin apex composed of two adjacent vesicle membranes bordering a little electron-bright space, and the basal ring component (Fig. 6C). Electron-grey material completely surrounds this component (Fig. 6C). The basal ring component faces the nucleus and measures 1.4 ± 0.42 µm (n=6) at its broadest diameter. The membrane bound subacrosomal space (subacrosomal vesicle) contains flocculent material. This vesicle measures 371.0 ± 67.0 nm (n= 6) in length and 503 ± 46.1 nm (n= 6) in diameter. The nucleus has a diameter of approximately 1.4 ± 0.32 µm (n=5) and consists of highly condensed material. The proximal centriole is perpendicular to the distal centriole (basal body) (Fig. 6A-B). The axoneme shows a 9 x 2 + 2
microtubular pattern. Its length could not be measured. The tail are provided with small lateral fin-like extensions of the plasma membrane, which dispose an angle of approximately 90° to an analogical longitudinal axis through the cross section of the flagellum (compare Lehrke and Bartolomaeus 2009).

Figure 6: Echiurus echiurus (Echiuridae), mature spermatozoon. A: Schematic 3D-view. B: Reconstruction (longitudinal section) based on a series of complete ultrathin sections. C: Details of the acrosome (acrosomal vesicle + subacrosomal material), longitudinal section (TEM). The conical acrosome is complanate. The subacrosomal space is filled with granular subacrosomal material encircled by a very electron-dense basal ring component. Note the subacrosomal space is membrane bound (arrowhead). Arrow indicates acrosomal membrane. av acrosomal vesicle, bb basal body, br basal ring component, ci cilium, mt mitochondrion, n nucleus, pc proximal centriole, sm subacrosomal material, ss subacrosomal space, v vesicle (modified from Lehrke and Bartolomaeus 2009).
3.2 Anal sacs

3.2.1 *Thalassema thalassemum* (Thalassematidae)

In order to identify and characterize anal sacs substructures in Echiura, the ultrastructure of the anal sacs in *T. thalassemum* was investigated by SEM, TEM, cLSM and light microscopy (azane staining). By comparing the anal sac morphology with the morphology of the hindgut in *T. thalassemum*, the next chapter (and the corresponding discussion chapters) is also intended to better understand the origin of the anal sacs.

General

All examined specimens of *T. thalassemum* have one pair of anal sacs that branch off from the cloaca, the posterior part of the hindgut, and extend into the trunk coelom (Fig. 7A). Each anal sac consists of a tubular end sac that is uniformly covered with numerous small ciliated funnels over the entire length of the organ (Fig. 7A; Fig. 9A). Since all funnels lack a stalk they will be termed “sessile ciliated funnels” (Fig. 7B). Generally, the end sac is significantly larger in diameter than the funnel at its broadest diameter. A circular sphincter muscle marks the transition between end sac and hindgut (Fig. 8A). Podocytes that line the ring vessel functionally interact with the anal sac, indicating that both podocytes and anal sac represent the metanephridial system in *T. thalassemum*.

In the following a detailed description of the hindgut morphology and the anal sac substructures, the end sac and the ciliated funnels, is given.
Hindgut

The hindgut lumen is lined by a simple epithelium consisting of intensely ciliated columnar cells (Fig. 8B). The cells are underlain by an extracellular matrix (ecm) measuring approximately 30 µm in thickness. Muscle fiber cells inside the ecm form a muscle grid that surrounds the gut. This grid consists of strong outer bundles of longitudinal muscle fibers and some single diagonal fibers (Fig. 8A). Circular muscle fibers form the inner part of the muscle grid. The circular fibers appear thinner and less frequent, especially compared to the outer longitudinal fibers.

End sac

In the SEM preparation, the end sacs measure about 5 mm in length, and thus about half of the length of the trunk. They are consistently wide (400-500 µm) over the entire length (Fig. 9A). Several thin, muscular mesenterial filaments attach the end sac to the cloaca as well as to the anterior portion of the hindgut. Ciliated funnels are regularly distributed over the entire surface of the end sac (Fig. 9A). The
end sac is covered by a peritoneum consisting of flat squamous peritoneocytes and a few podocytes, which are found particularly at the base of the ciliated funnels (Fig. 10A; Fig. 11B). The podocytes and peritoneocytes are interconnected by apical adherens junctions. Electron-dense diaphragms cover the clefts between the pedicels of the podocytes. The peritoneum rests on an ecm measuring approximately 5 µm in thickness.


Fiber muscle cells, isolated or in groups, form a muscle grid inside the ecm that is built up by inner longitudinal, outer circularly, and additional diagonal fibers (Fig. 9B-C). The diagonal fibers branch off of the circularly fibers, which appear thicker and more frequent than the longitudinal and diagonal fibers. Occasionally, muscle cells extend up to the base of the funnel. The ultrastructure of the muscle cells is uniform; generally, the myofibrils are surrounded by numerous oval mitochondria and small vesicles (Fig. 10). Myofilaments often concentrate on one side of the muscle cell. The muscle cells are never directly connected by cellular junctions, instead dense plaques adhere each cell to the matrix (Fig. 10A). If muscle cells form groups, dense plaques of neighbouring cells are opposite to each other and a small patch of *ecm* is located between both. Depending on the level of contraction of the organ, the inner epithelium may display irregular smooth involutions, which protrude into the lumen (Fig. 9C). Though it appears pseudostratified, the inner lining of the end sac is a simple epithelium that is composed of large, irregularly-formed cells. Clusters of microvilli emanate from the adluminal surface
of the aciliated or weakly-ciliated cells, which are connected by apical adherens junctions (Fig. 7B). Due to the size of the cells, the large oval nuclei of adjacent cells appear to be rather distant to each other.

Figure 9: End sac morphology of *Thalassema thalassemum* (Thalassematidae). **A:** External morphology (SEM), overview. Only one of the original two organs is depicted. Numerous ciliated funnels are regularly distributed over the entire length of the organ. **B:** View from above into the end sac. Phalloidin staining (red), and Sytox Green nucleic acid stain (bright cyan), projections of full confocal stack. Funnel are indicated by clustered Sytox green labelled nuclei. Nuclei of the end sac are less densely packed. The muscle fibers compose a muscle grid. *Arrow* indicate diagonal muscle fibers. **C:** Details of end sac epithelium, histological cross-section (azane staining, 5 µm section). The simple epithelium consists of aciliated or weakly ciliated irregularly-formed large cells. Inlet indicates arrangement of the musculature. *Arrow* depicts longitudinal muscle fibers. *Asterisk* indicates circularly muscle fibers. *aep* anal sac epithelium, *c* coelomocytes, *ci* cilia, *coe* coelom, *cmf* circularly muscle fibers, *ecm* extracellular matrix, *ep* epidermis, *es* end sac, *hg* hindgut, *lmf* longitudinal muscle fibers, *lu* lumen of end sac, *n* nucleus, *ptc* peritoneocyte (modified from Lehrke and Bartolomaeus 2011).

The cytoplasm of each epithelial cell contains many vesicles of different size, many small electron-dense spots, and numerous large, densely packed spheroid granules close to the apical surface (Fig. 11A). The granules have a mean diameter of 0.7 to 1.0 µm. They seem to be membrane-bound and inhibit median electron-dense material that has fallen out of the section, in most cases giving a conspicuous, irregular, electron-lucent or electron-dense appearance. Some appear to have a concentric layered structure. Occasionally, cells of the inner epithelium are broken and cell material seems to be deflated into the lumen of the end sac. Some adherens junctions are located about 2 µm off of the cellular border towards the lumen (Fig. 11A).
Figure 10: Muscle cells of the end sac (TEM, cross-sections). A: Diagonal muscle fiber cell (asterisk) adhered to the matrix via dense plaques (arrows). Arrowheads indicate pedicels of the podocytes. B: Longitudinal muscle fiber cells. cmc circular muscle fiber cell, coel coelom, ecm extracellular matrix, g pigmented granule, lmc longitudinal muscle fiber cell, mt mitochondrium, ptc peritoneocyte, v vesicle.

Figure 11: Cellular organization of the end sac epithel (TEM, cross-sections). Arrows mark adherens junctions. A: The cytoplasm contains numerous pigmented membrane-bound granules of various appearances. At the luminal margin the cytoplasm contains many electron-dense spots and vesicles of different size indicating presumably secretory processes in the end sac. B: Pedicels of podocytes lining the coelomic side of the end sac, near the base of the funnel. Arrowheads indicate electron-dense diaphragms covering the clefts between the pedicels of the podocytes. aep end sac epithelium, ci cilium, coe coelom, ecm extracellular matrix, g pigmented granule, mt mitochondrium, nu nucleus, ptc peritoneocyte, v vesicle (modified from Lehrke and Bartolomaeus 2011).
Ciliated funnel

The sessile funnels are reminiscent of an inverted bell directly attached to the end sac (Figs. 12A-C). The funnels open into the coelomic cavity with a maximal marginal diameter of 51.5±11.6 µm (n=7) and measure 50.7±4.9µm (n=8) in longitudinal extension, scaled from the apical margin of the funnel lip to the basal insertion of the funnel. When studied in both the SEM and the histological sections, the basal part of the funnel shows a basal neck-like constriction, here referred to as the neck region (Fig. 7B; Fig. 12A, C). Each funnel is composed of non-muscular, heavily-ciliated cells that rest on an ecm (Fig. 12). Resting on the opposite side of this ecm, flat squamous peritoneocytes form the coelomic lining of the funnel. All cells are interconnected by apical adherens junctions. Besides the nucleus and a few mitochondria, peritoneocytes contain small electron-dense spots and small vesicular structures. The funnel cells differ in shape according to their position. Those forming the rim or lip of the funnel are tall and polygonal (Fig. 7B; Fig. 12E), while those lining the neck region are flat and squamous (Fig. 7B; Figs. 12D, H). All funnel cells are multiciliated. The peripheral 9x2 microtubules arise from the basal body, while the central pair of microtubules adheres to the basal plate. Two rootlets, one vertical and one horizontal, adhere to the basal body and extend into the cell (Fig. 12F). The vertical rootlet is approximately 6 µm long; its inner tip often fuses with vertical rootlet tips of adjacent cilia. The horizontal rootlet is shorter and measures about the half the length of the vertical rootlet; among adjacent cilia the horizontal rootlets seem to fuse and form an apical meshwork of fiber-like structures (Fig. 12G). Cilia of the upper, outer, and inner margins of the funnel form groups of up to eight cilia, each group of cilia extends from a small ciliary pit (Fig. 7B; Figs. 12E-F). Towards the base of the funnel, the cilia are more and more evenly distributed along the entire cell surface (Fig. 7B; Fig. 12D). Microvilli are much more abundant in the latter region (Fig. 7B; Fig. 12D) than on the funnel margin, where they are restricted to the periphery of the cilia (Fig. 7B). Immunocytochemical localization of F-actin labelling assigns an apical actin-belt of the cytoskeleton of each single funnel cell (Fig. 12B). The actin net extends over the entire funnel including the upper rim, indicating the cell border of the funnel cells. Funnel cells contain a medially situated nucleus, numerous mitochondria (most of them close to the ciliary rootlets), and tiny electron-dense spots as well as many electron-lucent vesicles of different size. Occasionally, funnel cells also contain spherical electron-dense inclusions (Figs. 12D-E, G). These are different from the spheroid granules of the end sac in that they are more evenly electron-dense. Their mean diameter (0.6-1.0 µm) is almost identical to the granules observed within the epithelium of the end sac.
Figure 12: Funnel morphology of the anal sacs in Thalassema thalassemum (Thalassematidae). A: External morphology (SEM). The bell-shaped funnel is heavily ciliated and directly attached to the end sac. The basal part shows a neck-like constriction (= neck region, marked by arrowhead). B: Funnel with basis, lateral view, phalloidin staining (red), projections of full confocal stack. The funnel is composed of non-muscular cells. Phalloidin labelling assigns an apical actin-belt of the cytoskeleton of each single cell. The actin net extends over the entire funnel including the lip, indicating the cell borders of the funnel cells (arrow). Asterisk marks staining of diagonal muscle fiber within the end sac. C-H: Details of the cellular organization of the funnel (D, H: funnel cells of the neck region (TEM, sagittal-sections); E-G: funnel cells of the upper funnel lip (TEM, sagittal-sections). C: Histological sagittal-section (azane staining, 5 µm section). Asterisk indicates end sac musculature; arrowhead depicts neck region. D: Highly ciliated neck-region. The multiciliated cells are flat and squamous. Arrowhead indicates spherical electron dense inclusion. E: Funnel lip indicating polygonal shape of funnel cells. Cilia are forming groups at the outer and inner margin. Arrows mark adherens junctions. F: Close-up view of ciliary rootlets. G: Multiciliated funnel cell of the lip indicating a strong network of ciliary rootlets that proceed deep into the cell. The rootlets are interconnected. H: Funnel cells of the neck region with microvilli. aep end sac epithelium, c coelomocytes, cf ciliated funnel, ci cilia, cir ciliary rootlets, cmf circularly muscle fibers, coe coelom, ecm extracellular matrix, es end sac, fc funnel cell, flu funnel lumen, g pigmented granule, li lip, lmf longitudinal muscle fibers, mt mitochondrion, mv microvilli, n nucleus, ptc peritoneocyte, v vesicle (modified from Lehrke and Bartolomaeus).
3.2.2 Anal sac morphology in additional species

In order to survey the variability in the morphology of the anal sacs more comprehensively and systematically, the available data on the structure and composition of additional anal sacs were compiled and complemented by histological and scanning electron microscopical studies for a total of seven species that cover all high ranking subgroups of the Echiura, except for the Ikedaidae.

Scanning electron microscopy of the complete organs (including the ciliated funnels) was performed for an additional member of the Thalassematidae (*Anelassorhynchus adelaidensis*), members of the Bonelliidae (*Alomasoma belyaevi, Bonellia viridis, and Metabonellia haswelli*) and the Urechidae (*Urechis caupo, Urechis unicinctus*). In the thalassematid *Lissomeyema mellita* merely the morphology of the funnels was investigated. Histological studies (azane staining) of the anal sacs including their ciliated funnels were performed for *A. adelaidensis* (Thalassematidae), *M. haswelli* (Bonelliidae), and *U. caupo* (Urechidae).

General

All studied anal sacs are paired structures, each branching off from the cloaca and extending into the trunk coelom. Each anal sac consists of an end sac and numerous small ciliated funnels covering the end sac (Fig. 7). The investigated funnels usually consist of a conical segment including the ciliated lip and an externally visible neck like constriction basally, referred to as neck region. Generally, this neck region represents the basal most segment of the funnel and connects the funnel lumen with the lumen of the end sac. In case the funnel is set on a tubule, the neck region is also the most basal part of the funnel, but the larger tubule connects the funnel lumen with the lumen of the end sac (Fig. 15A).

Structural variation in the anal sac morphology was found regarding the shape of the end sacs (Figs. 13, 16, 19A) and the structure of the end sacs. This concerns the differentiation of the muscle net, the thickness of the *ecm*, and the filling of the epithelial cells with spherical orange-brown (azane staining) inclusions of various sizes (small or large granula-like inclusions) (Fig. 15; Fig. 17; Fig. 22). Variability was also detected in features that refer to the shape of the funnels and associated substructures such as tubules (= long funnel stalks) and neck regions (Tab. 5; Figs. 12A, C; Fig. 18; Fig. 23). Tubules may branch into additional smaller tubules or not. Such branching tubules are classified as follows (Figs. 13-15): The tubules that branch off first are here referred to as primary tubules; the tubules that branch off the primary tubules laterally are smaller in diameter and length and are consequently named secondary tubules. The tubules that branch off the secondary ones are referred
to as tertiary tubules and are the smallest regarding diameter and length. Funnels may be equipped with a tubule, or they are lacking such a stalk. In species that lack tubules the neck region may be present or not, in case it is present it may show different lengths. In two species a funnel dimorphism was detected regarding the general shape of the funnels (Tab. 5; Fig. 23).

In the following paragraphs a detailed description of the anal sac substructures, the end sac and the ciliated funnels, is given for the investigated bonelliid, thalassematid and urechid specimens.

Table 5: Total length of studied anal sac funnels and involved substructures, together with detected funnel dimorphism regarding the funnel shape. Measurements were taken from histological sections (azane staining) together with SEM and TEM micrographs, since autonomous measurements of all methods applied correspond to the standard deviation therein. The funnel length was scaled from the apical margin of the funnel lip to the basal insertion of the funnel (respectively the neck region (nr) or the stalk (= tubule, tu)). In case tubules of different hierachical levels occurred in one specimen, the primary tubules were chosen as a point of reference. The total length of the funnels set on primary tubules is an approximation based on SEM micrographs since it was impossible to assign the exact value due to masking of the primary tubules by other primary tubules or tubules of a higher hierachical level. cs conical segment; cc cylindrical segment; - = absent; + = present.

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<td><em>B. viridis</em> (Bonelliidae)</td>
<td>900</td>
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<td>-</td>
</tr>
<tr>
<td><em>M. haswelli</em> (Bonelliidae)</td>
<td>1000</td>
<td>Cs + nr + tu</td>
<td>-</td>
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<td><em>A. adelaidensis</em> (Thalassematidae)</td>
<td>42.51±6.96</td>
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<td><em>T. thalassemum</em> (Thalassematidae)</td>
<td>50.7±4.90</td>
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<td><em>L. mellita</em> (Thalassematidae)</td>
<td>64.18±4.28</td>
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<tr>
<td><em>U. caupo</em> (Urechidae)</td>
<td>64.67±16.18</td>
<td>Cs / cc</td>
<td>+</td>
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<tr>
<td><em>U. unicinctus</em> (Urechidae)</td>
<td>58.88±7.55</td>
<td>Cs / cc</td>
<td>+</td>
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3.2.2.1 Bonelliidae

End sacs

The end sacs in *A. belyaevi* are damaged, so that it was not possible to determine their shape (Fig. 13B). The end sacs in *B. viridis* and *M. haswelli* are sac-shaped (Figs. 13A, C-D). The end sacs of all bonelliid specimens branch many times into dendritic long tubules.

![Figure 13](image)

**Figure 13**: Anal sacs of examined Boneliidae, overview (*A: Bonellia viridis; B: Alomasoma belyaevi; C-D: Metabonellia haswelli*). Only one of the original two organs is depicted. Blue-white arrowheads mark primary tubules; arrows indicate ciliated funnels. **A**: SEM micrograph of the sac-shaped end sac. They branch many times into large primary and smaller secondary tubules. Asterisks indicate laminar mesenteries. **B**: SEM micrograph of the sac-shaped end sac. They branch many times into large primary and occasionally smaller secondary tubules. **C**: Specimen dissected in vivo showing the sac-shaped end sac (photograph provided by G. Rouse). The end sac is almost transparent, the same holds true for the primary tubules. Secondary tubules are coloured orange-brown. The funnels are of contrasting bright yellow colour. **D**: SEM micrograph. The primary tubules are masked by numerous secondary and tertiary tubules. as anal sac, es end sac, stu secondary tubule.
The three species mainly differ in the extent and pattern of the branching (Figs. 14C-E): in *B. viridis*, secondary tubules are confined to the basal half of the larger primary tubules, the distal half of which is devoid of secondary tubules (Fig. 13A; Fig. 14A); very rarely, primary tubules emerge from the end sac that entirely lacks any secondary tubules. In *M. haswelli*, secondary tubules are consistently distributed along the whole length of the primary tubules and sometimes additionally bifurcate into tertiary tubules (Fig. 14C; Fig. 15A). Generally, tubules of the different hierarchical levels decrease in length (and diameter) from large primary (about 900-1000 μm in longitudinal extension) over smaller secondary (about 100-300 μm in longitudinal extension) to very short tertiary tubules (about 60-80 μm in longitudinal extension) in all investigated species.

**Figure 14**: Anal sac tubules and their branching pattern in Bonelliidae, SEM micrographs (A-B: *Bonellia viridis*, C-D: *Metabonellia haswelli*). Arrowheads indicate ciliated funnels. Due to fixation artefacts the conical segment is not always clearly visible. A: Secondary tubules are confined to the basal half of the larger primary tubules, the distal half is devoid of secondary tubules. B: Details of primary tubule and neck region of the funnel. Due to fixation artefacts the cilia are lacking. C: Secondary tubules are distributed along the whole length of the primary tubules and sometimes additionally bifurcate into tertiary tubules (*yellow arrows*). D: Details of secondary tubule and neck region of the funnel. *cf* ciliated funnel, *es* end sac, *nr* neck region, *ptu* primary tubule, *stu* secondary tubule.
Laminar mesenteries were exclusively found in *B. viridis*, presumably emanating from various sides of the end sac and suspending it to the body wall and hindgut (Fig. 13A). In *M. haswelli* the end sac and all tubules are covered by a peritoneum consisting of flat squamous peritoneocytes. The peritoneum rests on an *ecm* measuring approximately 50 µm in thickness within the end sac (Fig. 15B, D). Inside the *ecm* of the end sac, a compact muscle grid built up by thick inner longitudinal and thick outer circularly fibers is existent (Fig. 15B). The inner epithelium displays irregular involutions, which protrude into the lumen (Fig. 15B). Generally, the inner lining of the end sac appears somewhat disintegrated, thus details of the epithelial cells were not observable. Similar to the state detected in *Anelassorhynchus adelaidensis* (Thallassematidae), the epithelial cells are broken and cell material seems to be deflated into the lumen of the end sac (compare Fig. 15B). The epithelium of the end sac proceeds into the primary and secondary tubules. Due to the disintegrated state of the epithelium within the end sac and within the primary tubules no assertion can be made on the ciliation of the epithelia within. The simple epithelium of the secondary tubules is devoid of cilia (Fig. 15E). Structural differences between the tubules are depending on their hierarchical level. The thickness of the *ecm* decreases from the primary tubules (about 8 µm) to the secondary tubules (about 3 µm) (Fig. 15G). In the primary and secondary tubules longitudinal as well as diagonal muscle fibers were detected (Figs. 15D, F). The frequency and the thickness of the muscular fibers decrease from several comparatively thick fibers within the *ecm* of the primary tubules (similar to those of the end sac) to a few filiform fibers in the secondary tubules (Fig. 15F). Spherical orange-brown (azane staining) granula-like inclusions, ranging from 1-5 µm in diameter, are abundant in the epithelial cells of the primary and secondary tubules (Figs. 15D-G). Generally, there are no histological data available for the tertiary tubules.

**Ciliated funnels**

Funnels of all investigated specimens are composed of a conical segment that includes the ciliated lip and a distinct neck region (Figs. 14B, D; Figs. 15E-F). Funnels of all investigated specimens are consistently set on a long tubule (Tab. 5; Figs. 13-14). Each funnel is demarcated from the tubule by a neck region that is externally (Fig. 14D), and histologically (Fig. 15E) discriminable from the conical segment of the funnel. In contrast to the tubule, the neck region features a smaller maximal diameter, a ciliation (especially at the upper part), and a high density of nuclei (Fig. 15E). Very rarely muscle fibers are present within the neck region, but never within the conical segment of the funnel (Fig. 15F). The funnels in *Metabonellia haswelli* are covered by the same peritoneum as the body of the end sac and all tubules. The funnels, including the neck regions, are lined by a simple ciliated epithelium.
The cells are small; their nuclei are relatively large compared to the cell size. Spherical orange-brown (azane staining) granula-like inclusions are lacking in all funnel cells (Fig. 15E, F).

Figure 15: Anal sac of Metabonellia haswelli (Bonelliidae). A: Schematic representation, overview. Only one of the original two organs is depicted. B-F: Histological sections (azane staining, 5 µm section). Arrows depict granula-like orange-brown inclusions. B: Cross-section of the end sac, posterior part. A compact muscle grid built up by thick inner longitudinal and thick outer circularly fibers has developed. The epithelium appears somewhat disintegrated. C: Primary tubule, sagittal section. D: Transition from the lumen of the end sac into the lumen of the primary tubule. The epithelium of the primary tubule appears somewhat disintegrated and cells seem to be released into the lumen. E: Ciliated funnel set on a secondary tubule, longitudinal section (medial). The funnel is composed of small, non-muscular, heavily-ciliated cells. A slender neck region is demarcated from the conical segment and the broader tubule. The tubule epithelium contains spherical granula-like orange-brown inclusions (arrows). F: Ciliated funnel set on secondary tubule, sagittal section. A few filiform muscular fibers are present within the ecm of the tubule and very rarely muscle fibers proceed into the neck region. Muscular fibers marked by arrowheads. G: Cross-section of primary and secondary tubules. aep anal sac epithelium, as anal sac, cf ciliated funnel, ci cilia, coe coelom, cmf circularly muscle fibers, cs conical segment of funnel, ecm extracellular matrix, ept tubule epithelium, es end sac, fl funnel lip, lmf longitudinal muscle fibers, lu lumen of end sac, lut lumen of primary tubule, nr neck region, ptc peritoneocyte (nucleus), ptu primary tubule, stu secondary tubule, tu tubule, ttu tertiary tubule.
Generally, funnels of all primary, secondary and tertiary tubules slightly differ in size dependent on the hierarchical level of the tubules (Figs. 14A, C). Thus, the funnels of the primary tubules are the largest compared to the secondary and tertiary tubules. Funnels of the primary tubules measure about 900-1000 µm in total length, scaled from the apical margin of the funnel lip to the basal insertion of the primary tubule (compare Tab. 5). Funnels of the primary tubules open into the coelomic cavity with a maximal marginal diameter of about 60 µm. Since the number of funnels correlates with the number of branches, the density of funnels is highest in *Metabonellia haswelli* (Fig. 13).

### 3.2.2.2 Thalassematidae

**End sac *Anelassorhynchus adelaidensis***

The end sacs are paired long tubes that branch off from the cloaca, and extend into the trunk coelom (Fig. 16). They are tapering slightly towards the free end: near the insertion the external diameter is about 500 µm, near the free end the external diameter is about 250 µm (Fig. 16; Fig. 17A). In total, the end sacs capture approximately two thirds of the total trunk length. Ciliated funnels are regularly distributed over the entire surface of the end sac (Fig. 16; Fig. 17A).

![Figure 16: Tubular anal sac of *Anelassorhynchus adelaidensis* (Thalassematidae), SEM. Only one of the original two organs is depicted. Due to specimen preparation the tip of the end sac is missing. Arrows indicate ciliated funnels. They are regularly distributed over the entire surface of the end sac. as anal sac.](image)

The end sac is covered by a peritoneum consisting of flat squamous peritoneocytes. The peritoneum rests on an *ecm* measuring approximately 20-30 µm in thickness (Figs. 17B-C). Inside the *ecm*, a muscle grid built up by inner longitudinal and outer circularly fibers is existent. Close to the apical surface, the *ecm* contains large accumulations of spherical orange-brown granula-like inclusions that
break the peritoneal lining on several sites (Figs. 17B-C). Single granula-like inclusions vary in size from about 1-6 µm. The granula-like inclusions are also detectable in large quantities in the lumen of the end sac (Fig. 17B). The inner epithelium displays irregular involutions, which protrude into the lumen (Figs. 17B-C). Though it appears pseudostratified, the inner lining of the end sac is a simple epithelium that is composed of aciliated irregularly-formed cells (Fig. 17C). Epithelial cells often contain many small orange stained spherical inclusions (Fig. 17C). Frequently, cells of the inner epithelium are broken and cell material seems to be deflated into the lumen of the end sac (Fig. 17B). This material is stained pale orange and blue-grey.

Figure 17: Tubular anal sac of *Anellassorhynchus adelaidensis* (Thalassematidae). A: Schematic representation, overview. Only one of the original two organs is depicted. Due to specimen preparation the tip of the end sacs could not be reconstructed. B-C: Histological cross-sections of the end sac (azane staining, 5 µm section). Arrows depict spherical orange-brown granula-like inclusions. B: Overview end sac. Asterisk marks cell material which seems to originate from the inner epithelium. C: Details of the ecm and inner epithelium. The ecm contains large accumulations of spherical orange-brown granula-like inclusions that break the peritoneal lining on several sites. The muscle fibers compose a muscle grid build up by outer circularly and inner longitudinal fibers. The simple epithelium consists of aciliated irregularly formed cells. aep anal sac epithelium, cf ciliated funnel, coe coelom, cmf circularly muscle fibers, ecm extracellular matrix, hg hindgut; lmf longitudinal muscle fibers, lu lumen of end sac.

Ciliated funnel *Anellassorhynchus adelaidensis*

The funnels are composed of a conical segment that includes the ciliated lip and a distinct neck region (Figs.18A-B). The neck region is best observable within the SEM micrographs (Fig. 18B). The funnels open into the coelomic cavity with a maximal marginal diameter of about 30 µm and measure 42.51±6.96 (n=8) in longitudinal extension, scaled from the apical margin of the funnel lip to the basal insertion of the funnel. Each funnel is composed of small, non-muscular, heavily-ciliated cells. Their
nuclei are relatively large compared to the cell size. Spherical small or larger orange-brown (azane staining) granula-like inclusions are lacking in all funnel cells (Fig. 18A).

Ciliated funnel *Lissomyema mellita*

The funnels are composed of a conical segment that includes the ciliated lip and a distinct neck region (Fig. 18C). The funnels measure 64.18±4.28 (n=3) in total length, scaled from the apical margin of the funnel lip to the basal insertion of the funnel. They open into the coelomic cavity with a maximal marginal diameter of about 30 µm.

**Figure 18:** Funnel morphology in the anal sacs in *Anelassorhynchus adelaidensis* (A-B) (Thalassematidae) and *Lissomyema mellita* (C) (Thalassematidae). A: Histological sagittal-section (azane staining, 5 µm section) of the conical funnel showing a composition of small, non-muscular, heavily-ciliated cells. A neck region is not externally distuingishable from the conical segment, the transition is smooth. B: SEM micrograph. A distinct neck region is externally discriminable giving the funnel a short-stalked appearance. Due to fixation artefacts the cilia are lacking. C: SEM micrograph of the conical funnel in *L. mellita*. The funnel consists of a conical segment that includes the lip and a distinct neck region giving the funnel a short-stalked appearance. Due to fixation artefacts the cilia are lacking. *aep* end sac epithelium, *cmf* circularly muscle fibers, *coe* coelom, *cs* conical segment of funnel, *ecm* extracellular matrix, *es* end sac, *li* lip, *lu* lumen of end sac, *n* nucleus, *nr* neck region.
3.2.2.3 Urechidae

End sacs

The end sacs of *Urechis caupo* and *Urechis unicinctus* are voluminous and sac-like (Figs. 19A, C; Fig. 22A). They capture each about one third of the trunk length of the fixed specimen. In both species the end sacs are tapering clearly distally towards the free end: in *U. caupo* with an external basal diameter of about 6.0 mm and about 0.4 mm at the apical tip, in *U. unicinctus* with an external basal diameter of about 3.0 mm and 0.5 mm at the apical tip. The external surface is covered with numerous irregular swellings, giving the end sacs in both species overall a cauliflower-like appearance. In *U. caupo*, the ciliated funnels are sitting predominantly apical upon the swellings, but some are found additionally inbetween (Fig. 19B). In *U. unicinctus*, the ciliated funnels are sitting predominantly inbetween the irregular swellings (Fig. 19D).

![Figure 19: External morphology of the anal sacs in Urechidae (A-B: Urechis caupo, C-D: Urechis unicinctus), SEM micrographs. Only one of the original two organs is depicted; the basal part at the insertion is missing. Arrowheads mark ciliated funnels. A: Overview. B: Detail of external surface, intermediate part. Ciliated funnels are predominantly sitting apical upon the swellings. C: Overview. D: Detail of external surface, intermediate part. Ciliated funnels are sitting predominantly inbetween the irregular swellings. as anal sac, es end sac.](image)

Generally, the number of the funnels in the latter species decreases towards the free end of the end sac compared to the other two thirds of the organ (funnels are more densely packed proximal) (Fig. 20B); in *Urechis caupo*, the funnels are more densely packed towards the free end in comparison to the remaining two thirds of the organ, where the distance between each funnel is more spacious (Fig. 22A).
Rope-like mesenteries were found in both species, arising directly from the end sacs (Fig. 21). In *U. caupo* they were distributed sporadical over two thirds of the organ; in *U. unicinctus* mesenteries were primarily observed over the basal first third of the length of the end sac.

**Figure 20**: Funnel distribution at the distal free ends of the anal sacs in Urechidae, SEM. **A**: *Urechis caupo*. The funnels are densely packed towards the free end in comparison to the remaining two thirds of the organ. **B**: *Urechis unicinctus*. The free end houses less funnels compared to the other two thirds of the organ. *Arrowheads* mark ciliated funnels. *as* anal sac.

**Figure 21**: Rope-like muscular mesenteries in Urechidae. **A**: *Urechis caupo*. **B**: *Urechis unicinctus*. In both species they arise from the end sacs. Due to specimen preparation it is not known where and in which way they are connected to the hindgut and/or body wall. *Arrowheads* mark ciliated funnels. *as* anal sac, *mes* mesentery.

In *U. caupo*, the end sac is covered by a peritoneum consisting of flat squamous peritoneocytes. The peritoneum rests on an *ecm*, which is very fine in the area of the apical swellings, and broadened in the area in which the muscle grid is embedded (Fig. 22B). Here, the *ecm* may reach a maximum thickness
of about 50-70 µm. The muscle grid is strong, consisting of thick longitudinal as well as thick circularly and diagonal fibers, all arranged in bundles or groups respectively (Figs. 22B-D). The big apical swellings that are responsible for the external cauliflower-like appearance of the organs emerge from elevations of the inner epithelium (Fig. 22B). The inner epithelium is simple and is composed of aciliated columnar cells. It displays irregular elongate involutions, which protrude into the end sac lumen (Fig. 22B-D). The involutions may show two different conditions: (1) their cells may be situated around a thin compact band of ecm located median within the involution (Fig. 22C), and (2) the epithelial cells are situated on a thin band of ecm, but this ecm is lined apically by flat peritoneal cells (Fig. 22D). All epithelial cells contain numerous small orange-brown stained inclusions. Larger orange-brown granula-like inclusions are found in the lumen of the end sac and consequently within the lumen of the apical bulges (Fig. 22B). The granula-like inclusions have a diameter of about 15 µm.

Ciliated funnels

In both species an inconsistent funnel shape was detected. The majority of funnels in U. caupo and U. unicinctus are conical and slender (Figs. 23A-B, D). A few funnels show a rather cylindrical form respectively (Figs. 23C, E). Each funnel is composed of a conical, or cylindrical segment, which includes the ciliated lip in both species. A distinct, externally visible neck region is not observable, neither within the azane stained sections of U. caupo (Fig. 23A), nor by the SEM micrographs that refer to U. caupo and U. unicinctus (Figs. 23B-E). The funnels of U. caupo open into the coelomic cavity with a maximal marginal diameter of about 20 µm and measure 64.67 ± 16.18 (n=20) in longitudinal extension, scaled from the apical margin of the funnel lip to the basal insertion of the funnel. Funnels of U. unicinctus measure 58.88 ± 7.55 (n=6) in longitudinal extension, scaled from the apical margin of the funnel lip to the basal insertion of the funnel. They have a maximal marginal diameter of about 20 µm. Each funnel in U. caupo is composed of small, non-muscular, heavily-ciliated cells. The basal area near the insertion to the end sac seems to be less ciliated compared to the upper part of the funnel (Fig. 23A). The nuclei of the funnel cells are relatively large compared to the cell size. Spherical small or larger orange-brown (azane staining) granula-like inclusions are lacking in all funnel cells (Fig. 23A).
Figure 22: Sac-like anal sac with a cauliflower-like external appearance in Urechis caupo (Urechidae). Arrows depict small orange-brown inclusions. Arrowhead marks orange-brown granula-like inclusions within the anal sac lumen. A: Schematic representation, overview. Only one of the original two organs is depicted. B-D: Histological cross-sections of the end sac (azane staining, 5 µm section). B: Overview. Asterisk indicates irregular elongate involution of the inner epithelium. C-D: Details of elongate involutions and muscle fibers. The involutions show different conditions: the aciliated epithelial cells may be situated on a thin band of ecm (C), or the involutions are lined inwards by flat peritoneal cells (D). Arrowhead marks ecm band. The muscle grid is is build of thick longitudinal, circularly and diagonal fibers, all arranged in groups. as anal sac; aep anal sac epithelium, cf ciliated funnel, coe coelom, cmf circularly muscle fibers, dmf diagonal muscle fibers; ecm extracellular matrix, hg hindgut; lmf longitudinal muscle fibers, lu lumen of end sac; mf muscle fibers; ptc peritoneocyte.
Figure 23: Funnel dimorphism in Urechidae. A-C: *Urechis caupo*. (B, C Scale bar = 25 µm). D-E: *Urechis unicinctus* (Scale bar = 25 µm). Due to fixation artefacts the cilia are largely lacking. 

A: Histological sagittal-section (azane staining, 5 µm section) showing a slender conical shape and a composition of small, non-muscular, heavily-ciliated cells, especially in the upper part. A neck region is not clearly distinguishable from the conical segment. *Arrows* indicate small orange stained spherical inclusions within the end sac epithelium. 

B: SEM micrograph showing a slender conical shape. A neck region is not clearly distinguishable from the conical segment. 

C: SEM micrograph showing a rather cylindrical shape, a neck region is not clearly distinguishable from the cylindrical segment. 

D: SEM micrograph showing a slender conical shape. 

E: SEM micrograph showing a rather cylindrical shape, a neck region is not clearly distinguishable from the cylindrical segment. 

3.3 Larval protonephridia in *Thalassema thalassemum* (Thalassematidae)

In order to reveal phylogenetically significant data, the ultrastructure of the larval protonephridia in *T. thalassemum* (Thalassematidae) was investigated by means of LM and TEM in collaboration with Kato et al. (2011).

The 98 h (and 122h) old larva of *T. thalassemum* features one pair of protonephridia. Each protonephridium is tubular in shape and measures about 44 µm in length (Fig. 24B, Fig. 25A). The nephridia extend straightly from the foregut anlage toward the anus and open to the exterior by piercing the epidermis close to the anus. The external opening is situated ventrolaterally close to the gastrotroch and ca. 40 µm anteriorly to the anus (Fig. 24B). A specialized nephridiopore cell that is embedded with most of its cell body into the epidermis is lacking. Both nephridia are composed of two cells only, a terminal cell and a duct cell (Fig. 25).

**Terminal cell**

The terminal cell is situated laterally to the anlage of the foregut and the mouth opening. The cell is elongate and measures about 16 µm in length (Fig. 25A). The proximal end of the cell tapers off into a small apex. At this apex the terminal cell is connected to a muscle cell via dense plaques (Fig. 25B). The nucleus is located within the proximal part of the terminal cell. With its distal most part the terminal cell forms a hollow, cylinder like compartment (Fig. 25A). This compartment is continuous with the lumen of the adjacent duct cell. The inner wall of the cylinder is formed by numerous microvilli that emanate from the margin of a small flattened area. Actin filaments are present within the microvilli (Fig. 25C-D). No anastomoses or interconnections are found between the microvilli. The microvilli are arranged in a ring-like area. They are arranged in at least two irregular circles (Fig. 25D). The outer circle of microvilli is surrounded by a thin layer of electron dense extracellular matrix. Occasionally, hemidesmosomes connect the extracellular matrix to the underlying microvilli (Fig. 25D). The matrix is continuous with the extracellular matrix surrounding the entire terminal cell. From the flattened cytoplasmic area between the bases of the microvilli several cilia protrude into the lumen of the terminal cell. In the region of the proximal apex and near the origin of the cilia, the cytoplasm contains numerous coated and uncoated vesicles (Fig. 25C). Membrane pits occur at the abluminal membran in the middle and distal part of the terminal cell.
Figure 24: DIC pictures of 122h old larva *Thalassema thalassemum* (Thalassematidae), in vivo, lateral view (specimens slightly compressed). Arrowheads indicate mouth opening; asterisks mark a particle within the intestinal pouch. A: Focus on external surface showing the prototroch, metatroch, gastrotroch and apical tuft. A telotroch has developed, but is difficult to detect in this illustration. The foregut shows through. B: Focus on internal structures. The head kidneys are located ventrolaterally beside the stomach and the intestinal pouch (only left head kidney is shown). Each head kidney extends from the level of the mouth opening towards the posterior third of the larva, where the nephropore is located. *at* apical tuft, *DIC* differential interference contrast microscopy, *fg* foregut anlage, *gt* gastrotroch, *ip* intestinal pouch, *hk* head kidney, *mt* metatroch, *nt* neurotroch, *pt* prototroch.

Duct cell

The duct cell forms a stretched tubule with a length of approximately 30 µm (Fig. 25A). The nucleus is located in a lateral bulge in the middle part of the duct cell (Fig. 25G). Vesicles of various sizes are densely distributed within the cytoplasm of the middle and distal sections of the duct cell (Figs. 25G, H). The adluminal and abluminal membranes of the duct cell possess numerous uncoated membrane pits (Figs. 25F-H). A thin electron dense layer of extracellular matrix surrounds the entire duct cell (Figs. 25E-F). This basal membrane is continuous with the basal membrane of the terminal cell and the adjacent epidermis cells. The lumen of the duct cell is percellular, i. e. the cytoplasm encompassing the lumen is interconnected by an adluminal zonula adherentes and septate junctions (Figs. 25E-F, H).
Figure 25: Tubular head kidney of the 98h-old larva of *Thalassema thalassenum* (Thalassematidae), modified after Kato, Lehrke and Quast (2011). A: Schematic 3D-representation reconstructed from a complete series of TEM sections. The head kidney is composed of one terminal cell and one duct cell. Inlet shows filter structure (see also TEM micrograph D). B-D: Series of representative cross-sections (TEM) of the terminal cell. E-I: Series of representative cross-sections (TEM) of the duct cell. B: The apex of the cell is connected to a muscle cell via dense plaques (arrowheads). C: Distal end of the cell forming a flattened area from which six cilia protrude into the nephridial lumen. Basal bodies with short rootlets and a basal footlet (arrow) anchor the cilia within this area. D: Filtration apparatus build of distally arranged microvilli that enclose the lumen of the terminal cell. The outermost microvilli are covered by a thin layer of extracellular matrix to which they are connected by hemidesmosomes (arrowheads). E: Proximal part of duct cell. Cilia and microvilli of the terminal cell extend into the duct lumen. Arrowhead points adluminal adherens junction. F: Cross section of duct cell proximally to the perikaryon. The lumen contains cilia of terminal cell. An adluminal adherens junction (arrowhead) forms a longitudinal seal along its whole length. Arrow refers to an uncoated pit indicating processes of transcytosis. G: Middle part of the duct cell with nucleus. Lumen contains cilia and numerous finger-like cytoplasmatic processes. H: Higher magnification of the duct lumen of the middle part showing finger-like cytoplasmatic processes lacking actin filaments. I: Distal most part of the duct cell with nephropore. Arrowheads indicate adherens junction. aj adherens junction, ci cilium, cp finger-like cytoplasmatic processes, dc duct cell, ecm extracellular matrix, ep epidermis cell, er endoplasmatisches reticulum, lu lumen of the duct, mc muscle cell, mi mitochondrium, mv microvilli, nu nucleus, ve vesicle, tc terminal cell.

In the proximal part of the duct cell the adluminal membrane forms neither cilia nor microvilli. Only the cilia and microvilli of the terminal cell extend into this part of the duct lumen (Figs. 25E-F). Because of their different length, the microvilli end successively at different levels in the proximalmost part of the duct lumen. The six cilia of the terminal cell project into the lumen of the duct cell and thus extend to the level of its perikaryon (Fig. 25G). The duct lumen widens from about 0.5 µm in the proximal part to a diameter of 2.5 µm in the region of the perikaryon (Figs. 25A, G-H). Here, several cilia project from the adluminal membrane into the lumen. From the region of the nucleus on, there also protrude numerous finger-like cytoplasmic processes into the nephridial lumen which are lacking actin filaments (Figs. 25A, G-H). The distalmost part of the duct cell passes through the epidermis and forms the external opening of the nephridium (Fig. 25I).
3.4 Gonostomal lips

To better distinguish between relevant morphological character states of the gonostomal lips the structure of sexually mature gonoducts was compared with sexually immature gonoducts in *Thalassema thalassemum* (Thalassematidae) by scanning electron microscopy (SEM) and histological studies (azane staining). In order to reveal phylogenetically significant data for additional species the morphology of the nephrostomal lips of primarily sexually mature gonoducts was studied using SEM in one additional thalassematid, *Anelassorhynchus adelaidensis*, two species of the Urechidae (*Urechis caupo*, *Urechis unicinctus*) and one member of the Bonelliidae (*Metabonellia haswelli*), which was exclusively studied in vivo by light microscopy.

The gonoducts in all investigated species are more or less sac-like vessels of different size that open behind the anterior ventral chaetae to the exterior (Figs. 26C-D). Each gonoduct opens to the coelomic cavity through a funnel (gonostome) and to the exterior via a genital pore, which lies ventrolaterally on the anterior trunk (Fig. 26C). The lip-like tissue that surrounds the gonostome is usually ciliated and is named gonostomal lips. It may be differentiated into various forms among the species. A survey of the observed structural differences of the investigated species is given in the following paragraphs.

3.4.1 Thalassematidae

*Thalassema thalassemum*

All investigated specimens of *T. thalassemum* possess four gonoducts, arranged in two pairs, one member on each side of the ventral nerve cord (Figs. 26C-D). The position of the gonostome in each gonoduct is basal, near the genital pore (Fig. 26C). The structure of the gonostomes varies among the studied specimens, depending on different levels of maturity and the content of gametes, respectively. During reproduction period (early April) sexually mature gonoducts are filled with masses of gametes, thus the large sacs extend to about two thirds of the length of the relaxed specimen trunk (Figs. 26A-B; Fig. 27A). The gonostome is directly connected to the gonoduct, without a stalk (sessile gonostome). It is equipped with two ciliated flap-like lips with a pointed tip (Figs. 27A-B). The lips insert laterally from the border of the funnel opening. They measure about 200 µm in length and posses a ciliated v-shaped groove running along the entire length of the lips. The groove is situated laterally on the lips, extending from the basal part of the gonostome (where it connected to the gonoduct) to the pointed tip (Fig. 27B). Sexually immature gonoducts have a vestigial appearance compared to the state observed
during the reproduction period. The length of the gonoducts is approximately one-fifth of the length of the gonoducts during reproduction period (Figs. 27C-D). The gonostome is sessile but the bi-lipped elongate structure is not apparent; the lips are short and feature a lip tissue that is ciliated all around and folded, resembling a rose petal. A v-shaped groove is not visible from exterior (Figs. 27C-D).

**Figure 26:** *T. thalassemum* (Thalassematidae). **A-B:** Gonoducts of in vivo dissected specimens. Scale bars = 5 mm. **A:** Two mature male gonoducts filled with masses of spermatozoa giving the entire organ a conspicuous white appearance. **B:** Mature female gonoduct filled with numerous ova giving the entire organ a characteristic orange colour. **C-D:** Histological cross-sections (azane-staining) of the anterior trunk region (5 µm section) showing the size and position of sexually immature gonoducts (C) and their gonostomal lips (D). The lumen of the gonoducts is devoid of gametes. Asterisks mark chaetal muscle strands. **C:** The gonoducts lie close to the anterior ventral chaetae in the coelom. Their distal free end lies in the body cavity; the basal end is attached to the ventral surface of the body wall. Arrow indicates genital pore which lies ventrolaterally on the anterior trunk. **D:** Higher magnification of the gonostomes showing short annular lobed lips: *c* coelomocytes, *ci* cilia, *coe* coelom, *cm* circular musculature, *ep* epidermis, *go* gonoduct, *gs* gonostome, *ibm* interbasal muscle, *lm* longitudinal musculature, *mes* mesentery, *om* oblique musculature, *vc* ventral chaetae, *vn* ventral nerve cord.

**Anelassorhynchus adelaidensis**

*A. adelaidensis* features four gonoducts, arranged in two pairs. The position of gonostome is basal near the genital pore (Fig. 28A). The gonostome is directly connected to the gonoduct, without a stalk. It is equipped with two filamentous spirally coiled lips of about 3 mm length which are slightly tapering towards their distal end (Figs. 28A-B). The lips become more and more coiled towards their distal end. A v-shaped ciliated groove is running along the entire length of the lips. The margin of the groove is formed by a broad (about 30 µm in maximal diameter) bulge extending along the entire length of the groove (Figs. 28B-C). The marginal bulge is scarcely ciliated on its apex and slightly more ciliated towards the inner side of the groove.
Figure 27: *T. thalassemum* (Thalassematidae). SEM micrographs of the gonoducts indicating different filling levels with gametes. **A-B:** Sexually mature female gonoduct during reproduction period (early April 2007) filled with masses of gametes. **A:** Overview. **B:** Sessile, bi-lipped gonostome featuring ciliated flap-like lips, frontal view. A ciliated v-shaped groove is running along the entire length of the lips (*arrowhead*). **C-D:** Sexually immature gonoduct of unknown gender showing a vestigial appearance of the organ (C lateral view; D frontal view). The sessile gonostome lacks a bi-lipped structure and features instead a heavy ciliated lip tissue that is folded, resembling a rose petal. *ep* epidermis, *go* gonoduct, *gol* gonostomal lips.

Figure 28: *A. adelaidensis* (Thalassematidae). SEM micrographs of gonoducts and sessile bi-lipped gonostomes. *Arrowhead* indicates basis of the gonostome. **A:** Overview on two of originally four gonoducts (slightly damaged during preparation). The gender and level of maturity is unknown. The gonostomal lips are filamentous and spirally coiled. **B:** Gonostomal lips with ciliated v-shaped groove with a broad bulging margin. **C:** Higher magnification of the scarcely ciliated groove. *ci* cilia, *go* gonoduct, *gol* gonostomal lips, *gr* ciliated groove, *om* oblique musculature of the trunk wall.
3.4.2 Urechidae

_Urechis caupo_ shows six sexually mature gonoducts arranged in three pairs; _Urechis unicinctus_ has four sexually mature gonoducts arranged in two pairs. The position of the gonostome in both species is basal near the genital pore and the gonostome is directly connected to the gonoduct, without a stalk (Figs. 30A, D). The sessile gonostome is equipped with two filamentous spirally coiled lips of about 3-4 mm length in _U. caupo_ (4-5 mm length in _U. unicinctus_) which are tapering slightly towards their distal ends (Figs. 30A, D-E). The lips of both species feature an almost consistent degree of coiling throughout their entire length. A v-shaped ciliated groove is running along the entire length of the lips (Figs. 30B, E). The margin of the groove is formed by a broad bulge (apex about 40-50 µm in diameter in both species) extending along the entire length of the groove (Figs. 30B-C, E). In _U. caupo_ it is heavy ciliated on its apex and on the inner side facing the groove (Fig. 30C). In _U. unicinctus_ it is mainly ciliated on the inner side of the groove (Fig. 30F). In both species the ciliated groove and its broad bulging margin converge into a common ciliated path that leads towards the entrance of the gonoduct (Fig. 29; Fig. 30D).

Figure 29: _Urechis caupo_ (Urechidae). SEM micrograph of the basis of the gonostome (frontal view). The ciliated groove and its broad bulging margin of the gonostomal lips converge into a common ciliated path that leads towards the entrance of the gonoduct (arrowhead). Asterisk marks oocyte. ci cilia
Figure 30: SEM micrographs of gonoducts and sessile bi-lipped gonostomes in Urechidae. Arrowheads indicate basis of gonostomes. Asterisk marks oocyte. A-C: Urechis caupo, D-F: Urechis unicinctus. A: Lateral view of sexually mature female gonoduct showing the basal position of the sessile bi-lipped gonostome. The gonostome is equipped with two filamentous spirally coiled lips. B: Lips with ciliated groove with broad bulging margin. C: Higher magnification of the groove showing a heavy ciliation on the apex and on the inner side. D: Top view of sexually mature gonoduct of unknown gender. The sessile bi-lipped gonostome displays a basal position and features two filamentous spirally coiled gonostomal lips. E: Lips with ciliated groove with broad bulging margin. F: Higher magnification of the groove showing a ciliation primarily on the inner side. ci cilia, go gonoduct, gol gonostomal lips, gr ciliated groove, om oblique musculature of the trunk wall.

3.4.3 Bonelliidae

Metabonellia haswelli

The specimen exhibits a single sexually mature gonoduct that features a globular specialized part or chamber where the dwarf male usually can be found (Fig. 31A). This basal part of the gonoduct is called “male sac” or androecium and presents the transition from the genital pore to the sac-like distal part of the gonoduct. In the specimen studied here, the male resides inside the sac-like part of the gonoduct (Fig. 31A). The entire gonoduct is attached to the coelomic wall of trunk, posterior to the level of the anterior chaetae. It measures about 1.0 cm in length, comprising about one third of the
length of the relaxed specimen without the proboscis. The gonostome is stalked and is situated laterally on the gonoduct in its proximal most third (Figs. 31A-B). The stalk of the gonostome measures 1.5 mm in length. The gonostomal lip is strongly crenated and resembles four frilled petals that are arranged annularly around the stalked gonostome (Fig. 31B). The external diameter of the gonostome including the lips is approximately 2 mm.

![Figure 31: Metabonellia haswelli (Bonelliidae). Sexually mature gonoduct during reproduction period (end of March 2006). Asterisk marks oocytes. A: Specimen dissected in vivo showing the single gonoduct inclusive the androecium (an) in which the dwarf males usually reside. Arrowhead indicates dwarf male. Inset marks the gonostome. The gonostome is situated laterally on the gonoduct in its proximal most third. B: Higher magnification of gonostome, lateral view. The gono-stome is stalked and is equipped with a strongly frilled lip that resembles petals arranged annularly around the gonostome. Photographs kindly provided by G. Rouse. ep epidermis, go gonoduct, gol gonostomal lips.](image-url)
3.5 Phylogenetic analysis of sequence data

The ML-analysis inferred from both, the “original dataset” (including 100% of the original positions) and the “restricted dataset” (including 76% of the original positions), resolve each one best tree with congruent topologies (Fig. 32). The analyzed alternative datasets display likelihood bootstrap support values (LBS) that do not differ substantially. For reasons of integrity both are indicated in Figure 32.

Monophyly of Echiura is highly supported, as evidenced by 100% LBS in both analyses. Echiura are retrieved as sister group of the Capitellidae (LBS from 91% to 97%). Within Echiura two monophyletic major clades are recovered, hereafter referred to as the Bonellia-group and the Urechis-group. Their sister group relationship is well supported (LBS from 86% to 93), as is the monophyly of the Bonellia-group (LBS from 86% to 95%); the Urechis-group retrieves LBS from 67% to 95%.

Within the Bonellia-group Protobonellia sp. branches off as sister to a clade comprising four traditional bonelliid taxa and Ikeda sp., a member of traditional Ikedaidae. This big clade includes two monophyletic groups: Ikeda sp. + Maxmuelleria lankesteri (LBS from 75% to 83%), and Metabonellia haswelli + Alomasoma belyaevi + Bonellia viridis which is only weakly supported (LBS from 57% to 59%).

The Urechis-group consists of two species of Urechis and Echiurus echiurus. Monophyly of Urechis receives high support in the “original dataset” (LBS 97%), as does the sister group relationship of E. echiurus + Urechis species (LBS 95%). Within the “restricted dataset” the same topology is recovered but the support is lower (LBS from 67% to 70%).

With regard to the well-supported clade comprising the Bonellia- and Urechis-group, paraphyly of basally branching “Thalassematidae” is weakly supported, especially by the original dataset (LBS from 42% to 61%). Thalassematid taxa are paraphyletic, comprising a highly supported clade of Ochetostoma erythrogrammon + the composite taxon Anelassorhynchus (LBS from 99% to 100%), Thalassema thalassemum and Arhynchite pugettensis branch off successively.
Figure 32: Maximum-likelihood tree of the multigene dataset (MT-CO1 + 16S rRNA + 18S rDNA) from RAxML analysis based on the GTR + I + Γ model of sequence evolution. Values separated by slashes at nodes represent ML bootstrap support of the “original dataset” (including 100% of the original positions, at left) and ML bootstrap support of the “restricted dataset” including 76% of the original positions, respectively. Taxa with asterisk mark composite taxa (Capitellidae: *Notomastus latericeus* (16S+18S rDNA) + *Dasybranchus* sp. DH1 (CO1); *Anellassorhynchus*: *Anellassorhynchus adelaidensis* (16S+CO1) + *Anellassorhynchus porcellus* (18S)). Branch lengths reflect evolutionary change in sequences (substitutions per site as indicated by scale bar). Taxon names with family rank on the right side refer to the traditional classification sensu Dawydoff (1959).
4 Discussion

The main objectives of the discussion are i) compiling potentially informative character states from the studied structural complexes and additional characters from the literature, ii) assessing the phylogenetic relationships within Echiura on the basis of morphological and molecular data and iii) reconstructing character evolution of the studied characters as well as the diagnostic traits used for taxonomic classification.

Therefore the discussion is composed of three main thematic complexes:

- Chapter 4.1-4.5 discusses the studied characters and their states as well as additional characters from the literature including the main taxonomic diagnostic traits (see chapter 1.2 General Introduction). The resulting character matrices are presented in Appendix 1, 2.

- Chapter 4.6-4.6.3 deals with the phylogeny of Echiura. It includes a cladistic analysis of the generated morphological data set (Appendix 2) and discusses the topology of the favoured morphological tree in comparison with the molecular tree.

- Chapter 4.6.4-4.6.5 covers the character evolution of all considered main character complexes (spermatozoa, anal sacs, larval protonephridia, gonoducts, chaetae, probosces). Furthermore, it is concerned with stem species reconstruction in Echiura and concluding remarks on traditional “Thalassematidae”

The first part of the discussion is the longest one, because in order to discriminate between potentially informative and uninformative morphological characters it was first necessary to get an overview on the morphological data available. After a subsequent critical evaluation of all relevant character states these were compiled within a data matrix considering representatives of all echiuran subgroups. Due to problems in obtaining a variety of echiuran specimens it was not possible to follow the same taxon sampling consequently for each newly studied character complex. Though, in order to provide lacking information for representatives of each echiuran subgroup the data matrix was complemented by literature data whenever possible (Tab. 4). Molecular and morphological taxon sampling differ slightly from one another not only due to the difficulties to obtain the relevant specimens. This was also due to
preliminary results from the cladistic analyses indicating an unresolved morphological tree in case the same species from the molecular analysis were included into the morphological analysis.

Basically, for each new character complex considered, the discussion is composed of an introducing paragraph to elucidate the relevance, followed by a character discussion and the assembly of potentially informative states, and finally a summarizing conclusion. In case of the spermatozoa character discussion was already done in the corresponding manuscript (Lehrke and Bartolomaeus 2009). Special emphasis has been laid on the apomorphic anal sacs. Prior to the character discussion, a chapter dealing with the question of the identity of the organs is integrated (chapter 4.2.1). In addition, a characterization of substructures is included to identify which substructures of the anal sacs are homologous and therefore comparable (chapter 4.2.2.1).

4.1 Spermatozoa

4.1.1 Comparison within Echiura

To date, only few studies on gametogenesis and sperm ultrastructure are available for Echiura (Lehrke and Bartolomaeus 2009, Tab. 1). These studies comprise species of the Bonelliidae, as well as Thalassematidae and species of the Urechidae. No data are available for Ikeda taenioides (Ikedaidae), and ultrastructural data for Echiuridae are lacking as well. In order to get a broader database for the spermatozoa across Echiura, the sperm ultrastructure of mature spermatozoa in Echiurus echiurus (Echiuridae) and Thalassema thalassemum (Thalassematidae) was analysed. By comparing these new ultrastructural data with already known data on echiuran spermatozoa from the literature, a survey of the spermatozoa morphology in Echiura is provided (Fig. 33). Terms used in this study referring to the acrosomal substructures are explained in Lehrke and Bartolomaeus (2009). Characters and character states which presently seem to be potentially informative are marked in brackets and have been included into the matrix (compare Appendix 1, 2). An elaborate discussion of the characters and character states is given in the published paper (Lehrke and Bartolomaeus 2009).

1. Shape of the whole spermatozoon (character 1): (0) longitudinal axis in line with ciliary axoneme; (1) longitudinal axis oblique relative to ciliary axoneme axis. The spermatozoa of both T. thalassemum and E. echiurus show characteristics of ect-aquasperm (sensu Rouse and Jamieson 1987), which indicates external fertilization. Like most other echiuran spermatozoa, their longitudinal axis (i.e., from acrosome to distal centriole) is straight relative to the ciliary axoneme (Fig. 33). Spermatozoa of
Bonellia viridis and Harmingia arctica clearly differ in that the head and midpiece are curved and the longitudinal axis is oblique relative to the axoneme (Franzén and Ferraguti 1992: Fig. 4). The aberrant state of the spermatozoa in the two bonelliid species correlates with the internal mode of fertilization, characteristic for all Bonelliidae (Franzén and Ferraguti 1992; Ruppert et al. 2004). Since all known Bonelliidae share this internal fertilization along with introsperm (sensu Rouse and Jamieson 1987), it is assumed that the remaining bonelliid species share a similar aberrant ultrastructure like B. viridis and H. arctica.

2. Acrosomal vesicle (=acrosome) (character 2): (0) acrosome wider than long (oblate); (1) longer than wide (elongate); (2) extremely longer than wide (filiform). In all echinurans the acrosome is differentiated into a more or less conical, bell-shaped acrosomal vesicle and the inner subacrosomal space (Fig. 33). Unlike the state in E. echiurus, the acrosome of T. thalassemum is quite elongate, but not as long as in B. viridis and H. arctica (Franzén and Ferraguti 1992). The acrosome of Listriolobus pelodes (see Pilger 1993) has almost the same length as in T. thalassemum. In order to describe the different states of the acrosome length more precisely, the relation between the length of the acrosome and its broadest diameter was chosen, using the TEM micrographs of the original descriptions (Lehrke and Bartolomaeus 2009, Tab. 2). Based on these longitudinal–transversal ratios of acrosomes a classification that becomes already obvious from the TEM illustrations appears to be useful: The acrosome may be wider than long (rather oblate) as in E. echiurus, Urechis caupo, Ochetostoma caudex, and Ikedosoma gogoshimense, or elongated as in L. pelodes and T. thalassemum, or much longer than wide (filiform, extremely elongated) as in the bonelliids B. viridis and H. arctica. Further resolution via the extension of the acrosomes seems promising (very small longitudinal–transversal ratios exclusively observed within E. echiurus and U. caupo, compare Lehrke and Bartolomaeus 2009, Tab. 2), but cannot be achieved unless additional metrical data are collected and analysed, also regarding intra-specific variation.

3. Distribution of electron dense material in the acrosome (character 3): (0) restricted to basal ring component; (1) overall. The bonelliid species B. viridis and H. arctica uniquely show electron-dense material filling the entire acrosomal vesicle (Franzén and Ferraguti 1992: Fig. 8, 11). In all other echinuran species studied, the electron-dense material is restricted to the basal ring component in the basal portion of the acrosomal vesicle. It may extend towards the apex, but without filling the acrosome completely. The extension seems to be species specific.

4. Subacrosomal space-acrosomal rod (=perforatorium sensu Franzén and Ferraguti 1992) (character 4): (0) absent; (1) present. The two studied bonelliids share another striking character of their acrosome: the subacrosomal space includes a long acrosomal rod of unknown function, which is absent in all other echinurans studied thus far (Franzén and Ferraguti 1992).
Figure 33: Survey of echiuran spermatozoa based on TEM micrographs presently available. Representative sperm types from each taxon are shown as schematic drawings except for Ochetostoma caudex to demonstrate the structural diversity within Echiura, especially the varieties of the acrosome (marked in red). Blue line indicates membrane-bound subacrosomal space (= subacrosomal vesicle). Spermatozoa of Hamingia arctica differ marginally in structure from those in Bonellia viridis, so that only a scheme of B. viridis is exemplarily given. All sperm type schemes are modified from the literature (Thalassema thalassemum and Echiurus echiurus modified from Lehrke and Bartolomaeus, 2009). O. caudex: modified from Bisewar, 1991, but it was not possible to deduce a scheme from the TEM pictures; Ikedosoma gogoshimense modified from Sawada et al., 1975; Listriolobus pelodes modified from Pilger, 1993; B. viridis modified from Franzén and Ferraguti, 1992; Urechis caupo modified from Tyler, 1965; Cross, 1984; Cross et al. 1985). Scale bar = 1µm. ar acrosomal rod, av acrosomal vesicle, br basal ring component of acrosomal vesicle, km “Kern-Mantel”, mi mitochondrion, n nucleus, pc proximal centriole.

5. Subacrosomal space-membrane bound subacrosomal vesicle (character 5): (0) absent; (1) present. 
E. echiurus proved to be unique in having a membrane bound subacrosomal vesicle. All remaining known echiuran spermatozoa seem to lack such a membrane bound subacrosomal vesicle.

6. Shape of nucleus (character 6): (0) ovoid; (1) ellipsoid; (2) barrel-shaped; (3) spherical; (4) spherical, but indented; (5) sausage-shaped. Some slight variation in the shape of the nuclei of echiuran spermatozoa was detected. The nucleus is spherical in T. thalassemum and E. echiurus (Lehrke and Bartolomaeus 2009), spherical but slightly indented apical in U. caupo (e.g. Cross 1984: Fig 1; Tyler 1965: Fig. 2), ovoid (Savada et al. 1975 for Ikedosoma gogoshimense), ellipsoid (Pilger
1993 for *Listriolobus pelodes*), or barrel-shaped (Biswar 1991 for *Ochetostoma caudex*) in the remaining thalassematid species. In *B. viridis* and *H. arctica* the nucleus is somewhat elongate ("sausage-shaped" Franzén and Ferraguti 1992) compared to the other echiurans (Fig. 33).

7. "Kern-Mantel": (0) absent; (1) present. A "Kern-Mantel" (*sensu* Leutert 1974 and Franzén and Ferraguti 1992), i.e. electron-dense material forming a cylinder around the nucleus has only been described for the bonelliids *B. viridis* and *H. arctica* so far (Franzén and Ferraguti 1992: 29).

8. Centrioles: (0) co-axial; (1) laterally displaced. The 9 x 2 + 2 axoneme of the spermatozoal flagellum emanates from the distal centriole. In all echiurans a second centriole, the proximal centriole, is present perpendicularly to the distal centriole. This proximal centriole is located directly in front of the distal centriole in all known echiuran species, except for the bonelliids *B. viridis* and *H. arctica* (see Franzén and Ferraguti 1992: Fig. 11). In these two species the proximal centriole is laterally displaced to the distal one, which is not coaxial to the nucleus (Fig. 33).

9. Flagellum: (0) without fins; (1) with fins. Among the studied spermatozoa exclusively *T. thalassemum* and *E. echiurus* have a flagellum provided with small lateral fin-like extensions of the plasma membrane (Lehrke and Bartolomaeus 2009, Fig. 2D, Fig. 3D).

4.1.2 Conclusion

The comparative study reveals characters and character states which seem to be phylogenetically informative partly at the species level and partly for higher taxonomic entities. The number and shape of mitochondria, in contrast, proved to underly individual variation in *T. thalassemum* and *E. echiurus* (compare Lehrke and Bartolomaeus 2009, chapter 4.4, Fig. 2M-P, Fig. 3F). This inconsistent appearance of mitochondria may be explained by incomplete fusion of these organelles during spermiogenesis. Thus, the number and shape of spermatozoa is a disputable phylogenetic marker and questions the view by Franzén and Ferraguti (1992) that the number and shape of mitochondria are phylogenetic informative. Accordingly, the number and shape of mitochondria is not included into the matrix. Based on the present survey, potentially synapomorphic transformations of spermatozoal structures within Echiura are as follows:

Bonelliidae uniquely share (i) filiform spermatozoa with a head and midpiece that is curved along with internal fertilization (introsperrm); (ii) extremely elongate acrosomes with electron-dense material entirely filling the acrosomal vesicle; (iii) a perforatorium (acrosomal rod) within the subacrosomal space; (iv) an elongate, sausage-shaped nucleus that is partly encircled by electron-dense material.
(‘Kern-Mantel’); and (v) a distal centriole that is not coaxial to the nucleus. All other studied echiurans are characterized by ect-aquat sperm (‘round-headed’ spermatozoon sensu Schmidt-Rhaesa 2007) with shorter acrosomes and a variable nucleus structure. Potential synapomorphies of Echiuridae, Thalassematidae and Urechidae are (i) a straight longitudinal spermatozoal axis relative to the ciliary axoneme; (ii) a conical acrosome with a basal ring component and electron-dense material restricted to the basal portion of the acrosomal vesicle; and (iii) a coaxial position of the distal centriole to the nucleus.

A close relationship between the two thalassematids *T. thalassemum* and *L. pelodes* is supported by a similar acrosome length and a special characteristic of the basal ring component (electron gray margins). Alternatively, a closer relationship between *T. thalassemum* and *E. echiurus* is indicated by a corresponding spherical shape of the nucleus and a flagellum provided with small lateral fin-like extensions of the plasma membrane.

Among the echiurans studied, *E. echiurus* proved to be unique in having a membrane bound subacrosomal vesicle, and *U. caupo* proved to be unique in having a slightly indented nucleus, whereas the nuclei are spherical or ovoid in the remaining species (Fig. 33).

### 4.2 Anal sacs

#### 4.2.1 Identity of anal sacs

Within protostome taxa the anal sacs are unique excretory organs that originate from the hindgut (rectum or cloaca) and characterize Echiura as monophyletic taxon (Harris and Jaccarini 1981; Ax 1999). By discharging waste products into the cloaca they are assumed to serve their excretory function (e.g., Baltzer 1931; Bock 1942; Stephen and Edmonds 1972; Harris and Jaccarini 1981; Ruppert et al. 2004; Schmidt-Rhaesa, 2007). In addition they may function, to some extent, in gas exchange and osmoregulation (Brusca and Brusca 2003). To date, neither the development nor the evolutionary origin of these organs is well understood. A century-old discussion on the topic has focused on two alternative hypotheses:

1. anal sacs are modified metanephridia (Hatschek 1880, Goodrich 1945, Datta-Gupta and Singh 1976, and Bartolomaeus and Quast 2005), and
anal sacs develop from hindgut diverticula (Spengel 1879; Newby 1940; Brusca and Brusca 2003; Ruppert et al. 2004: Fig. 14-6, cited from Pilger 1978, although not mentioned within). These two different hypotheses originate in contradictory data on the early development of the anal sacs. Spengel (1879), Newby (1940), and Ruppert et al. (2004: Fig. 14-6, cited from Pilger 1978, although not mentioned within) state that the anal sacs develop from a pair of endodermal hindgut evaginations which later acquire a terminal funnel. Salensky (1908), Hatschek (1880), Baltzer (1917), and Baltzer (1931) describe that the anlage of the anal sac, consisting of the end sac and one terminal ciliated funnel, appears shortly before metamorphosis. This anlage is situated among prospective muscle cells and has no connection to the hindgut. Later in development the connection to the hindgut is formed while additional funnels arise and the end sac enlarges. This origin of the anal sac anlage largely corresponds to that for polychaete metanephridia, which also differentiates from a single anlage embedded into mesodermal cells of the prospective coelomic lining (Bartolomaeus 1999; Bartolomaeus and Quast 2005). In order to find support for one of the two above mentioned hypotheses, the histology and ultrastructure of the anal sacs were studied in T. thalassemum (Thalassematidae) and compared with the histology of the hindgut of the same species (Lehrke and Bartolomaeus 2011). The comparative study in T. thalassemum as well as the limited information on anal sac formation supports the assumption that anal sacs are modified metanephridia.

The term metanephridial system was introduced by Ruppert and Smith (1988) to describe an excretory system that is composed of two different spatially separated substructures: podocytes that serve in filtration and a metanephridium that modifies the ultrafiltrate during its passage to the exterior. The metanephridium consists of a duct and usually one ciliated funnel that open into the coelom (Ruppert and Smith 1988; Bartolomaeus and Ax 1992; Bartolomaeus and Quast 2005). These structural as well as functional demands are fulfilled by the anal sacs. Following the hypothesis that the anal sacs are modified metanephridia, the end sac most likely presents a modified metanephridial duct that connects the coelomic cavity with the outer medium via hindgut and anus. Though, the number of the ciliated funnels is increased. Metanephridia that possess more than one ciliated funnel are also known from individuals of the polychaete Capitella capitata (Fabricius, 1880 see Eisig 1887) and from some clitellates (e.g., Tonoscolex sp. and Pheretima posthuma (Vaillant, 1868 see Goodrich 1945).

Podocytes that allow selective fluid transfer from one compartment into another (Ruppert and Smith 1988) are found among the cells of the peritoneum covering the anal sac in T. thalassemum, and additionally have been discovered on the coelomic side of the blood vessels (ring vessel in T. thalassemum; ventral vessel in E. echiurus, Bartolomaeus 1993). While podocytes resting on the coelomic side of the perivascular ecm is in accordance with the concept of metanephridial systems
(Ruppert and Smith 1988), the role of podocytes resting on the coelomic side of the ecm of the anal sacs remains unsolved. The podocytes at least guarantee nutrient (amino acids, glucose, etc.) transfer from the coelom into the matrix to supply the musculature of the anal sacs. Podocytes surrounding the metanephridial duct have also been previously described from some polychaetes in various subgroups (in: Sabellidae, Pectinariidae and Serpulidae, Bartolomaeus and Quast 2005), as well as in some sipunculids (e.g. Serrano and Angulo 1989 for Phascolosoma granulatum Leuckart, 1828; Adrianov et al. 2002 for Thysanocardia nigra (Ikeda, 1904), Bartolomaeus and Quast 2005 for Golfingia minuta(Keferstein, 1862)). In the latter, the nephridia possess an independent muscular system embedded in the perinephridial ecm like in T. thallasseum.

Furthermore, evidence for modification of the ultrafiltrate within the end sac has been found in T. thallasseum by the presence of numerous different kinds of vesicles within the cytoplasm of the epithelial cells indicating endo-, exo-as well as transectosis. Datta-Gupta and Singh (1976) showed that in the bonelliid Acanthobonellia pirotanensis the anal sacs are generally rich in urates, so that the anal sacs also serve in storing of excretions. The high number of pigmented spheroid granules, which were found in the inner epithelium of the end sac in T. thallasseum and in additional species (Echirus echiurus, Baltzer 1931; Bonellia viridis, Harris and Jaccarini 1981), presumably contain such excretions. In T. thallasseum and in B. viridis (Harris and Jaccarini 1981) spheroid granules were increasingly found towards the adluminal surface of the end sac and granules of the same size were also present within samples of the anal sac fluid in the latter species. This led Harris and Jaccarini (1981) to assume that the granules are excretory products produced intracellularly by the secretory epithelium of the end sac, and that they are removed periodically by continuous flushing into the lumen where they can be eliminated with the faeces via the cloaca. The structure of the muscular grid allows the assumption that the anal sac can perform contractions needed for such a manner of elimination. The outflow into the hindgut is finally controlled and accomplished by the relaxation of the anal sac sphincter. Muscular mesenteries which attach the end sac to the hindgut and body wall support the movements. Although the cellular process of granula secretion is not completely understood, the observations in T. thallasseum support the idea of secretory processes in the end sac. Moreover, the lack of structural correspondences between hindgut and anal sac support the hypothesis of anal sacs as being modified metanephridia (Lehrke and Bartolomaeus 2011). These structural differences are: the absence or presence of podocytes (on the hindgut or end sac), the thickness of the ecm with an opposed arrangement of embedded musculature, and differing characteristics of the epithelia in both organs (degree of ciliation, form and size of cells). Podocytes adjoining the peritoneum were occasionally detected in the end sac. Menon and Arp (1992) did not find any podocytes in the hindgut of U. caupo. Although not studied here, this is expected for the hindgut in T. thallasseum. Within the end sac a muscle grid built up by inner longitudinal, outer circular, and
additional delicate diagonal muscle fibers is present. The muscle cells of the end sac are embedded in a relatively thin ecm (about 5 µm thick) compared to the ecm of the hindgut (approximately 30 µm). Contrary to the end sac, the hindgut is devoid of ciliated funnels. Compared to the muscle grid of the end sac, the hindgut displays a stronger musculature, consisting predominantly of thick outer longitudinal fibers arranged in bundles, lined by a layer of diagonal fibers, followed by innermost circular muscle fibers. The inner epithelium of the end sac in *T. thalassemum* is composed of large, irregularly-formed, usually non-ciliated or scarcely ciliated cells, since only one single cilium was observed in the sections. The epithelium of the hindgut, in contrast, is composed of smaller heavily ciliated columnar cells with a uniform appearance.

4.2.2 Comparison of anal sacs within Echiura

In the past, the excretory organs have played a contradictory role for traditional classification and identification. Some authors refer to the anal sacs as being potentially important for classification (e.g. Bock 1942; Saxena 1986) and their structure as being consistently uniform within species (Saxena 1986), while others state they bear less on the taxonomy of the group (e.g. Datta-Gupta and Singh 1976). This contradictory view may be due to their variable external appearance, which on the one hand is reported from many studies (e.g. Bock 1942; Fisher 1946; Stephen and Edmonds 1972; Saiz-Salinas et al. 2000) and on the other hand this variability lacks a detailed comparative approach among the taxonomic subgroups considering all anal sac substructures. The majority of the literature refers to the anal sac external appearance mostly as being branched or unbranched (e.g. Bock 1942; Fisher 1946; Stephen and Edmonds 1972). Other studies in which information about the external structure of anal sacs could be found are often restricted to terms such as, “swollen”, “well developed“, “elongated”, “short“, or “relatively small with no conspicuous characteristics“ (e.g. Stephen and Edmonds 1972). Such short notes are subjective and include imprecise as well as confusing information. Even when different authors are cited for one species inconsistent descriptions may occur. Occasionally, the ciliated funnels are mentioned, but this information is also not comparable in many cases, since their structure is mostly inadequately described (e.g. “small”, “minute”), or their presence is not mentioned at all (e.g. Fisher 1946; Stephen and Edmonds 1972; Saxena 1986; Saiz-Salinas et al. 2000). But at least there are a few detailed descriptions on the internal morphology of the anal sacs. Ultrastructural studies of the anal sacs are limited to *Bonellia viridis* (Bonelliidae) (Harris and Jaccarini 1981). With the recent study of the anal sacs in *T. thalassemum* (chapter 3.2.1, see also Lehrke and Bartolomaeus 2011) a second species, a member of the Thalassematidae, is investigated ultrastructurally. Light microscopical data are available for *B. viridis* (Baltzer 1917, 1931), *Echiurus*
Echiurus (Spengel 1880), Echiurus abyssalis (Baltzer 1931), Urechis caupo (Seitz 1907) and T. thalassemum (Bock 1942).

Due to the scattered data on the anal sacs, previous information was generally not applicable for phylogenetic inferences. Since a consistent terminology was also missing to date, the objectives of this study are (1) to identify and to characterize anal sac substructures to find out which substructures are actually comparable among the taxa, and (2) to establish primary homology hypotheses for these substructures by comparing them systematically across Echiura. A character discussion provides an evaluation of the character states. By doing so, this study also aims in contributing towards a starting point to use anal sac data in future studies for cladistic analyses.

To identify anal sac substructures the literature was thoroughly studied and a wide range of methods (histology, SEM, TEM, cLSM) was applied to species that were available. Although it was not always possible to treat every studied species in the same accurate manner or method, all specimens (except for Urechis unicinctus) were relaxed prior to fixation in order to ensure comparability. Since data referring to the relative size and length of the anal sacs seem to correlate generally with age (Fisher 1946) and size of the individual (Saiz-Salinas et al. 2000), the anal sac length and size are generally not considered in this study.

The new data on the anal sac morphology in various species (chapter 3.2.1, chapter 3.2.2) together with a broad survey of literature data indicate that anal sacs can usually be subdivided into an end sac, the tubules (funnel stalks) and the ciliated funnels. The funnels in turn can be further subdivided into their neck regions and funnel lips. These substructures are hypothesized to be homologous among one another and therefore comparable.

4.2.2.1 Characterization of substructures

**End sac:** Usually, each anal sac consists of an end sac and numerous small ciliated funnels covering the end sac. Generally, the end sac is anteriorly directed within the coelom and significantly larger in diameter than the funnel or its stalk at its broadest diameter. A circular sphincter muscle usually marks the transition between end sac and hindgut (Fig. 8A). End sac cells can be distinguished from hindgut cells by the presence of podocytes occasionally adjoining the peritoneum (Fig. 10A; Fig. 11B), by the presence of a muscle grid built up by inner longitudinal, outer circular, and additional delicate diagonal muscle fibers (opposed arrangement in hindgut), and large, irregularly-formed, usually non-ciliated or scarcely ciliated epithelial cells facing the end sac lumen (hindgut with smaller heavily
ciliated columnar cells with a uniform appearance). These inner epithelial cells have the capability to comprise small as well as larger granula-like inclusions that are presumably part of the excretory process (Harris and Jaccarini 1981; Lehrke and Bartolomaeus 2011).

**Tubules:** The tubule consistently displays the same maximal diameter as the maximal marginal diameter of the funnel, but a significantly smaller maximal diameter than the end sac. The tubule generally presents the stalk of an associated funnel; it is demarcated from the funnel by a neck region. The lumen of the tubules is connected with the lumen of the end sac, either directly or indirectly via additional tubules. Such branching tubules are classified as follows: The tubules that branch off first are here referred to as primary tubules; the tubules that branch off the primary tubules laterally are smaller in diameter and length and are consequently named secondary tubules. The tubules that branch off the secondary ones are referred to as tertiary tubules and are the smallest regarding diameter and length (Fig.14C, D; Fig. 15A). On the cellular level, tubules basically share the same structural characteristics as the end sacs: large, aciliated cells with their nuclei widely spaced, and have the capability to comprise small as well as larger granula-like inclusions. Since end sac and tubule epithelium share similar characteristics it is adopted here that both are generally homologue structures.

**Funnels:** Each funnel presents a filtration unit associated to the anal sac via certain structures: merely a neck region or a tubule, i.e. the lumen of the funnel is connected with the lumen of the end sac either through a slender canal through the end sac epithelium or tubules attached to the end sac. Funnel cells are not clearly discriminable from cells of the neck region since both share the same gross characteristics of their epithelia (small, non-muscular cells, large nuclei closely-packed, ciliation present). But on the ultrastructural level some slight differences were detected (compare following paragraph “neck region”). Funnel cells can be more easily separated from cells of end sac / tubules due to structural differences (large, aciliated muscular cells, with nuclei widely spaced). In addition, all funnel cells generally lack small as well as large granula-like inclusions (Fig. 7B); occasionally they may inhibit spherical electron dense inclusions (Fig. 12 E).

**Neck region:** Generally, the neck region represents the basal most part of the funnel and connects the funnel lumen with the lumen of the end sac, either directly or indirectly via a tubule. The neck region is an externally visible neck like constriction that has a significantly smaller external diameter as the funnel and tubule, i.e. a straight segment that is externally discriminable from the funnel segment. Since funnel and neck region cells are almost similar in structure (small cell size, large nuclei closely-packed, ciliation present), putative neck region cells cannot be separated from the remaining funnel cells by histology. But ultrastructurally they show a slightly different shape: neck region cells seem to be more flat and squamous compared to the remaining funnel cells (shown for *T. thalassemum*). In addition their cilia are more uniformly arranged compared to the upper funnel cells where they are
forming groups at the outer and inner margin. However, as the funnel cells, neck region cells can be more easily distinguished from the cells of the tubule and end sac in the histological sections. All neck region cells generally lack small as well as large granula-like inclusions (Fig. 7B); occasionally they may inhibit spherical electron dense inclusions (Fig. 12D, G).

The finding of a somewhat muscular neck region in the bonelliid *M. haswelli* (Fig. 15F) stands in contrast to the observations on the neck regions in all remaining echiuran subgroups. Due to a lack of ultrastructural data in *M. haswelli*, it cannot be excluded that the neck region in this species is possibly interspersed with contractile projections of muscular cells which originally have to be assigned to the tubules. The only presently available ultrastructural study on bonelliid anal sacs reports on such contractile projections of muscular cells for *B. viridis* (Harris and Jaccarini 1981). However, due to the above mentioned major cellular similarities it is proposed here, that the neck regions in all subgroups are homologue structures. This hypothesis is argued although slight differences on the ultrastructural level in funnel and neck region cells regarding cell shape and assembly of cilia have been observed in *T. thalassemum* (Thalassematidae).

**Lip:** The lip is usually heavily ciliated and encircles the conical or cylindrical funnel segment. Its tissue is slightly bulging outward towards the coelomic cavity (Fig. 12A-C).

**Nephridial muscles:** The muscle cells found underneath the inner lining of the anal sacs are termed nephridial muscles here. As in the end sacs they have a musculature in the tubules, but this is only weakly developed compared to the muscle net of the end sacs. Occurrence and thickness of these muscular fibers decreases from several comparatively thick fibers within the primary tubules to a few filiform fibers in the secondary tubules. At present no histological data available for the tertiary tubules. In species that develop tubules, the neck region may contain occasionally single muscular fibers (Fig. 15F); species without tubules are devoid of muscular fibers within the neck region. Harris and Jaccarini (1981) report on irregular funnel contractions in freshly dissected specimens of *Bonellia viridis* (Bonelliidae) that may serve to seal the funnel. The same movements (“stretching and retracting”) were observed by Greef (1879) in *Echiurus echiurus* (Echiuridae). 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data U. Steinmetz) reveal unambiguously that the funnel is composed of non-muscular cells. Muscle cells may occur in *T. thalassemum* and *E. echius*, but do not exceed the base of the funnel (Lehrke and Bartolomaeus 2011; unpubl. data U. Steinmetz). Thus, Greefs (1879) observations of muscular funnel cells in *E. echius* are rejected.

### 4.2.2.2 Characters and character discussion

The following discussion is based on a cautious evaluation of the available literature data together with the results in chapter 3.2. Since there are generally very scattered data on the anal sac development (Spengel 1879; Hatschek 1880; Salensky 1908; Baltzer 1917, 1932; Newby 1940; Fisher 1946) and the intraspecific variation of the anal sac morphology (Menon et al. 1964), the characters and character states presented here, still have to be treated with caution, but are intended as a starting point for further studies on the intra-/ interspecific variability and phylogenetic significance of the excretory organs in Echiura. Characters and character states which presently seem to be potentially informative are marked in brackets and have been included into the matrix (Appendix 1, 2). For identification of substructures that are here referred to compare previous chapter (characterization of substructures).

1. **Anal sacs (character 10):** (0) absent; (1) present. Usually, all echiurans possess one pair of anal sacs attached to the hindgut (e.g. Pilger 1993). These excretory organs are unique among protostome taxa and thus are regarded as apomorphic for Echiura (Harris and Jaccarini 1981; Ax 1999). Quotations in the literature that report on the absence of the anal sacs in Echiura may be explained by damaged and poorly preserved specimens (e.g. *Bruunellia bandae*; *Listriolobus riukiuensis*; *Sluiterina sibogae*; *Thalassema antarcticum*; see Stephen and Edmonds 1972), or juvenile specimens where small anal sacs might have been overlooked (e.g. *Thalassema ovatum* see Stephen and Edmonds 1972). In addition many of these species descriptions are based on a single individuum only, which are thus problematic. Although it is presently not possible to re-investigate these species, it is assumed that all adult echiurans have anal sacs. References that deviate from the paired occurrence of the organs are very rarely found in the literature (e.g. Biseswar 1988 reported on three tubular anal sacs in *Ochetostoma natalense* Biseswar, 1988) and may be explained by incomplete development.

2. **Composition of anal sacs (character 11):** (0) end sac absent; (1) end sac present. The majority of known echiuran taxa develop end sacs. Species that seem to lack a uniting end sac are uniquely observed in a few Bonelliidae (e.g. *Alomasoma noradpacificum* see Stephen and Edmonds (1972, Fig. 44E); *Ikedella misakiensis* Fig. 36E; *Pseudobonellia biuterina* see Johnston and Tiegs 1919, p. 220;
some specimens of *Acanthobonellia pirotanensis* Menon et al. 1964, Fig. 3A). In these species the tubules seem to arise directly from the wall of the hindgut, and the anal sac is exclusively composed of ciliated funnels sitting atop their tubules. An end sac is not visible from the exterior (Fig. 36D, E). Compared to all remaining anal sacs, anal sacs lacking an end sac, are generally shorter, thus they are often described as tuft-like organs (Stephen and Edmonds 1972). Limited information is available for these tuft-like forms, presumably lacking end sacs. This published information is hitherto restricted to the external morphology of these forms. Thus applying the recent proposed definition for the identification of end sacs (see “characterization of substructures”), merely the external diameter of the structure in question can be used at present, i.e. to distinguish among tubules and end sacs. Consequently, in several cases it remains arguable whether an end sac or its remains had originally developed or not. This issue becomes obvious by comparing the available illustrations of the anal sacs in *Acanthobonellia pirotanensis* which vary considerably (José 1964; Menon et al. 1964). According to Menon et al. (1964) illustrations some of the specimens featuring a common duct (from which the tubules arise) have approximately the same diameter as the corresponding tubules. This observation would support the lack of an end sac, but in some individuals the ducts and the main branches are expanded; this on the other hand would support the hypothesis that these specimens develop end sacs. The latter observation is supported by José (1964) because his illustration clearly shows the presence of an end sac contrary to the inconsistent specimens of Menon et al. (1964). So, in some cases, based on the external morphology alone and without re-investigations of the respective specimens, it is not possible to distinguish between tubules and end sacs. Promising are histological and cLSM studies of the corresponding tissues which may detect differences in the specification of the musculature (e.g. weak versus strong musculature, sphincter muscle present or not; compare characterization of substructures). Nevertheless, the presence or absence of an end sac is basically coded here, because in some species, there is no trace of an end sac, though this decision is made on external characters alone (e.g. Fig. 36D, E). Such short tuft-like forms seem basically related to the absence of end sacs, thus, as a consequence, merely the absence or presence of an end sac is coded, not the shape (“tuft-like).

3. Connection between end sac and hindgut (character 12): (0) via two pores; (1) via one pore. In most species the connection of the end sac to the hindgut is realized via two pores, i.e. the end sacs open separately into the hindgut (Fig. 34A). In two thalassematids (*Arhynchite inamoenus* see Stephen and Edmonds 1972, p. 418 and *Ochetostoma australiense* see Saxena 1986, p. 66) merely a single pore is present (Fig. 34B).
Figure 34: Opening modes of the end sacs to the hindgut. For reasons of clarity the end sacs are illustrated as simple sacs and the funnels are omitted. **A**: State as observed in the majority of echiuran species. The connection to the hindgut is realized via two pores, i.e. each end sac opens separately into the hindgut. **B**: State very rarely observed within Thalassematidae (*Arhynchite inamoenus* see Stephen and Edmonds 1972; *Ochetostoma australiense* see Saxena 1986. The end sacs open via a short common duct, respectively a single pore into the cloaca. *an* = anus, *cl* = cloaca, *es* end sac, *hd* = hindgut.

A single pore is so far also known from several specimens of *Acanthobonellia pirotanensis* (Bonelliidae). In these specimens the single pore presents the ending of a narrow duct from which the tubules arise (the primary tubules coming from two sides unite into the duct on the ventral side of the hindgut). At present it remains unresolved whether this duct presents an end sac or not (compare character 11). Surprisingly, Menon et al. (1964, Fig. 3A) noticed one additional condition in some *A. pirotanensis* individuals: an opening mode via several pores, i.e. several primary tubules open directly into the hindgut, seemingly lacking a common duct (or end sac). Given the fact all investigated specimens actually belong to the same species, this would indicate an enormous intraspecific variation regarding the opening mode, i.e., single pore vs. several pores (Menon et al. 1964). On the other hand, following Baltzer’s (1932) and Fisher’s (1946) hypothesis that the number of tubules increases with age, it seems also possible, that the different states may be an age-related phenomenon because in the specimens lacking a common duct, an increased branching of the tubules is noticed. This higher amount of tubules may have hampered the identification of the opening mode in these specimens. However, it seems also likely that the specimens featuring several pores actually belong to a yet undescribed species. Supportive for this hypothesis is the finding that a direct opening mode via several pores (presumably lacking a common duct or end sac) is found in several other bonelliid species (*Ikedella misakiensis, Amalosoma paradolum, Amalosoma nordpacificum*) (compare character 11). In addition, Menon et al. (1964) state that the *A. pirotanensis* specimens came from different collection sides (Pirotan Island, NW India, Arabian Sea and Andaman Islands, SE India, Indian Ocean). Anyhow, without a re-investigation of *A. pirotanensis* individuals as well as without further information on individual variation and developmental studies of the anal sacs in general, it is
presently difficult to assess Menon’s hypothesis (1964). Thus, a final statement is not possible at present. But since the opening mode via several pores (each pore is associated with one tubule respectively) generally seems dependent to the absence of an end sac, this state is presently not coded into the matrix. The connection between end sac and hindgut via one or two pores in contrast is coded into the matrix, because it is assumed that these states are more consistent and possibly contain some phylogenetic signal.

4. Shape of end sacs (character 13): (0) sac-like; (1) tubular. Although there are several additional short characterizations for the anal sacs in some species such as “ball shaped” (Stephen and Edmonds 1972), “voluminous at the base, distally slender” (Annandale and Kemp 1915; Fisher 1948), “elongated” (Stephen and Edmonds 1972) or “crescent-shaped pouches” (Fisher 1946), the shapes which are coded here as potentially phylogenetic informative characters are simply sac-like (Fig. 35C-E) and tubular (Fig. 35A, B, Fig. 36A-C; for further information see “problematic characters”). This is on the one hand due to the limited material available, and on the other hand based on in-vivo observations of the sac-like end sacs in Metabonellia haswelli (Bonelliidae) and the tubular end sacs in T. thalassema (Thalassematidae). After a comparison of relaxed in-vivo material and relaxed fixed material in these two species, both character states seem to be independent from the fixation process implemented in this study. Specimens in both species fixed for SEM, histology (azane staining) as well as TEM, cLSM in T. thalassema displayed the same states as the in-vivo material (Fig. 9, Fig. 13C, D). Additional support for both shapes comes from many quotations in the literature for sac-like or tubular forms (Stephen and Edmonds 1972; Bisewar 2006, 2010). The tubular form in T. thalassema is clearly supported by Bock (1942) and Stephen and Edmonds (1972). Saiz-Salinas et al. (2000) used the classification into sac-like and tubular forms before for the identification of a few bonelliid species. However, their sac-like forms are rather reminiscent of the anal sacs lacking an end sac.

Comparison of available literature and own results reveals, that hitherto, all valid thalassematid species share a tubular end sac with all presently known members of Ikedaidae (Ikeda pirotensis, Ikeda taenioides), with some members of Bonelliidae (e.g. Torbenwolfia galatheae, Jakobia densopapillata; Pseudoikedella achaeta, Maxmuelleria lankesteri), and presumably with all Echiuridae (shown for Echiurus echiurus, e.g. Baltzer 1931 or unpubl. data U. Steinmetz), but not with Urechidae (Stephen and Edmonds 1972) (Fig. 35, 36). Urechis species generally have a sac-like end sac with a conspicuous external morphology, which seems to be caused by rounded apical extensions of the inner end sac epithelium (Fig. 22; Fig. 35C). The majority of Bonelliidae has sac-like end sacs (Stephen and Edmonds 1972).
Figure 35: Survey on the gross-morphology of the studied anal sacs (schematic representations), based on material fixed for SEM and histology. A-B: Thalassematidae; C: Urechidae; D-F: Bonelliidae. Only one of the original two organs is depicted. A: Tubular end sacs with a constant external diameter and conical funnels in *T. thalassemum*. B: Tubular end sacs with conical funnels in *A. adelaidensis*, presumably tapering towards the tip. C: Sac-like end sacs with a cauliflower-like surface and slender funnels in *U. caupo* and *U. unicinctus*. D-E: Sac-like end sacs branching into different orders of tubules (funnel stalks), each tubule terminating into a conical funnel. D: *B. viridis*. The end sac branches into long primary (*ptu*) and shorter secondary tubules (*stu*). E: *M. haswelli*. The end sacs branches into long primary, shorter secondary and occasionally very short tertiary tubules (*ttu*). hd hindgut.

Figure 36: Survey on the gross-morphology of the anal sacs (schematic representations) in Ikedaidae (A), Echiuridae (C) and Bonelliidae (A-E) compiled from the literature. A: Tubular end sac with numerous long tubules, each terminating into a conical funnel as observed in some bonelliid species (e.g. *Sluitierina kaikourae*, *Maxmuelleria lankesteri* as well as in all known *Ikeda* species (*Ikeda pirotransis*, *Ikeda taeirioides*). Differences may occur in the extent of branching; modified from Edmonds (1985), Bock (1942), Menon and Datta-Gupta (1962) and Ikeda (1904). B: Tubular end sac with well spaced short tubules as observed in *Torbenwolffia galathea*, each terminating into a funnel of unknown shape (modified from Biseswar (2010) and Zenkevitch (1966)). C: Tubular end sac with well spaced funnel stalks of unknown identity as observed in the bonelliid *Jakobia densopapillata* (modified from Biseswar, 2006) or the echiurid *Echiurus echiurus* (modified from Baltzer 1931). D-E: Tuft-like anal sacs presumably lacking an end sac. The tubules seem to arise directly from the wall of the hindgut. D: *Alomosoma nordpacificum* with unbranched tubules; modified from Zenkevitch (1958). E: *Ikedella misakiensis* showing branched tubules; modified from Stephen and Edmonds (1972). hd hindgut, *ptu* primary tubule, *stu* secondary tubule.
5. Muscle net within the end sac

In *T. thalassemum* a muscle grid built up by single inner longitudinal, single outer circular, and additional single delicate diagonal muscle fibers have developed (Fig. 9B, C; Lehrke and Bartolomaeus 2011). Other thalassematine taxa are described without having such diagonal fibers (e.g., *Ochetostoma septemyotum* see Datta-Gupta and Singh 1976). Additional diagonal muscle fibers have hitherto also been detected in *Echiurus echiurus* (Echiuridae) (Baltzer 1931) and within the bonelliids *Maxmuelleria lankesteri* (see Bock 1942) as well as in *Metabonellia haswelli* (Fig. 15B, D). The fibers seem to be quite thick and numerous in the latter species, and only sparsely distributed and thin within *E. echiurus* end sacs. Spengel (1880) did not recognize any diagonal fibers in his studies on *E. echiurus*. However, this may be due to the filiform structure of the diagonal fibers which may be easily overlooked. This may also be true for additional species in which diagonal fibers have not been yet detected (e.g., *Bonellia viridis*, Harris and Jaccarini 1981). Longitudinal fibers were found frequently in other bonelliids (Bock 1942; Datta-Gupta and Singh 1976) and *Anelassorhynchus adelaidensis* (Thalassematidae) (Fig. 17B, C). The significance of these observations, however, remains to be evaluated. Presently, it seems likely that all echiurans have developed a muscular net composed at least of outer circular and inner longitudinal fibers that are able to contract the entire anal sac.

Arrangement of muscle fibers (character 14): (0) single (isolated) fibers; (1) fibers concentrated in groups (bundles). Compared to the hitherto described end sac musculature in other species *Urechis caupo* (Urechidae) shows some specific characteristics. The fibers are concentrated in groups (bundles) in *U. caupo* (Fig. 22B-D), which stands in contrast to Seitz (1907) who reports on a weakly developed musculature in *Urechis chilensis*. However, grouped muscle fibers within the end sacs are so far also known from *Ikeda pirotansis* (Ikedaidae) (Datta-Gupta and Singh 1976). The remaining species studied here exclusively have single (isolated) fibers. Thus, the presence of grouped or isolated fibers is included into the matrix.

Texture of muscle net (character 15): (0) fine meshed; (1) wide-meshed. The muscle net in *U. caupo* seems to be wide-meshed (Fig. 22B) compared to the fine-meshed net in the remaining studied species (Fig. 9B, C). Comparative data from the literature for additional species are missing in this respect.

6. Mesenteries (character 16): (0) rope-like; (1) laminar. Data on the shape of the mesenteries have not been described in previous studies. In this thesis the general structure (SEM data) was investigated in *B. viridis* (Bonelliidae), *U. caupo* (Urechidae) and *U. unincinctus* (Urechidae). Within the *Urechis* species their appearance is rope-like contrary to the expanded laminar attachments in *B. viridis*. Due to specimen preparation we lack presently any information about the termination of the mesenterial
strands within these specimens. In the remaining bonelliid and thalassematid species analysed here, no noticeable attachment structures have been observed, but it cannot be excluded that inconspicuous mesenteries in these species have got lost somehow during preparation.

While studying the literature about the anal sacs it turned out that the kind of attachment of the end sacs via muscular mesenteries may be potentially phylogenetic informative, too, because various patterns mainly regarding the attachment mode are described in the literature at least for a few taxa. These patterns basically refer to (i) the absence and presence of mesenteries in general, (ii) the location of the attachment on the end sac (e.g. attached only basal or distal, or both), (iii) their extension over the organ (e.g. anchored at about two thirds of the sac or fastened for the whole length etc.), and (iv) the fixation of the mesenteries on the various components within the coelomic cavity and/or the body wall. Although, mesenteries seem to have developed in the majority of known species, only scattered data on the noticed patterns (i-iv) are available: Presently, mesenteries seem to be absent in some Thalassematidae (e.g. Anelassorhynchus dendororhynchus andsee Fisher 1946; this study; Arhynchite inamoenus see Fisher, 1946; T. thalassemum, Bock 1942, this study) and in some Bonelliidae (e.g. Pseudoikedella achaeta see Zenkevitch, 1958). However, it cannot be excluded that these may have also been generally overlooked. Mesenteries may be joined exclusively to the body wall as known from many Bonelliidae and Thalassematidae, or to the alimentary canal, as it has been reported so far only for a few thalassematid species (e.g. Arhynchite hiscocki see Edmonds 1960). Within species that have mesenteries exclusively fastened to the body wall some additional patterns are apparent: End sacs may be attached only at their distal ends in some Bonelliidae and Thalassematidae (e.g. Bengalus longiductus; Anelassorhynchus inanensis see Ikeda, 1904), or end sacs may be fastened only at their base, which is thus far known exclusively from a few Thalassematidae (e.g. Anelassorhynchus adelaidensis). But thalassematid anal sacs may also be anchored at about two thirds of the length from their base (e.g. Arhynchite californicus see Fisher, 1949). Within bonelliid species most of the anal sacs (inclusively the branches) are joined to the body wall (e.g. Ikedella misakiensis). Although the data on the distribution and shape of the anal sac mesenteries are presently too scattered, it is proposed here, that these characteristics seem to serve as promising characters, as soon as additional comprehensive data for more species are available. So far, data on the distribution of the mesenteries are not included into the matrix.

7. Tubules (character 17): (0) absent; (1) present. Based on the present data, merely a tubule can be unambiguously demarcated from the neck region and its funnel by their external and internal structure (compare chapter “characterization of substructures”). Thus, a tubule is here referred to as a stalk of an associated funnel, a term which is meanwhile broadly accepted in the literature (Ikeda 1904; Fisher 1946; Menon and Datta-Gupta 1962; Hughes and Crisp 1976; Ruppert et al. 2004). A tubule is here
regarded as homologous among taxa featuring such stalks (“branches” sensu Nishikawa 2002). Traditional classification already recognizes the presence and absence of tubules (“stalked or sessile funnels” sensu Bock 1942, Datta-Gupta 1976, Datta-Gupta and Menon 1976) as well as the branched or unbranched nature of the anal sacs (Bock 1942; Fisher 1946; Datta-Gupta 1976). Both characters were used to distinguish at the family level between Bonelliidae and Echiuridae (including Thalassematidae) by simply recognizing “anal sacs usually consisting of branched tubules” (Fisher 1946) or anal sacs as being “unbranched elongated sacs bearing usually sessile funnels” (Bock 1942, Fisher 1946, Datta-Gupta 1976). The survey on the morphology of the anal sacs showed that branched end sacs always develop tubules and vice versa that unbranched end sacs never develop tubules. So, the presence of tubules is equivalent to the occurrence of the branched or unbranched nature of the end sacs which is also reflected by the corresponding structure of the epithelia (compare chapter “characterization of substructures”). As a consequence, merely the presence or absence of tubules is coded here.

Tubules have so far developed in the majority of Bonelliidae (Bock 1942, Menon et al. 1964, Datta-Gupta and Menon 1976) and all known Ikedaidae. Tubules are lacking in all known Thalassematidae (Lehrke and Bartolomaeus 2011), Urechidae (Stephen and Edmonds 1972) and putatively Echiuridae (Stephen and Edmonds 1972; Datta-Gupta 1976; Datta-Gupta and Menon 1976). In some taxa the structure of the funnel stalks remains an arguable character since their composition is not known (Bonelliidae: e.g. Jakobia densopapillata, Fig. 36C), or the data are contradictory (Echiuridae: Echiurus echiurus compare Baltzer 1931, Datta-Gupta 1976, unpubl. data U. Steinmetz; Fig. 36C). Thus, it is presently difficult to assess whether the stalks in the above mentioned species, which vary between short-stalked and somewhat elongated (Fig. 36B, C), originally are reduced tubules or somewhat elongated neck regions. For the bonelliid J. densopapillata it is assumed that the stalks present reduced tubules. The inconsistent descriptions on the length of the funnel stalks in E. echiurus may be due to the investigation of different developmental stages.

Tubules may display a great variation in their branching (Fig. 35D, E; Fig. 36E). This variation has not been included into the matrix here but has been critically discussed in the chapter “problematic characters”.

8. Funnels

It is adopted here that all completely developed anal sacs are beset with ciliated funnels. Sporadic reports that certain species have no funnels at all (e.g. Anellassorhynchus vegrandis see Stephen and Edmonds 1972, Listriolobus bahamensis see Stephen and Edmonds 1972) are seriously doubted here, because the anal sacs cannot take up their excretory function without the ciliated funnels. Additionally,
the species descriptions of the latter two taxa are based on a single or on damaged specimens; it appears also likely that the funnels have been overlooked.

There is some confusion in the literature especially on the length of the funnel stalks which do not show the characteristics of tubules. This is due to the inconsistent usage of terms in the literature without providing a definition. The results of this study together with the investigation of available literature data suggest that there is more variation between the states “sessile” and “stalked” which have been used in traditional classification (Bock 1942, Datta-Gupta 1976). Therefore, in order to improve the recognition of certain character states, the characterization of substructures resulted in the implementation of partly new terms. The term “sessile” was retained but further specified (see character 18, 19). The term “neck region” which was already used by Harris and Jaccarini (1981) for B. viridis funnels is recovered in almost all studied funnels, thus it is regarded as homologous among the taxa and it is included into the matrix.

**Funnel shape**

The majority of known anal sac funnels is bell-shaped, i.e. more or less conical (e.g. Stephen and Edmonds 1972) (Fig. 37). Deviations from this shape have been merely found in one member of the Bonelliidae (Stephen 1956 for Amalosoma paradolum) and in two species of the Urechidae (this study and Seitz 1907 for Urechis caupo; this study for Urechis unicinctus). Differentiations of the funnel structure in Urechis species are considered by characters 18 and 19. Regarding the findings in A. paradolum (Stephen 1956), these are doubted here because they are not supported by other authors who have investigated this species (e.g. Fisher 1946). Consequently they are not coded into the matrix. According to Stephen (1956) the funnels are “vase-shaped” (widest at funnel base) in A. paradolum and conical in Amalosoma eddystonense.

**Funnel polymorphism (character 18):** (0) absent; (1) present. Within this study it turned out that U. caupo and U. unicinctus proved to be unique regarding their funnel shape. All funnels in the Urechis specimens share a similar slender shape based on a similar maximal diameter, which is basically lower than within the other studied species (Fig. 37). Thereby in each specimen, slender-conical and slender-cylindrical funnels occur simultaneously. These findings are partly supported by Seitz (1907) who reports on a funnel dimorphism in Urechis chilensis. But contrary to the findings in this study, Seitz (1907) reports on short and broad funnels as well as on slender and elongated funnels to be simultaneously present. Since the slender form of the funnels in U. caupo and U. unicinctus seems to be dependent on the structure of the funnels (compare character 19), the form itself is not coded here. However, the absence or presence of a funnel dimorphism is included into the matrix, although for the majority of species no detailed information on the funnel structure is available.
Funnel structure (character 19): (0) neck region present; (1) neck region absent. Generally a funnel may be equipped with a neck region (Fig. 37A, B, E), or not. This case has been exclusively found in Urechidae (Fig. 37C, D). In the latter case the funnel is exclusively composed of a slender-conical or slender-cylindrical segment which lacks an externally assignable neck segment (shown for U. caupo and U. unicinctus). Although it is neither externally (SEM), nor observable within the histological sections (shown for U. caupo, Fig. 23A-C), it cannot be excluded that a neck region with a smooth transition may be detected by ultrastructural (TEM) investigation in these species in the future. In T. thalassemum funnels in which the neck region is only very short and difficult to identify externally, TEM data improved the differentiation significantly (compare Lehrke and Bartolomaeus 2011; chapter “characterization of substructures”). However, for the majority of species no detailed information on the funnel structure is available.

Funnel neck region (character 20): (0) short/ inconspicuous (sessile appearance of funnel; (1) distinct (short-stalked appearance of funnel). By comparing the neck regions within the investigated thalassematid species, it turned out that the neck region may display different lengths (Fig. 37A, B): The neck region may be short/ inconspicuous giving the funnel a sessile appearance (Fig. 37A; Lehrke and Bartolomaeus 2011 for Thalassema thalassemum), or it may be distinct, giving the funnel a short-stalked appearance (shown for Lissomyema mellita, Anelassorhynchus adelaidensis; Fig. 37B). Both states are supported by literature data also for additional thalassematid taxa. Sessile funnels are reported from several Ochetostoma species, e.g., Ochetostoma indostinense see Stephen and Edmonds (1972) or Ochetostoma baronii see Biseswar (1988), Fisher (1946), Lanchester (1905) and Arhynchite arhynchite see Stephen and Edmonds (1972). It may be that quotations like “tiny little funnels”, which occur comparatively often within the literature (e.g. Stephen and Edmonds 1972), actually present sessile funnels. However, according to Stephen and Edmonds (1972) Anelassorhynchus mucosus and Thalassema fuscum have “short-stalked funnels” suggesting that these taxa may be equipped with a distinct neck region. Contradictory are the information available for Echiurus echiurus. Datta-Gupta (1976) refers to “sessile funnels” whereas the illustrations given in Spengel (1880), Baltzer (1931) and U. Steinmetz (unpubl. data) show rather somewhat elongated stalks that seem to lack characteristics of tubules. The inconsistent descriptions for E. echiurus may be due to the investigation of different developmental stages, but this cannot be unambiguously determined prior to a re-investigation of adult E. echiurus specimens. However, the species studied here showing differences in their neck region lengths at the adult stage. It remains to be seen whether the detected differences can be sustained by additional metrical data, or whether developmental studies will weaken the significance of this character. Interestingly, in the studied bonelliid species the neck regions are distinct and display approximately the same lengths provided that the same hierarchical tubule levels are compared respectively.
Nevertheless, the presently included character states “inconspicuous” neck region (“sessile” funnel) and “distinct” neck region (“short-stalked” funnel) have to be discretized in the future more precisely on the basis of additional metrical data and developmental studies.

Figure 37: Survey on the various funnel shapes (schematic representations) and associated substructures observed within the studied anal sacs, broadly based on material fixed for SEM and histology. A-B: Thalassematidae (scale bar = 20 µm); C-D: Urechidae with funnel dimorphism (scale bar = 20 µm); E: Bonelliidae (scale bar = 80 µm). A: Thalassemata thalassemum. Funnel with short, inconspicuous neck region (gray), giving the funnel a sessile appearance. B: Lissomyema mellita and Anelassorhynchus adelaidensis. Funnel with distinct neck region, giving the funnel a short-stalked appearance. C: Slender conical funnel shape in Urechis caupo and Urechis unicinctus. D: Slender cylindrical funnel shape in U. caupo and U. unicinctus. Note the lack of the neck region. E: Secondary tubule terminating into a conical funnel with distinct neck region in Metabonellia haswelli. cf ciliated funnel, cs conical segment, cys cylindrical segment, li lip, nr neck region.

Arrangement of funnels upon the end sac (character 21): (0) mostly distal; (1) mostly proximal; (2) decrease from proximal to distal; (3) increase from proximal to distal; (4) uniform; (5) arranged in rows. A great variation has been found in the literature on the distribution of funnels (lacking a tubule) upon the end sac within Thalassematidae (Prashad and Awati 1929, Jones and Stephen 1955, Stephen and Edmonds 1972) and Urechidae (this study; Seitz 1907). In the majority of taxa the arrangement of funnels upon the end sac is uniform as observed here in the thalassematids T. thalassemum and A. adelaidensis. Deviations from this arrangement are so far known from some additional thalassematid taxa (e.g., distal arrangement as observed in Ochetostoma capense or proximal arrangement as observed in Ochetostoma bombayense. Funnel arrangements with a rather smooth decrease from
proximal to distal are reported from *Echiurus echiurus* (Spengel 1880 and Baltzer 1931) and *Urechis unicinctus* (Figs. 20B). Surprisingly in *Urechis caupo* it is the other way round: the funnels are generally more densely packed towards the distal free end compared to the other two thirds of the organ (Fig. 20A). In addition to these distribution patterns Seitz (1907) reports on a peculiarity in *Urechis caupo* that is associated with the external cauliflower-like swellings of the end sacs. The dimorphic funnels are said to be present in different areas on the end sac. The slender elongated forms are said to have developed in-between the irregular swellings, the shorter ones are stated to be exclusively present apical upon the swellings. However, in *U. caupo* and *U. unicinctus*, such a separation of the different funnel forms was not traceable. Instead, the dimorphic funnels of both species were found predominantly in different areas on the end sac. The slender elongated forms are said to have developed in-between the irregular swellings, the shorter ones are stated to be exclusively present apical upon the swellings. However, in *U. caupo* and *U. unicinctus*, such a separation of the different funnel forms was not traceable. Instead, the dimorphic funnels of both species were found predominantly in different locations: in *U. caupo* the funnels sit predominantly apical upon the swellings and additionally in-between, whereas in *U. unicinctus* both funnel forms where predominantly restricted to the space between the irregular swellings (Fig. 19D). Whether the different funnel spreading noticed in the studied species as well as in *U. chilensis* (Seitz 1907) turns out to be apomorphic for the respective species, presently remains unclear. Thus, it is not coded into the matrix. Further studies are necessary to unravel the significance of the distribution of funnels, especially in the Urechidae. Except for Seitz (1907) no literature data on this topic is presently available. Another peculiarity is reported from some Thalassematidae, where the funnels are arranged in rows (e.g. two rows in *Anelassorhynchus branchiorhynchus*, *Anelassorhynchus dendrorhynchus* and *Anelassorhynchus microrhynchus*; three rows in *Ochetostoma hornelli*, see Stephen and Edmonds 1972).

4.2.2.3 Problematic character states

External morphology

**Shape:** Since the anal sacs are expandable organs which eliminate their content by contractions into the cloaca (Harris and Jaccarini 1981), it seems likely that the inconsistent literature data for some species may be explained by different structural artefacts recorded during the fixation process. In a few thalassematids the anal sacs are described as swollen at the base and distally slender (e.g., Stephen and Edmonds 1972 for *Anelassorhynchus porcellus* Stephen and Edmonds 1972 for *Anelassorhynchus dendrorhynchus* and Fisher 1946 for *Listriolobus pelodes*). Since a grid-like muscular system was found underlying the end sac epithelium, this peculiar shape may represent an artefact resulting from fixing the animal during anal sac contraction. Artificial preservation could also explain differing illustrations of the end sac shape in *L. pelodes*, which show either a more tubular or a basally swollen
anal sac (Amor 1971 versus Fisher 1946). Contradictory information is also given for *Pseudobonellia biuterina* (Bonellididae): In Stephen and Edmonds (1972) the anal sacs are described as simply being “tuft-like masses”...with tubules “opening directly into the cloaca”, which is clearly illustrated by Johnston and Tiegs (1919) (see Stephen and Edmonds 1972, Fig. 51E). Edmonds (1960) in contrast reports on a “slight outpocking of the cloacal wall” from which the tubules arise, which might be interpreted as small end sac pouch. In the light of the comparative data on the anal sac morphology it seems more likely that the end sac is missing as observed in several bonellid species (character 11), and the described “outpocking” may presumably present an artefact resulting from fixing the animal during hindgut sphincter contraction. Ambiguous descriptions like “elongate or tubular sacs” are comparatively often found in the literature (Stephen and Edmonds 1972), and can presently not be unambiguously ascertained for many species. However, without a re-examination of many species, especially those featuring the last named characterizations and those presumably lacking an end sac, the definite shapes are hard to evaluate.

**Asymmetries:** Unusual modifications of the anal sacs that cannot be easily explained by contraction artefacts are mainly known from Bonellididae. According to Fisher (1946, Pl. 31, Fig. 6) the paired end sacs in *Amalosoma paradolum* are differently constructed: on the left side a “common chamber” which can be interpreted as tubular end sac, on the right side in contrast a “common chamber” seems to be lacking and the tubules arise directly from the wall of the cloaca. Fisher (1946) concluded on this that the number of tubules increases with age, the heterogenic state being somehow age-related. The specimen he investigated was sexually mature, however, whether this implies that the number of tubules increases throughout ontogeny remains unknown. Additional unusual anal sac shapes that are described in the literature on the basis of merely one specimen and/or species cannot be assessed so far until re-investigations of the specimens (e.g. “ball shaped” anal sacs in *Bonellia thomensis* see Stephen and Edmonds 1972; or “crescent-shaped” anal sacs in *Nellobia eusoma* see Fisher 1946: Pl. 29, Fig 3; or tubular anal sacs branching at the tip for *Archibonellia michaelseni* see Stephen and Edmonds 1972, Fig. 45C).

**Diameter:** Another arguable character in Thalassematidae is the significance of the mean diameter at the distal free end of tubular end sacs. In contrast to the constantly wide end sac in *Thalassema thalasseum* (Lehrke and Bartolomaeus 2011), the end sac in *Anelassorhynchus adelaideensis* is tapering towards the distal end so that the tip is presumably pointed (Fig. 17A). Literature data support a tapering end in *A. adelaideensis* (Edmonds 1987, Fig. 9). Furthermore, pointed tips of tubular end sacs are also known from additional thalassematid species (e.g. *Ochetostoma erythrogrammon* Fig. 55B in Stephen and Edmonds 1972; *Ochetostoma baronii* see Biseswar 1988). However, this character state remains to be evaluated on a basis of additional data.
**Branching pattern:** In the investigated bonelliid specimens a high variation regarding the branching pattern of the tubules is noticed that generally corresponds to the literature data for additional species. Tubules in both, Bonelliidae and Ikedaidae, may be generally branched, featuring tubules of consecutive hierarchical levels (i.e. primary, secondary, tertiary tubules etc.), or the tubules may be generally unbranched. In the latter case exclusively primary tubules are present. In taxa featuring branched tubules the branching may be more or less intense as described in *Ikedella misakiensis* with at least three branches (Stephen and Edmonds 1972), or with up to five branches as described in *Ikeda taenioides* (Ikeda 1904). The branching may also be less intense (e.g. *Sluiterina kaikourae*, see Edmonds 1985) or the tubules are not branching at all (e.g. *Sluiterina flabellorhynchus* see Saiz-Salinas et al. 2000; *Choanostomellia bruuni* see Zenkevitch 1964; *Ikeda pirotantis*). Thereby, it turns out that the pattern of branching is usually not restricted to a certain end sac shape. In tubular as well as tuft-like forms unbranched or branched tubules occur. However in sac-like end sacs it appears as if exclusively branched tubules have developed. In *Bonellia viridis* primary and secondary tubules have predominantly developed, but also very few primary tubules lacking any secondary tubules emerge from the end sac. These results together with the hypothesis that the number of tubules generally increases with age (Baltzer 1931, Fisher 1946) generally questions the view of a consistent character and supports the idea of an age-related phenomenon. But likewise, it cannot be excluded that the inconsistent branching patterns are also due to intra-specific variation. There are some conflicting notes in the literature that refer to a varying branching pattern in some species. According to Herdmann (1898) the tubules never branch in *Maxmuelleria lankesteri*. Bock (1942) in contrast reports for the same species that a branching sometimes occurs. However, the significance of these states presently seems not assessable and therefore the branched or unbranched nature of the tubules is not included into the matrix. If ontogenetic impact or intra-specific variation may also explain the various distribution pattern of secondary tubules (Fig. 38) remains to be resolved, too. In *Metabonellia haswelli* and several other bonelliids (e.g. *Bonellia viridis* in Greef 1879; *Ikedella bogorovi* in Zenkevitch 1964; *Ikedella misakiensis* in Ikeda 1904) the secondary tubules are uniformly distributed (Fig. 38A); in *B. viridis* they concentrate mainly basally (Fig. 38B) and in *Protobonellia annularis* the secondary tubules are exclusively distally concentrated (Fig. 38C) (Biseswar 1992). Contrary to own observations on *B. viridis*, previous studies report on alternative branching patterns for this species. A more or less uniform distribution of the secondary tubules is indicated in Greef (1879, Tafel 7, Fig. 76, 79) whereas in Shipley (1901) the secondary tubules concentrate more distally. Therefore, the distribution of secondary tubules seems to be a doubtful character that may be generally age-related or a subject of intra-specific variation, at least in *B. viridis*, and possibly in other species. As a consequence the three described character states (Fig. 38A-C) are not included into the matrix.
Internal morphology

Epithelial cells: Characteristics of the end sac epithelium are presently also hard to evaluate. The epithelial cells of the investigated species generally resemble each other in being aciliar, (or if at all sparsely ciliated), in being relatively large compared to their funnel cells and in featuring an irregular shape. The epithelia are simple. Since the epithelium in Metabonellia haswelli (Bonelliidae) appears somewhat disintegrated nothing can be stated on the characteristics of these cells (Fig. 15B). Referring to the ciliation and the shape of the epithelial cells in additional echiurans described in the literature inconsistent notes are available. The ciliation is quoted as either being ciliated in some bonelliids (e. g. Acanthobonellia pirotanensis see Datta-Gupta and Singh 1976; B. viridis see Harris and Jaccarini 1981) or as being not ciliated in Echiuridae (Echiurus echiurus in Spengel 1880; Baltzer 1931; unpubl. data U. Steinmetz) and Urechidae (Urechis chilensis in Seitz 1907). In many quotations the epithelial cells are described as cuboidal or columnar (e. g. Bisewar 1983, Datta-Gupta and Singh 1976). However, various forms of the epithelial cells were also reported in E. echiurus (unpubl. data U. Steinmetz) and Urechis chilensis (Seitz 1907).

Undulations of epithelia: Undulations of different degrees in the epithelia are found hitherto in various taxa, also in the specimens studied here. They may be very low as observed in T. thalassemum or extremely elongated as detected in U. caupo. According to Spengel (1880) these inner mounds are
due to contraction artefacts at the time of fixation (shown for *E. echiurus*). Although the entire specimen of *T. thalassemum* and *U. caupo* were relaxed prior to fixation, some slight undulations were observed in *T. thalassemum* and elongated undulations were present in *U. caupo*. However, it cannot be excluded that the specimens where not completely relaxed despite the treatment with magnesium chloride and thus the differences in the degree of undulation are here proposed to be not consistent character states. Spengel (1880) proposes on the other hand that these undulations may also be partly responsible for the elasticity of the organ, which would imply that they also might have developed differently among the different taxa. A final conclusion is impossible until comparative physiological studies are made testing different filling conditions and their impact on the end sac epithelium. Making the process more difficult is the fact that contradictory azane stained sections were found in *U. caupo*. The undulations may show two different conditions: (i) their cells may be situated around a thin compact band of *ecm* located median within the involution (Fig. 22C), and (ii) the epithelial cells are situated on a thin band of *ecm*, but this *ecm* is lined apically by flat peritoneal cells (Fig. 22D). The first state can be interpreted as a rather constant character; the second state would imply that the undulations rather present temporary foldings of the inner epithelium.

Besides the inner elongate undulations, *U. caupo* and *U. unicinctus* have apical rounded bulges into the surrounding coelom giving the end sac overall a cauliflower-like appearance (Fig. 35C). In *U. caupo*, these bulges seem to emerge from elevations of the epithelium. This is supported by Seitz (1907) who has studied this species by light microscopy. Since the underlying muscle net is comparatively strong and wide meshed, the apical bulges may also be explained by contractions during fixation as already noticed by Seitz (1907) and Fisher (1946). Within the somewhat relaxed specimen of *U. caupo* studied here, it cannot be excluded that, the anal sacs showed some residual contractions during the fixation process, too. However, *U. unicinctus* which was not relaxed at all displays also these bulges, so it may be alternatively independent from the usage of magnesium chloride.

**Ecm:** Observed differences in the thickness of the *ecm* (20-70 µm) within the end sacs of the investigated specimens ranging from 20 µm in *Anelassorhynchus adelaidensis* to 70 µm in *U. caupo*, can presently not be discretisized. There is no information on this in the literature.
4.2.3 Conclusion

For the first time, based on the given identification of anal sac substructures, it is possible to compare anal sac morphology systematically. It turns out that on the one hand there is more variation within the anal sac structures as previously noticed, which may be phylogenetically informative, and on the other hand a wide range of character states already described in the literature seem to be caused by artefacts, may be age related or may due to intra-specific variation. Discrimination between informative and uninformative characters remains difficult especially due to the lack of developmental data and studies on intra-specific variation. Characters that presently appear not applicable for phylogenetic inferences are some states that refer to the general shape of the end sacs, the branching pattern of the tubules (funnel stalks) and characteristics of the end sac epithelium as well as the thickness of the underlying ecm. Generally promising characters but presently too scattered data available refer to i) the structure and arrangement of the funnels, ii) partly the gross morphology of the end sacs, iii) the differentiation of the muscle net within the end sacs and iv) the sort of anchorage of the end sac via mesenteries. Based on some of these characters a few limited statements are possible; these seem to be phylogenetically informative partly at the species level and partly for higher taxonomic entities, but probably not at the generic level as previously suggested by Saxena (1986).

Generally, Bonelliidae are most diverse in their anal sac gross-morphology compared to the remaining subgroups; but merely some bonelliid taxa (of various genera) uniquely share (i) anal sacs lacking an end sac; (ii) short tuft-like anal sacs with a direct opening mode of the primary tubules into the hindgut via several pores (each pore is associated to one primary tubule respectively). Furthermore, it turned out that Bonelliidae and Ikedaidae uniquely share (i) end sacs accompanied by tubules (long funnel stalks) which was already tentatively formulated by Nishikawa (2002, “branches” which are “…highly reminiscent” to one another).

All known Thalassematidae seem to possess a tubular end sac which is shared by all known members of Ikedaidae as well as with some members of Bonelliidae and Echiuridae. Apomorphic for Thalassematidae may be the presence of funnels with a short/ inconspicuous neck region giving the funnel overall a sessile appearance. Urechidae uniquely share (i) a strong wide meshed muscle net within the end sacs which is presumably responsible for the conspicuous cauliflower-like external appearance of the anal sacs in a slightly contracted state; (ii) a funnel dimorphism (slender conical + slender cylindrical funnels simultaneously present); (iii) funnels presumably lacking a neck region or rather featuring a indistinguishable neck region with a smooth transition from the conical (or cylindrical) segment to the funnel base.
4.3 Larval protonephridia

Like most polychaetous annelids, Echiura show a biphasic life cycle with a planktonic trochophore larva (Baltzer 1917, 1931; Newby 1940; Miner et al. 1999; Rouse 1999). These can either be planktotrophic like in most echiurans, or lecithotrophic, which exclusively occurs within Bonelliidae. Basically, trochophore larvae are characterized by a specialized circumlarval ciliary belt (the prototroch), a sensory apical organ, and one pair of transitory protonephridia (Rouse 1999; Nielsen 2004). These protonephridia (head kidneys sensu Hatschek 1878, 1880) are located in the periphery of the larval blastocoel anteriorly to the anlagen of the trunk mesoderm (Hatschek 1880; Goodrich 1945). During metamorphosis the head kidneys disintegrate and become functionally replaced by segmentally arranged nephridia in annelids or by the anal sacs in most echiurans (Baltzer 1931; Goodrich 1945; Bartolomaeus and Ax 1992). For Bonellia viridis (Bonelliidae) dwarf males it was shown that the head kidneys are functionally replaced by definite protonephridia (metanephridia sensu Baltzer 1931) in the posterior region of the male (Schuchert 1990). Anal sacs are lacking presumably in all bonelliiid dwarf males (e.g. B. viridis Baltzer 1912, 1931; Schuchert and Rieger 1990).

4.3.1 Comparison within Echiura

Only scarce information on echiuran larval protonephridia is published (compare Kato et al. 2011, Tab. 1). These studies are exclusively based on the light microscopical level and comprise one species of the Bonelliidae (Bonellia sp., Baltzer 1914), as well as one species of the Echiuridae (Echiurus sp., Hatschek 1880, Goodrich 1910). No studies are available for Ikedaidae and Urechidae (but see Fig. 1D, E in Hessling 2002), data for Thalassematidae were lacking as well. In order to get a broader database for the head kidneys across Echiura, the ultrastructure of larval head kidneys in Thalassema thalassemum (Thalassematidae) was analysed in collaboration by Kato et al. (2011, co-author J. Lehrke), providing the first ultrastructural data on larval protonephridia in Echiura. By comparing our ultrastructural data with already known data from the literature, a survey of the head kidney morphology in Echiura is provided in the following. Despite the very limited data on the structure of echiuran head kidneys the characters and character states presented here are regarded as potentially phylogenetic informative and have thus been included into the matrix (compare Appendix 1, 2). Nevertheless, their phylogenetic significance remains to be evaluated. Within Annelida, structural data on the head kidneys provide a number of discrete characters and some of these have found to be characteristic for high-ranking subtaxa within the Annelida (Bartolomaeus 1995, 1998; Quast 2007).
Thus, the proposed characters for echiuran head kidneys are included into the matrix (marked in brackets).

The definite protonephridia of the dwarf male in *B. viridis* are not included into the following survey of potentially informative characters and character states because these protonephridia are not homologue to the larval head kidneys in other species. Thus, they are unsuitable for phylogenetic inference. The definite protonephridia in *B. viridis* arise in postlarval stages and are differently positioned at the posterior end of the trunk (Schuchert 1990). This is in contrast to the larval protonephridia which are located in the presumptive head region.

1. **General shape of head kidney (character 22):** (0) branched; (1) tubular (unbranched). The head kidneys in *T. thalassemum* (Thalassematidae) are tubular (Fig. 39B). They are composed of two elongate cells, a terminal cell comprising the filtration structure and a duct cell leading to the exterior via a nephridiopore (simple opening). The duct cell is almost double the length of the terminal cell (Kato et al. 2011). Basically, this tubular structure resembles the state found in *Bonellia* sp. (Bonelliidae) (Fig. 39A). Baltzer (1914) described the head kidneys in late larvae of *Bonellia* sp. as unbranched tubes with a blind terminal end. Dawydoff’s (1959 Fig. 711 A) illustration of a head kidney in *Bonellia* sp. shows overall also a tubular form. In *Echiurus abyssalis* and *Echiurus* sp. (Echiuridae) in contrast, the head kidneys are branched, consisting of short-branched tubular ducts (Hatschek 1880; Goodrich 1910; Baltzer 1917; Korn 1960). Arguable information is available for *Urechis caupo* (Urechidae). According to Hessling’s (2002, Fig. 1D, E) immunocytochemical study the nephridia could be interpreted as branched, but this can presently not be substantiated. His eLSM–micrograph rather leaves room for speculation than providing an unambiguous picture of the head kidneys. Though Hessling’s (2002) results disproved Newby’s (1940) finding *U. caupo* larva do not develop any protonephridia. Data are completely missing for Ikedaidae.

2. **Terminal structure**

The terminal structure generally comprises the terminal cell(s) and the filtration structure. Characters which could be potentially informative are the number of cells involved, the number of cilia per cell, the absence or presence of circumciliary microvilli and the composition of the filter (Kato et al. 2011).

*Number of cells (character 23):* (0) several; (1) one. In *T. thalassemum* the terminal structure is built up by one terminal cell only (Kato et al. 2011). In *E. echiurus* in contrast several terminal cells are involved (Hatschek 1880; Goodrich 1910). Nothing is known on the number of terminal cells in other echiurans.
Cilia per cell (character 24): (0) several (=multiciliated terminal cell); (1) one (=monociliated terminal cell). In *T. thalassemum* the terminal cell has 6 cilia and is thus multiciliated (Kato et al. 2011). One single cilium in contrast is reported in *E. echiurus* head kidney, rendering the terminal cell monociliated (Hatschek 1880; Goodrich 1910). Contradictory information is available for *Bonellia* sp. According to Baltzer (1914) the terminal end of the tube bears solenocytes, i.e. terminal cells with a single cilium. This stands in contrast to Dawydoff’s (1959) illustration which suggests instead a multiciliary tuft. Additional data for the remaining echiurans are still missing.

![Figure 39: Survey on echiuran larval protonephridia presently available. Schematic representations not to scale (A-B: tubular, unbranched organs; C: branching protonephridia). A: *Bonellia* sp. (Bonelliidae) modified from Dawydoff (1959). The terminal structure is multiciliated, although it is not known how many cells build up the terminal structure. No further details on the structure of the duct, the filter structure or the nephridiopore are known. B: *T. thalassemum* (Thalassematidae) modified from Kato et al. (2011). The organs are composed of two elongate cells, a terminal cell building the terminal structure and a duct cell leading to the exterior. The filter is formed by layers of elongate microvilli which surround the lumen of the terminal cell in a tubular manner. C: *Echiurus* sp. (Echiuridae) modified from Goodrich (1910, 1945). The protonephridia ("solenocytes") are composed of several short-branched tubular ducts. The terminal structure is composed of several monociliated terminal cells. The tubular filter structure is presumably composed of a perforated cytoplasm (pers. comm. B. Quast). No further details on the composition of the filter structure, the duct or the nephridiopore are known. ci cilium, cp cytoplasmatic protrusion, cr ciliary rootlet, mv microvilli, n nucleus, np nephridiopore, tc terminal cell.](image-url)
Circumciliary microvilli (character 25): (0) absent; (1) present. Circumciliary microvilli are absent in *T. thalassemum*. Since the studies of Goodrich (1910, 1945) and Dawydoff (1959) were based on light microscopic data, that hardly permit an identification of microvilli, a final statement regarding the absence or presence of cmv within the head kidneys of *Bonellia* sp. and *E. echiurus* is not possible to date. Nothing is known for the remaining echiurans.

Filter structure (character 26): (0) Solenocyte sensu Goodrich (1910, 1945); (1) ring of elongate microvilli emerging from the terminal cell. In *T. thalassemum* the filter structure is formed by two to three layers of elongate microvilli which surround the lumen of the multiciliated terminal cell in a tubular manner. A thin layer of extracellular matrix encloses the outer microvilli of the tubular structure. The tips of the microvilli project into the lumen of the adjacent duct cell but are not directly connected to it (Kato et al. 2011). In *E. echiurus* the filter structure is not ultrastructurally known, but according to Goodrich’s (1910, 1945) light microscopic studies the head kidneys are composed of solenocytes, i.e. terminal cells with a single cilium surrounded by an elongate tubular filtration structure composed of cytoplasmatic protuberances. Because Goodrich was not able to assign the fine structure of the cytoplasmatic protuberances, it remains to be investigated whether the latter structures are microvilli after all in *E. echiurus*. These can only be identified on the basis of ultrastructural data by the detection of actin filaments within the microvilli (pers. comm. B. Quast). The filter structures of the remaining echiurans remain unknown.

3. Duct. The duct generally comprises the duct cell(s), their cilia and microvilli. Characters which could be potentially informative are the number of cells involved, the number of cilia per cell and the absence or presence of microvilli. To date, only data for *T. thalassemum* are available in this context (Kato et al. 2011). Anyhow, for reasons of clarity they are coded into the matrix.

Number of cells involved into the composition of the duct (character 27): (0) several; (1) one. In *T. thalassemum* the duct is composed of one cell only. Nothing is presently known on the number of cells involved within the remaining echiurans. As soon as additional data are available character state (0) could be differentiated more precisely.

Number of cilia per duct cell (character 28): (0) several; (1) one. In *T. thalassemum* about 15 cilia project from the adluminal membrane into the lumen. Data are missing in this respect for other echiurans; but as soon as additional data are available character state (0) could be discretized more precisely and state (1), which is presently just a speculation, could be reassessed.

Microvilli emerging from the duct cells (character 29): (0) absent; (1) present. In *T. thalassemum* the duct lacks any microvilli that insert from the duct cells. Instead some finger-like cytoplasmatic processes of unknown function have developed. Data for additional species are still missing.
4. Nephridiopore (character 30): (0) via a specialized nephropore cell; (1) nephropore cell absent. In *T. thalassemum* a specialized nephropore cell is absent. The most distal end of the duct cell leads to the exterior via a simple opening. Nothing is known on this structural detail in the remaining echiurans.

4.3.2 Conclusion

Since to date only light microscopical data were available for one species of the Bonelliidae and one species of the Echiuridae, our study in collaboration with Kato et al. (2011) provides the first (ultrastructural) data for a member of the Thalassematidae. Due to the fact that only the very few data are hitherto published, limited statements are possible. The comparison reveals some structural correspondences of the larval head kidney of the thalassematid *T. thalassemum* and the bonelliid *Bonellia* sp. These are: i) the general tubular, unbranched shape of the organs, ii) the multiciliarity of the terminal structure (although it is not known how many cells build up the terminal structure in *Bonellia* sp.) and iii) the absence of circumciliary microvilli in the terminal structure. These correspondences have to be treated with caution, because a consistent comparision was not possible due to a lack of sufficient data on the filter structure, the duct and the nephridiopore in *Bonellia* sp. The same applies to the available data on *Echiurus* sp. Anyway, the general shape of the organs in *Echiurus* sp. and some present information on the terminal structure indicate enormous structural differences compared to the other two echiurans. These are i) the general arborescent morphology (several short-branched tubular ducts), ii) the monociliarity of the terminal structure, iii) the composition of the terminal structure of several cells, and iv) the presence of a so called “solenocyte” (according to Goodrich 1910) with a tubular filtration structure. Strikingly, the definitive protonephridia in the dwarf males of *B. viridis* (Bonelliidae) are similar to to the head kidneys in *Echiurus* larvae since they possess several terminal cells (Schuchert 1990). Still these organs are not homologue to the larval head kidneys in other echiuran species because the definite protonephridia arise in postlarval stages and are differently positioned. Moreover, unlike the state in *T. thalassemum*, the definite protonephridia in *B. viridis* possess an unbranched multicellular duct, a higher number of cilia and one differing structural detail of the filter. Contrary to the filter structure of the definite protonephridia in *B. viridis*, anastomotic interconnections between the microvilli-like cell protrusions are lacking in the larval protonephridia of *T. thalassemum*.

Although based on very few structural data, the larval protonephridia seem promising for providing a comparatively high number of discrete potentially phylogenetic informative characters and character
states within Echiura, provided that the taxon sampling will be enlarged in the future. Within some polychaete groups ultrastructural characters (with states regarding the terminal cells and filtration sites) already have been proven to be phylogenetically significant (compare Kato et al. 2011). Thus, on the basis of the enlargement of comparative ultrastructural data within Polychaeta and Echiura by future studies, characters and character states of the head kidneys might be helpful to contribute to the search on the sister group of Echiura within polychaetes, and to clarify their evolution within Echiura (compare Kato et al. 2011).

4.4 Gonostomal lips

General

In the older literature the gonoducts of Echiura are often referred to as “anterior nephridia” (Herdman 1897), “mixonephridia” (Goodrich 1945), “segmental organs” (Spengel 1879; Greef 1879; Baltzer 1931) or gonoducts (Baltzer 1931; Bock 1942; Dawydoff 1959). Additional terms are “genital pouches” (Rietsch 1886; Stewart 1900) or “genital sacs” (Ruppert et al. 2004). Based on histological and histochemical studies, Datta-Gupta and Singh (1976) revealed that the sac-like organs are exclusively responsible for reproduction, i.e. the temporary storage and release of gametes. Thus, acting exclusively as gonoducts, it was postulated that they do not play a role in excretion as previously suggested by others (e.g. Goodrich 1945). Although this view is widely accepted today, the term “nephridia” or “metanephridia” still occurs in many recent textbooks (e.g. Stephen and Edmonds 1972; Edmonds 2000; Brusca and Brusca 2003; Ruppert et al. 2004), which somehow reflects the ongoing discussion on their origin and the arguable hypothesis that they are homologue to annelid segmental nephridia (Baltzer 1931, Goodrich 1945). However, within this study the term gonoduct is adopted, since it is the most appropriate term related to its function (Datta-Gupta and Singh 1976; Saxena 1983; Pilger 1993). Each organ possesses a gonostome and a sac-like duct with an external pore. The gonostome is usually equipped with ciliated lips by which the sac-like organs are able to select mature gametes from the coelomic cavity. The gametes are stored in the duct and released via their genital pores. These are usually located on either side of the ventral midline.

Characters of the gonoducts have always played an important role in traditional taxonomy of the group (Bock 1942; Fisher 1946; Stephen and Edmonds 1972; Saxena 1983; Datta-Gupta 1974, 1976; Datta-Gupta and Menon 1976). This includes the number of gonoducts, their paired or unpaired arrangement, common or separate genital pores and characters related to the gonostomes (position and structure). All these characters have been used in traditional classification, either in combination
among one another, and together with other morphological characters. Regarding gonoductual characters, primarily the number of gonducts (Fisher 1946; Stephen and Edmonds 1972; Datta-Gupta 1976), the structure and position of gonostomes (Fisher 1946, 1949; Datta-Gupta 1976); Datta-Gupta and Menon 1976) and the presence of common or separate genital pores (Saxena 1983) were generally considered as key features.

However, as many other taxonomic characters used in echiuran classification the gonoduct data could not contribute to a phylogenetic system so far. Due to the fact that the data were not standardized throughout any taxonomic entity. In addition similar character states are often used on multiple taxonomic levels, so that taxonomy based on gonoduct data appears contradictory. Moreover, although for the majority of species comparable data are still missing, many studies report on some variation especially for the number of gonducts within several species (e.g. Lacaze-Duthiers 1858; Stewart 1900; Ikeda 1904; Baltzer 1931; Bock 1942; Edmonds 1963; Stephen and Edmonds 1972; Datta-Gupta 1974). This variation is either due to the sex of the specimen (e.g. Ikedosoma gogoshimense see Ikeda 1904, Sato 1934; Ikedosoma elegans see Ikeda 1907), or varies considerably within individuals of a single species (e.g. six to 11 gonducts in Anelassorhynchus fisheri, see Datta-Gupta 1974).

Outstanding is, however, the comparatively low number of gonducts in the majority of Bonellioidea (usually one, sometimes two, rarely three) and the extremely high number of gonducts in Ikeda taenioides (Ikedaidae) which is stated to be 200-400 (e.g. Ikeda 1904; Stephen and Edmonds 1972). Compared to the second Ikeda species, I. pirotansis, the number is much smaller and varies between 16-40 gonducts (Datta-Gupta and Menon 1976; Stephen and Edmonds 1972). Since the paired or unpaired arrangement of gonucts seems to be also affected by some variation and mixed asymmetrical arrangements including pairs, clusters and single gonucts within a single species, this character is also hard to evaluate satisfactorily at present (compare Datta-Gupta 1974; Stephen and Edmonds 1972). In addition, sometimes incorrect data occur with regard to the arrangement of gonucts: Ruppert et al. (2004) use the assumed unpaired arrangement of gonucts in Bonellioidea and Ikedaidae as a synapomorphy for a clade comprising both subgroups, although the second member of the Ikedaidae, I. pirotansis, is quoted for featuring pairs of gonucts (Stephen and Edmonds 1972; Datta-Gupta and Menon 1976).

Thus, altogether, a re-examination of gonoductual characters seems appropriate prior to phylogenetic inference, especially because of the discrepancies in the literature, a broad lack of detailed data but also because of the remarkable variation observed in many echiuran gonucts.

As a starting point, this study focuses on one character, the shape of the gonostome and their lips. To better distinguish between relevant character states of the shape of adult gonostomes has been comparatively studied during reproduction period and outside the season in Thalassema thalassemum
(Thalassematidae) by scanning electron microscopy (SEM) and histological studies (azane staining). The results for *T. thalassemum* compared with literature data of the shape of the gonostomal lips in additional species indicates that their shape is not consistent. As the character discussion shows, the lip structure is rather dependent on various influences in these species (compare chapter “problematic characters”). Nevertheless, together with the investigation of additional species (*Anelassorhynchus adelaidensis, Urechis caupo, Urechis unicinctus, Metabonellia haswelli*), it turned out, that the differentiations of the lips may provide promising character states in future, given that some basic conditions concerning data recording are regarded.

4.4.1 Comparison within Echiura

The following character discussion refers to characters related to the gonostome. Characters and character states which presently seem to be potentially informative are marked in brackets and have been included into the matrix (compare Appendix 1, 2).

1. General appearance of gonostome (character 31): (0) sessile; (1) stalked. The stalked or sessile character of a gonostome is often referred to for certain species in the literature (e.g. Stephen and Edmonds 1972). Stalked gonostomes with a conspicuous tubular stalk are known from very few Thalassematidae (e.g. *Arhynchite pugettensis* see Fig. 53F in Stephen and Edmonds 1972), many Bonelliidae (e.g. *Maxmuelleria lankesteri*, Fig. 42A-C, *Bonellia viridis*, Fig. 42D-E; *Acanthobonellia rollandoe* and see Fig. 43G in Stephen and Edmonds 1972) and all Ikedaidae (*Ikeda taenioides* see Fig. 24 in Ikeda 1904; *Ikeda pirotansis* see Fig. 58B in Stephen and Edmonds 1972). Sessile gonostomes in contrast indicate, if at all, in some cases a very short, inconspicuous stalk (e.g. *U. caupo*, Fig. 40A), but normally even a short stalk is not visible from exterior (e.g. *Thalassemata thalassemum*, Fig. 27A-B; *Echiurus echiurus*, Fig. 41A, C). So-called sessile gonostomes occur within all Urechidae, presumably all Echiuridae, and in many Thalassematidae. For several Bonelliidae, the morphology of the gonostomes is still unknown, so it remains unresolved whether this subgroup also develops sessile gonostomes (compare Stephen and Edmonds 1972). Bonelliid species for which data are known hitherto have all stalked gonostomes. Occasionally, a distinction into “short” or “long” stalks is made within the literature for a few bonelliid taxa, but these relative terms or states are presently not assessable due to the limited data available.

However, since this study has shown for *T. thalassemum* that the sessile appearance of a gonostome is not affected by different grades of sexual maturity (Fig. 27), which is also reported from additional thalassematid and urechid species (*Listriolobus pelodes* in Fisher 1946; *U. caupo*, Fig. 40), the general
appearance of a gonostome as stalked or sessile is assessed here to be a consistent character, as already suggested by Saxena (1983). Thus, it is included into the matrix, although being aware that data for many taxa are still missing.

2. Shape of gonostomal lips (character 32; compare also chapter “problematic characters-shape”): (0) not spirally coiled (not filamentous); (1) spirally coiled (filamentous). Within the investigated species, one member of the Thalassematidae, *Anelassorhynchus adelaidensis* and two members of the Urechidae, *Urechis caupo* as well as *Urechis unicinctus* show characteristic elongate spirally coiled lips (Fig. 30, Fig. 40). The degree of coiling varies slightly among the species, mainly in being less strongly coiled in *A. adelaidensis* compared to the two urechid specimens. This difference cannot be assessed due to a lack of comparative data. The general coiled or uncoiled nature of the lips is described for numerous species, including the studied species here (e.g. Stephen and Edmonds 1972). Basically, coiled elongate filamentous lips occur in several thalassematid genera (*Anelassorhynchus*, *Ikedosoma*, *Listriolobus* and *Ochetostoma*, but see exceptions below), within all Urechidae, but are unknown from Bonelliidae or Ikedaidae (e.g. Stephen and Edmonds 1972).

Considering the traditional classification exclusively on the basis of spirally or not spirally coiled lips, the devisions on family, sub-family and generic level lack a reasonable ground of classification because they are inconsistent. Stephen and Edmonds (1972) separate traditional Bonelliidae and Echiuridae by characterizing Bonelliidae with gonostomal lips that are never spirally coiled (besides other characters) and Echiuridae by lips that may or may not be spirally coiled (besides other characters). Datta-Gupta and Menon (1976) used the differentiation of the longitudinal muscles and the shape of lips to separate traditional Ochetostomatinae and Thalassematinae within traditional Thalassematidae: Ochetostomatinae are characterized by spirally coiled lips, whereas members of the Thalassematinae may or may not have spirally coiled lips. The inconsistent distribution of spirally coiled lips within the Thalassematinae also continues within Ochetostomatinae, in contrast to the definition of the sub-family (sensu Datta-Gupta and Menon 1976). There are species described that are said to lack spirally coiled lips although these should possess such lips according to their generic description or their membership to Ochetostomatinae (e.g. *Ochetostoma indosinense*, *Ochetostoma. decameron*, *Listriolobus hexamyotus* see Stephen and Edmonds 1972). On the basis of the presently available literature data, the genus *Ikedosoma* appears to be the only thalassematid taxon that develops spirally coiled lips consistently within its corresponding species.
Based on Fishers observations (1946) that the coiled nature does not change significantly within different maturity conditions (Fig. 40A, B) this state is regarded here as a permanent character. Thus, it is coded into the matrix. The length of spirally coiled lips which is quoted occasionally in the literature as being “short” (Ikedosoma elegans see Stephen and Edmonds 1972) or “long” (several Ochetostoma species, see Stephen and Edmonds 1972), cannot be assessed to date, and generally requires more metrical data for phylogenetic inference. Characters and character states referring to the angle formed by the edge of the spirally coiled lips, the diameter of the lips and the diameter of their ciliated groove in contrast provided evidence that these are dependent on the sex of the specimen (shown for Urechis caupo, in MacGinitie 1935).

States that fall into the category “not coiled” are discussed separately, because it is presently not possible to determine the various shapes of the remaining taxa unambiguously (compare “problematic characters”).

3. Position of gonostome (character 33): (0) basal; (1) central; (2) near distal end; (3) terminal. According to Fisher (1946, 1948, 1949) the position of the gonostome is an important taxonomic character, a view that is adopted by Stephen and Edmonds (1972) and Saxena (1983). It is said to be especially useful to differentiate between the genera of the Bonelliidae. This subgroup is separated into genera largely on the basis of a total of six general morphological characters, one of which refers to the position of the gonostome (Fisher 1946). Datta-Gupta (1967) questions the position of the gonostome as a permanent character but he neither specifies his hypothesis nor proves it on the basis of his data. For T. thalassemum it was shown that the position of the gonostome does not change with the sexual maturity of the specimen (Fig.27). Thus, the author follows here basically the view of Fisher (1948) until reliable studies on the intra-specific variation or the effects of the sexual maturity are analyzed.
All known Thalassematidae, Echiuridae and Urechidae develop basal gonostomes, i.e. coelomic openings near the genital pore, within the basal most third of the gonoduct. The majority of bonelliid taxa possess such basal gonostomes, too, but several genera also develop so called distal gonostomes (e.g. Bonelliaopsis, Eubonellia, Ikedella, Metabonellia, Pseudobonellia, Vitjazema). According to Stephen and Edmonds (1972) a distal position means “towards the coelomic extremity”. The comparison of the positions based on illustrations in the literature together with the own results on Metabonellia haswelli indicate that this is a very elastic term, masking additional potential character states. Eubonellia valida should have a so called distal position, but actually the gonostome is located at the tip, respectively the terminal distal end (Stephen and Edmonds 1972, Fig. 47D). The genus Metabonellia is quoted in the literature for having a distal gonostome, too (Stephen and Edmonds 1972). But as available species descriptions (e.g. Edmonds 1987, see Fig. 2) together with the own results for M. haswelli unambiguous prove is that the gonostome is situated some distance away from the terminal distal end of the gonoduct (in its proximal most third). So a more precise classification of the possible positions of the gonostomes is required. But it remains unknown how many as distally described gonostomes should be rather classified as the Metabonellia-type. In addition, precise or unambiguous data are lacking for several other bonelliid species (e.g. Archibonellia michaelseni see Fisher 1948; Bruunellia bandae, Jakobia birsteini, see Stephen and Edmonds 1972). So the consistency of the different positions within a genus was not unambiguously determinable in many cases due to the doubtful and deficient data presently available.

Following in part Fisher (1948), it is suggested here to classify into (i) basal gonostomes (near the genital pore, within basal most third of gonoduct; all Thalassematidae (e.g. T. thalassemum, Fig. 27A), Echiuridae and Urechidae; several Bonelliidae, (e.g. Bonellia viridis, Hamingia arctica, Maxmuelleria lankesteri), (ii) central placed gonostomes (a few Bonelliidae, e.g. Ikedella bogorovi Stephen and Edmonds 1972; Sluiterina album see Edmonds 1987), (iii) gonostomes near the distal end (within proximal most third of gonoduct but not terminal; Bonelliidae, hitherto only known from Metabonellia haswelli (Fig. 31A) and Pseudobonellia biuterina see Stephen and Edmonds 1972; and (iv) terminal situated gonostomes (at distal tip; several Bonelliidae, e.g. Bonelliosis alaskana Fig. 46D in Stephen and Edmonds 1972; Charcotus charcotus see Biseswar 2006; Jakobia densopapillata see Biseswar 2006 and all Ikedaidae (Ikeda 1904, Datta-Gupta and Menon 1976). Anyhow, due to the lack of additional data, it cannot be excluded that the proposed character state “basal” possibly still includes two additional states with only slight differences in position: “basal, in direct vicinity of the genital pores” (like in T. thalassemum) or “sub-basally, still within the basal most third of the gonoduct” (like in Achaetobonellia maculata see Fig. 44C in Stephen and Edmonds 1972). However, since these are only slight differences in position and the lack of data on the development of the gonostomes these
latter subdivisions are not included in the present character matrix. It may also be possible that such slight differences in position are also due to intra-specific variation.

4.4.2 Problematic characters – not spirally coiled gonostomal lips

**Shape:** Besides the classification into spirally coiled or not spirally coiled lips, generic distinctions on the basis of the lip morphology have been tried to make on descriptions such as e.g. “flap-like”, “petaloid”, “leaf-like” or “inconspicuous” and many more (compare Stephen and Edmonds 1972; Saxena 1983), but since an integrative terminology is missing it is presently not possible to determine the various shapes of the remaining taxa unambiguously. Moreover, for many taxa data on the lip structure are missing, or already available data seem not to contain sufficient structural details and are therefore not comparable. The investigation of the gonostomal lips in this study for *T. thalassemum* (Thalassematidae) has shown that the shape of the lips in this species is not a permanent character, since it is rather dependent on seasonal changes, i.e. the filling level with ripe gametes within the gonoduct. During reproduction period sexually mature gonoducts are filled with masses of gametes and the organs enlarge to about two thirds of the length of the relaxed specimen trunc. Then, the gonostome is sessile and equipped with two flap-like lips that display a v-shaped groove. Sexually immature gonoducts in contrast are less voluminous and show only about one-fifth of the length of the gonoducts during the reproduction period. Furthermore, the shape of the gonostomal lips has changed tremendously, merely the sessile basal appearance of the gonostome is retained. The lips are short and have a heavy ciliated lip tissue that is folded, resembling a rose petal; a v-shaped groove is not visible from exterior. These findings together with the seasonal dependency may explain the different descriptions on *T. thalassemum* gonostomal lips that are present in the literature: the “two lobed funnel” described by Bock (1942) is highly reminiscent of the bi-lipped structure of the gonostomal lips during reproduction period found in the specimen studied here; “lips that form a semicircular frill” (Stephen and Edmonds 1972) could be reminiscent of the folded lip tissue resembling a rose petal in the specimen studied here outside reproduction period. Such inconsistent descriptions of the lip shapes in single species have never been related to the filling condition of the gonoduct with ripe gametes. Contrary to the varying size of the gonoducts which was shown to be dependent on the reproduction condition of the specimen (e.g Seitz 1907; Baltzer 1917; Fisher 1946; Edmonds 1987). The comparison within available *Echiurus* illustrations demonstrates also an inconsistent picture of the shape on the gonostomal lips which may be due to fixation artefacts and/or the varying age of the specimen and/or disparities on subspecies level (Fig. 41).
Figure 41: Survey on the varying lip morphologies compiled from the literature within *Echiurus echiurus* (Echiuridae) (A, B: subspecies *echiurus*, C: subspecies *alascanus*). A: Presumably adult specimen and sexually mature according to the size of the gonoduct (modified from Greef 1879). B: Not fully grown-up specimen and sexually not mature (compiled from Steinmetz unpublished data). C: Presumably adult specimen and sexually mature according to the size of the gonoduct (modified from Fisher 1946). *go* gonoduct, *gol* gonostomal lips, *gr* ciliated groove.

Another good example of high intra-specific variability of the lips is given in Bock (1942) for the bonelliid *Maxmuelleria lankesteri* (Fig. 42A-C). Based on his investigation on a few specimens he states that the stalked gonostome either has a “brim-like border with frilled lips”, or that “sometimes two lips have developed, one larger than the other which is lacking a frilled border”. The lack of information on the maturity of the specimens as well as on the fixation conditions hampers the evaluation of the illustrated character states found in the literature. Seasonal influences (reproduction period), state of sexual maturity or fixation artefacts, all aspects seem supposable. The comparison of the in vivo studied gonostomal lips of *Metabonellia haswelli* (Fig. 31B, Fig. 42G) with the fixed specimen shown in Dartnall (1976) and Edmonds (2000) rather indicates that artefacts occurred during the fixation process and may have modified the external structure of the delicate lips (Fig. 42F, G). The size of the gonoducts in the fixed specimens suggests that they were sexually mature and/or possibly fixed during reproduction period. Both, fixed and in vivo studied specimens are generally comparable. The comparison of available illustrations on *Bonellia viridis* gonostomal lips supposes a similar coherency (Fig. 42D, E).

**Length:** Bock (1942) detected that in *T. thalassemum* the lips are “usually” unequal in length. But he does not comment further on this observation. It may be that his observation supports the findings of this study for *T. thalassemum* by presenting an intermediate state of maturity of the gonoducts (reflected by lips of unequal length). However, Bock (1942) recognized such lips of unequal length
Figure 42: Compilation of some examples from the literature for the documented intra-specific variability of the gonostomal lips among Bonelliidae. **A-C:** *Maxmuelleria lankesteri.* (A modified from Herdman (1897); B modified from Bock (1942), C compiled from Stephen and Edmonds (1972, but originally after Bock 1942). **D-E:** *Bonellia viridis* (D modified from Fisher 1946, E modified from Greef 1879). **F-G:** *Metabonellia haswelli* (F modified from Dartnall 1976, G scheme deduced from in vivo studied specimen, own observation). _an_ androecium, _e_ egg, _ec_ egg channel, _go_ gonoduct, _gol_ gonostomal lips, _gr_ ciliated groove, _st_ stalk of gonostome.

also in *E. echius*, which is approved by Baltzer (1931) and by Steinmetz (1989, unpublished diploma thesis). Baltzer (1931, Fig. 83) states nothing on the age or the maturity condition of his studied specimen, but according to his illustration, which shows no gametes inside the gonoduct, it seems likely that this specimen was at least outside the reproduction period. The specimen
investigated by Steinmetz showing an analogue lip morphology was probably not fully grown (pers. comm. T. Bartolomeus) suggesting likewise that the lips were at an early ontogenetic stage (Fig. 41B). But according to Baltzer (1931) the different morphology of the two lips seems to be generally related to functional aspects and not to conditions of sexual maturity or age of the specimen. Based on his light microscopic investigations, it turned out that in the larger dorsal portion the lip tissue is contractile and has a well-developed ecm and in the ventral lobe the cells seem to lack any muscle fibrils and the ecm has only weakly developed. Thus, Baltzer’s (1931) hypothesis that depending on the degree of “swelling” of the lips, caused by an controlled fluid exchange inside the lip, the inner channel that leads to the gonoduct may be kept open or closed. Generally, this sounds logical, also with respect to the sexually immature gonducts in T. thalassemum studied here. With their short folded lip tissue it does not appear as if this structure would be capable to select gametes from the coelomic cavity. The longer and thicker lips observed during reproduction period seem to accomplish this function easily. Anyhow, Baltzer’s (1931) hypothesis neither explains the two different states observed within T. thalassemum nor the inconsistent information on gonostomal lips within additional species satisfactory.

4.4.3 Conclusion

Although generally difficult to trace, solely on literature data alone, the presented examples of inconsistently described character states on the gonostomal lips indicate that the information available is only applicable in a very limited scope regarding cladistic analyses or a consistent taxonomy. It turned out that character states affiliated to the shape of the gonostomal lips may be affected by the age of a specimen, and/or may be heavily affected by fixation artefacts. Thereby it appears as if spirally coiled lips are less affected to the last mentioned impacts compared to non-spirally coiled lips. Considered together with the new results for T. thalassemum that suggest a dependence of the lip morphology on seasonal influences (reproduction period), it is demonstrated that the gonostomal lips, especially non-spirally coiled, are insufficient studied and generally need a broad re-investigation based on the usage of specimens of similar age, data acquisition during reproduction period (fully functional lips), and the application of similar or comparable fixatives causing a minimum of artefacts. Though, in vivo studies during reproduction period seem most promising for future analyses as shown here exemplarily for M. haswelli. Another supposable impact on the shape of the lips may be the sex of the specimen. Such an influence causing slight distinctions between the sexes was shown beforeonly for spirally coiled lips (MacGinitie 1935). Whether such a dependency also exists in taxa that have non-spirally lips remains unclear.
Nevertheless, the shape of the gonostomal lips is here generally regarded as a consistent character. It may be useful in future also for taxa featuring non-spiral lips as soon as additional data and a standardized terminology are adopted. At present, regarding lip morphologies, simply the classification into spirally-non-spirally coiled lips seems to be potentially phylogenetic informative respectively unambiguously discriminable. Generally, all Urechidae and numerous Thalassematidae share elongate spirally coiled lips. Echiuridae, Bonelliidae and Ikedaidae lack such lips.

Additional potentially informative characters of the gonostome refer to i) the general appearance of the gonostome (sessile or stalked) and ii) the position of the gonostome (basal, central, near distal end, terminal). Generally, all Urechidae, presumably all Echiuridae and many Thalassematidae have sessile gonostomes. Bonelliid species for which data are presently known develop all stalked gonostomes, a state they share with all Ikedaidae, but only with very few Thalassematidae. The variation within Bonelliidae regarding the position of the gonostomes is comparatively high. A few bonelliids share basal placed gonostomes, while others share central placed ones. A gonostome near the distal end (proximal most third of gonoduct) is thus far only known from very few Bonelliidae. Some bonelliid taxa and all Ikedaidae uniquely share terminal gonostomes. Basal gonostomes as detected in some Bonelliidae also occur within all Thalassematidae, Echiuridae and Urechidae.

Besides characters of the gonostome, further potentially promising gonoductal characters are iii) the position of gonoducts (pre-or postechaetal) and iv) the opening mode of the genital pores (separate openings or common duct with single genital pore). Coding of these characters, however, presently makes no sense, since information on these characters is too scattered in the literature.

Characters that presently appear not useful for phylogenetic inference are the number of the gonoducts by exact counting. This is due to a high intra-specific variation documented for many species covering all subgroups and a dependency on the sex of the specimen in a few thalassematid taxa. The arrangement of gonoducts into paired and unpaired is presently also not usable (dependent on number of gonoducts, intra-specific variation and mixed arrangements), but is included into the matrix to test the hypothesis of Ruppert et al. (2004) of being a phylogenetic informative character.)
4. 5 Additional characters

In order to test the phylogenetic significance of the morphological characters proposed by Ruppert et al. (2004) and Stephen and Edmonds (1972) for certain subgroups, the following characters are included into the present analysis: the anterior ventral chaetae, the posterior rings of anal chaetae, a so-called post-pharyngeal diaphragm, the proboscis, a glandular girdle on anterior trunk, the hemal system, a specification of the cloaca (“water lung”), the presence of dwarf males (pronounced sexual dimorphism) and the arrangement of gonoducts. Additionally, a character referring to the colour pattern of the proboscis is here regarded as potentially phylogenetic significant and therefore included into the analysis (character 42). The corresponding character states of all additional characters are explained in more detail within the following paragraphs (characters 34 to 47).

Characters that were not included from Ruppert et al. (2004) refer to the potential apomorphies of traditional Ikedaidae. They are listed in the following two paragraphs together with a short explanation of the reasons for their exclusion.

(i) “Non-segmental multiplication of gonoducts”: The term implies that all members of the Ikedaidae have an unpaired arrangement of a conspicuous high number of gonoducts. Regarding the number of gonoducts, this suggested apomorphy is not included into the matrix, because the term already includes an interpretation of the evolution of the gonoducts that presently can not be reassessed unambiguously. In addition, the number of gonoducts is here basically regarded as uncertain character due to its documented high intra-specific variability, which also occurs within traditional Ikedaidae (compare chapter 4.4.1 and character 47). Furthermore, Ruppert et al. (2004) assumption is based on the characteristics of only one member of the subgroup, *Ikeda taenioides*, although there are additional *Ikeda* species known that differ significantly in the number of gonoducts (compare Nishikawa 2002; World Register of Marine Species, http://www.marinespecies.org.). *Ikeda taenioides* is described with 200-400 gonoducts (Ikeda 1904), *Ikeda pirotansis* has a number that varies between 16-40 gonoducts (Stephen and Edmonds 1972; Datta-Gupta and Menon 1976), and within yet unknown *Ikeda* species the number is not known (*Ikeda* sp. in Edmonds 1987), or it is comparatively low with respect to the additional *Ikeda* species (10 in *Prashadus* sp. compare Saiz-Salinaz 1996). So the extremely high number of gonoducts in *I. taenioides* seems to be species specific, but not characteristic for all *Ikeda* species respectively traditional Ikedaidae as suggested by Ruppert et al. (2004).

(ii) Arrangement of the body wall muscles: Ruppert et al. (2004) and others (e.g. Stephen and Edmonds 1972) consider an alternative arrangement of the body wall muscles into outer longitudinal and inner circular layers as a further apomorphic character for traditional Ikedaidae. But re-
examinations of the body wall musculature in *Ikeda taenioides* by Nishikawa (2002) revised previous false information about the arrangement which is regardless still included in many textbooks (e.g. Edmonds 2000, Brusca and Brusca 2003, Ruppert et al. 2004). Like in all other echiurans it is consisting of outer circular, middle longitudinal and inner-most oblique layers (Nishikawa 2002). Consequently, this thesis follows Nishikawa (2002). The opposed arrangement as an apomorphy for traditional Ikedaidae in Ruppert et al. (2004), and Stephen and Edmonds (1972) is abolished and therefore not included into the matrix.

### 4.5.1 Chaetae

Chaetae are present in the body wall of most echiurans, though in different locations. Their structure and composition is similar to those of Annelida (Baltzer 1931; Orrhage 1973; Storch 1984), indicating a homology (Hausen 2005). As in annelids, echiuran chaetae develop within a chaetal follicle by assembly of chaetal material on the outer surface of the microvilli of the basal-most cell of the chaetal follicle, called the chaetoblast (Baltzer 1931, compare Hausen 2005). Chaetae developing in juveniles are situated laterally to the adult chaeta, also within a young but smaller chaetal follicle. When the young chaeta protrudes from the epidermis next to the adult chaeta, the latter drops out and is replaced in the same place by the young chaeta including its follicle. The old follicle had already collapsed (Spengel 1880, Baltzer 1931).

**Anterior ventral chaetae (character 34):** (0) absent; (1) present. Within Echiura, usually, a pair of short hooked chaetae occurs ventrally on the anterior part of the trunk on each side of the ventral midline just posterior to the mouth (Ruppert et al. 2004). They assist in locomotion, digging and holding the animal in place within the burrow. Unusual modifications of the paired occurrence are present within very few Thalassematidae and several Bonelliidae. Ventral chaetae have so far not been observed in the thalassematid *Ochetostoma senegalense* (see Stephen and Edmonds 1972) and numerous bonelliids, all living not less than 130 meters deep, including also several deep-sea species (*Alomasoma belyaevi*, *A. nordpacificum*, *Choanostomellia bruuni*, *Eubonellia valida*, *Ikeda* species, *Nellobia eusoma*, *Sluiterina sibogae*, Stephen and Edmonds 1972). In these species the male lacks ventral chaetae, too, or the male is unknown. From a few bonelliiid species it is reported that the males have paired ventral chaetae, contrary to the state in their corresponding females, which are lacking these structures (e.g. *Hamingia arctica*, *Amalosoma eddystonense*, *Amalosoma paradolum*, see Stephen and Edmonds (1972)). These species live in greater depths from 220-347 compared to the former (Stephen and Edmonds 1972).

**Number of anterior ventral (adult) chaetae (character 35):** (0) two; (1) more than two; (2) one (single). Variation from the ordinary paired occurrence of two ventral chaetae has been described in one thalassematid species (*Anelassorhynchus chaetiferus*) and several bonelliids (Stephen and Edmonds 1972). Generally, these numerous chaetae can either been arranged within a muscular pad, or within a so-called genital groove, a depression that extends from the gonopores to the mouth on the ventral surface in some bonelliids (eight chaetae in *Acanthohamingia shiplei* and nine in *Acanthohamingia ijimai* Stephen and Edmonds (1972)). Basically the chaetae embedded within a muscular pad are irregularly scattered over a small area posterior to the mouth and anterior to the gonopores. The numbers of chaetae therein can vary considerably (e.g. two-14 in *Acanthobonellia pirotanensis*; 29 in *Acanthobonellia miyajimai* according to Ikeda (1904), 16-20 according to Menon et al. (1964); eight “spinlets” in *Anelassorhynchus chaetiferus*; six chaetae in *Prometor gracilis*, compare Stephen and Edmonds (1972)). In the majority of species featuring muscular pads, these are paired (one pad on each side of the ventral nerve cord). A species of *Acanthobonellia* (*A. rollandoeand*) is unique in possessing only one chaeta embedded within a single muscular pad (Stephen and Edmonds 1972). A single muscular pad (including several chaetae) is also described from *A. pirotanensis* (Menon et al. 1964).

Except for the thalassematid *A. chaetiferus* for which the bathymetric range is not exactly known the mentioned bonelliids occur in greater depths from 220 meters (*Acanthobonellia*) to the deep sea (*Prometor* and *Acanthohamingia*).
Posterior rings of anal chaetae (character 36): (0) absent; (1) present. In addition to the anterior ventral chaetae, members of traditional Echiuridae and Urechidae have rings of slightly curved, pointed chaetae around the posterior extremity of the trunk (e.g. Fig. 59B, E, F in Stephen and Edmonds 1972). The Latin name Echiura ("spine-tails") is deduced from this arrangement of chaetae. The conspicuous rings are likely used for burrow maintenance and anchorage (Ruppert et al. 2004).

Number of posterior rings of anal chaetae (character 37): (0) one; (1) two. Two almost complete rings of anal chaetae encircle the posterior extremity of the trunk in all Echiurus species (Fig. 44). Within Urechis species in contrast only one almost complete ring of anal chaetae has developed (Stephen and Edmonds 1972). The number of chaetae involved varies slightly between seven to 13 within individuals of Urechis species (Stephen and Edmonds 1972). In E. echiurus it is usually seven (five to nine) in the anterior row, and usually six (five to eight) in the posterior row (Stephen and Edmonds 1972). In other species the numbers vary, too (e.g. eight to 10 in anterior row and five to six in posterior row shown for Echiurus antarcticus).

4.5.2 Post-pharyngeal diaphragm

Post-pharyngeal diaphragm (character 38): (0) absent; (1) present. The absence and presence of a so-called post-pharyngeal diaphragm is coded into the matrix, because Stephen and Edmonds (1972) presume this thin-walled, funnel shaped septum to be apomorphic for traditional Echiuridae. It incompletely separates the anterior (peripharyngeal) coelom from the general body cavity and exclusively occurs in Echiurus (Fig. 52E in Stephen and Edmonds 1972). All remaining echiuran taxa are said to lack such a conspicuous septum (Stephen and Edmonds 1972).
4.5.3 Proboscies

Proboscies (character 39): (0) absent; (1) present. All echiurans are usually characterized by a ciliated, protrusible anterior prostomium (Baltzer 1931, Korn 1982), called proboscis. The proboscis is highly mobile and capable of great extension in many species, but contrary to the introvert in sipunculans, it cannot be retracted into the trunk. Externally, it is the most distinctive characteristic of the echiuran body and it is apomorphic for Echiura (Ax 1999). It is the source of the common name “spoon worm”. Functionally, it acts like a sensory and respiratory organ, but primarily it is used to collect sediment from around the burrow as most species are burrowing deposit feeders. Food particles are transported on the ventral surface, which is usually heavily ciliated, and subsequently these particles are carried to the small mouth located basally. When disturbed the proboscis retracts quickly. Quotations about echiurans that are said to lack this unique sensitive food gathering organ should be critically assessed on the basis of re-investigations of the relevant species, because it can be sometimes readily detached from the trunk and easily been lost (Stephen and Edmonds 1972). Although there are species that have very short proboscies (Urechis species), this explanation seems likely to elucidate descriptions where the organ is completely missing. Proboscies are unknown in: the bonelliid genera Amalosoma and Nelloobia and some thalassematid species (e.g. three of six Arhynchite species, such as Arhynchite arhynchite, Arhynchite inamoenus, Arhynchite rugosus, or a few Thalassema species, e.g. Thalassema elapsum, Thalassema mortensi).

Proboscis length, relaxed condition (in vivo) (character 40): (0) “short”; (1) “elongate”; (2) “very long”. The length of the proboscis is significantly short in Urechis species (Urechidae) and significantly long in some Bonelliidae and all Ikedaidae (Stephen and Edmonds 1972; Ruppert et al. 2004) (Fig. 54). Within Urechis species the short stout-like organ is not clearly separated from the trunk and measures, if at all, only a few centimeters (depending on the size of the specimen). In Bonellia viridis in contrast, it can reach a length of about 1.50 meters during feeding (Baltzer 1931) and in Ikeda species similar lengths are reported (0.75-2.0 meters, Ikeda 1904, Hughes and Crisp 1976, Edmonds 2000). In B. viridis and the Urechidae the length of the proboscis is particularly noticeable in proportion to the size of the trunk. This is in contrast to Ikeda taenioides and Ikeda pirotansis where also the trunks are comparatively long and slender. However, Ruppert et al. (2004) use the term “very long” for members of the Bonelliidae and Ikedaidae which is adopted here. Besides B. viridis, it seems highly probable that additional bonelliid species have proboscies of such enormous lengths. But at present no comparative studies are available dealing with this topic. Regarding Urechis species Ruppert et al. (2004) use the term “reduced proboscis”. In order to avoid any primary interpretations, the term “short” is included into the matrix as it seems more appropriate. These short
proboscies are related to the specific filter feeding process in *Urechis* species. It is assumed that the “rudimentary” proboscis assists in retrieval and ingestion of a food-laden mucous net that is produced by slime glands on the anterior trunk (compare *character 42*) (Ruppert et al. 2004). Ruppert et al. (2004) assign “elongate proboscis” to the echiuran ground plan, without giving a corresponding definition or length specification. Thus, a clear statement based on metrical data is presently not possible. But they can be distinguished to some degree from the two other states: somewhere in-between “short” and “very long” (Fig. 54).

**Proboscis shape (in vivo) (character 41):** (0) not bifid (tongue-like); (1) bifid (forked). In the majority of echiuran species the proboscis is not bifid, it is rather tongue-like (Fig. 54). Exclusively within bonelliiid taxa bifid, forked proboscies occur (e.g. *Achaetobonellia*, *Bonellia*, *Metabonellia*, *Hamingia*; compare Tab. 16 in Stephen and Edmonds 1972). The arms so formed may be long or short. Although not present in all bonelliids the development of a forked proboscis is used by Ruppert et al. (2004) as an apomorphic character for Bonelliidae; it is accordingly coded here. Ruppert et al. (2004) includes an “elongate flattened” proboscis into the echiuran ground plan which corresponds to character state (0) in this study. Nevertheless, additional characteristics of the proboscies are known which may be useful for future investigations. At present the diversity of proboscis shapes is reflected by a variety of descriptive terms that are presently hard to classify. This includes peculiarities of the anterior tips and lateral margins, mainly in tongue-like proboscies. The tips may be rather broadened, fan-shaped, spatulate, or narrow and tapering towards the end. Very unusual differentiations of the lateral edges are present in a few Thalassematidae (e.g. gill-like processes in *Anelassorhynchus branchiorhynchus*, or fused margins at the base forming a cup-like structure in *Ikedosoma elegans*, compare Stephen and Edmonds 1972). Other specifications that surround the mouth are observed in some deep-sea bonelliids (e.g. lips in *Jakobia birsteini*, funnel like collar in *Choanostomellia* species, basal cup in *Prometor benthophila*, compare Stephen and Edmonds 1972). Their function is presently not exactly known, presumably they are linked to the feeding and/or sensory process. There may be even more structural modifications which are presently unknown to science.

**Proboscis colour pattern (in vivo) (character 42):** (0) absent; (1) present. *Ikeda* sp. used in this study for molecular analysis was tentatively identified as an undiscribed species of *Ikeda* on the basis of the conspicuous colour pattern and length of the proboscis (Fig. 3F, Fig. 54A-B). This was because Edmonds (1987) assigned a proboscis from almost the same locality (South Australia) with exactly the same colour pattern to the genus *Ikeda*. Like the tissue sample of the proboscis used in this study, the dorsal surface of the proboscis in Edmond’s (1987) specimen is pale grayish to white and has numerous, transverse brown to black bands or spots (Fig. 54B; compare plate 11.5 and 11.6 in Edmonds 2000). Due to difficulties in digging out the entire specimen it was only possible to get a
piece of proboscis for this study (kindly provided by G. Rouse). However, Edmonds (1987) was able to collect additionally a badly damaged trunk, and could thus add very few details, such as an indication of size (proboscis: 40 cm; trunk: 29 centimeters long, 0.7-1.1 centimeters wide) and information on the longitudinal musculature (five longitudinal muscle bands) in his *Ikeda* sp. specimens. Besides proboscis and trunk size the colour pattern was certainly also a decisive factor for Edmonds’s (1987) choice, because monotypic Ikedaidae (based on *Ikeda taenioides*) are traditionally classified as large animals with a very long trunk and proboscis which is decorated with narrow transverse brown stripes (Fig. 54A-B; Ikeda 1904; Fig. 58C in Stephen and Edmonds 1972). The habitus of the proboscis in the second valid species, *Ikeda pirotansis*, supports this colour pattern (Menon and Datta-Gupta 1962; Hughes and Crisp 1976, plate 2). The comparison with available information on the colour patterns in the remaining echiurans leads to the conclusion that the presence of numerous transverse brown to black bands or spots on a pale grayish to white subsurface may be apomorphic for *Ikeda* species. Thus, this character is included into the matrix.

In fact, a few echiuran species are known that also display spots or stripes on the dorsal proboscis surface, but contrary to the state in *Ikeda* species, the underground is never pale grayish to white (e.g. black spots in *Acanthobonellia miyajimai* on grayish brown surface, or small green spots and yellow margins in *Ikedosoma gogoshimense*; compare Stephen and Edmonds 1972). Given the fact that the proboscis is white it always lacks dots or stripes (e.g. *Bonellia pumicea* see Stephen and Edmonds 1972). It seems that the majority of echiuran species lack a definite colour pattern. Many species have single-coloured proboscies, or in case they are multicoloured the colours show a smooth transition (compare Stephen and Edmonds 1972).

### 4.5.4 Glandular girdle

*Glandular girdle on anterior trunk (character 43):* (0) absent; (1) present. Members of traditional Urechidae differ from other echiurans in their feeding action by entrapping fine particles in a mucous net. This way, the animals are able to filter food from the water that pumps through its U-shaped burrow for irrigation. The net traps food particles and is then consumed periodically (Fisher and MacGinitie 1928). Several slime glands that open on the anterior part of the trunk near the gonopores are responsible for the production of the mucous net.
4.5.5 Hemal system

Hemal system (character 44): (0) absent; (1) present. A hemal system is present in all echiurans except in *Urechis* species (Urechidae) (e.g. Stephen and Edmonds 1972). The vascular system usually consists of a hemal sinus (ring vessel) around the foregut, from which blood is transported to the proboscis by a mid-dorsal vessel. Blood returns to the trunk in a pair of lateral prostomial vessels, which unite in the trunk to form a midventral, longitudinal vessel. Branches from the ventral vessel unite with the sinus to complete the circuit. Because there is no respiratory pigment in the colorless blood, it has been hypothesized that the blood transports and allocates exclusively nutrients (Ruppert et al. 2004). Gas exchange presumably occurs across the general body wall of both trunk and proboscis. In the latter probably by simple diffusion, within the thick trunk, however, oxygen diffusing is transported internally by hemoglobin-containing coelomocytes (Ruppert et al. 2004).

4.5.6 Cloaca

Enlarged cloaca (“water lung”) (character 45): (0) absent; (1) present. In *Urechis* species body wall gas exchange occurs primarily by exchange across the thin-walled cloaca and hindgut, which are enlarged to form a “water lung” (Fisher and MacGinitie 1928, Fisher 1946, Stephen and Edmonds 1972, Menon and Arp 1992, Ruppert et al. 2004). By the pumping action of the cloaca, oxygenated water is forced from the environment into the hindgut (often in a series of movements), and from time to time it is discharged through the anus. Gas exchange additionally proceeds via the hemoglobin-carrying coelomocytes.

4.5.7 Sexual dimorphism

Sexual dimorphism (dwarf males) (character 46): (0) absent; (1) present. In all echiurans the sexes are separated. Within traditional Echiuridae, Thalassematidae and Urechidae females and males are broadly indistinguishable externally (but see differently coloured gametes in *T. thalassemum* Fig. 26A-B). In traditional Ikedaidae male specimens never have been found so far (Ikeda 1904, Datta-Gupta and Menon 1976, Nishikawa 2002). Within traditional Bonelliidae, reproduction and sex determination differs extremely from that of the remaining echiurans. A ciliated dwarf male, usually 1-
6 millimeters long, lives in or on the much larger female (trunk up to 15 centimeters, Edmonds 2000). Usually, the male lives permanently in the gonoduct of the female (often within a specialized chamber called “male sac”, androecium), but may also reside on the proboscis and body wall or in a specialized tube within the female (“male tube” in *Pseudobonellia*, Stephen and Edmonds 1972). It has been also detected within the body cavity, the oesophagus and pharynx (Stephen and Edmonds 1972). Although it is generally accepted that all bonelliids feature dwarf males, there are numerous bonelliid species in which the male remain unknown (compare Stephen and Edmonds 1972). However, based on the available information from the known species important characteristics can be noticed: The male body is flat, planariform or nematoform, usually lacks pigment and a proboscis and develops an adhesive organ with which they attach themselves to the surface of the female (Ruppert et al. 2004). Besides the proboscis, several other organs present in adult animals are lacking in the dwarf males, such as a mouth, anus, anal sacs and sometimes ventral chaetae (compare Fig. 60A, in Stephen and Edmonds 1972 (after Baltzer 1931)). Generally, the alimentary, excretory, vascular and nervous system is much reduced; the male is dependent on the female for food and protection. They usually meet their metabolic needs by exchange with the female coelomic fluid in which they are bathed (Ruppert et al. 2004). The reproductive system is well-developed; the body includes a gonad, a seminal vesicle and a pair of prostonephridia (Schuchert 1990; Schuchert and Rieger 1990; Ruppert et al. 2004). As the dwarf male retains some larval/juvenile characters (i.e. minute size, ciliation of body, prostonephridia, metanephridia posterior to the prostonephridia, some characteristics of nervous and alimentary canal), but also contains gametes that mature into ripe cells, this is an example of progenesis in Echiura (Baltzer 1924 for *Bonellia viridis*). The male not only fertilizes the eggs internally, at oviposition it also secretes a gelatinous material used to bind the eggs together. Thus contrary to the remaining echiurans, fertilization is internal in Bonelliidae. The yolky eggs develop into lecithotrophic trochophores. In case these short-lived larvae settle on an adult female proboscis, most become dwarf males. If they settle apart from a female, they metamorphose into juvenile females (Baltzer 1914; Michel 1930; Bridges 1963). It is thought that substances produced by adult females are basically responsible for sex determination. Although several others (e.g. Baltzer 1925; Leutert 1974; Jaccarini et al. 1983) have studied sex determination of the bonelliid larva, the mechanism is not entirely understood. Undetermined environmental factors may also have an impact on sex determination (Jaccarini et al. 1983; “environmental sex determination” compare also Pilger 1978).
4.5.8 Arrangement of gonoducts

Arrangement of gonoducts (character 47): (0) paired; (1) unpaired. Ruppert et al. (2004) include conclusions regarding the arrangement of gonoducts by implementing “segmental metanephridia” for the echiuran ground plan and an “unpaired metanephridium” as an synapomorphy for a clade consisting of Bonelliidae + Ikedaidae. Although the arrangement as an independent consistent character is regarded as controversial by the author and yet not usable for cladistic analyses, nevertheless, it is here included using the terms paired (“segmental metanephridia”) and unpaired. This is because not only to test Ruppert et al. (2004) apomorphies but also to reveal the contradictory character states (polymorphism in *Hamingia arctica* (Bonelliidae) (Baltzer 1931, Stephen and Edmonds 1972); polymorphism in *Anelassorhynchus adelaidensis* (*A. porcellus adelaidensis* sensu Edmonds (1987); Thalassematidae); assumed paired arrangement in *Ikeda pirotensis*, Stephen and Edmonds (1972), Datta-Gupta and Menon (1976), assumed unpaired arrangement in *Ikeda taenioides* Stephen and Edmonds (1972)). Within *Ikeda* species it is also conceivable that based on the extremely high number of gonoducts the arrangement has been incorrectly assessed. However, generally, the arrangement of the gonoducts seems hard to evaluate satisfactory, also due to its presumed dependency on the number of gonoducts, which is often affected by a high intraspecific variability (Lacaze-Duthiers 1858; Stewart 1900; Ikeda 1904, 1907; Baltzer 1931; Bock 1942; Edmonds 1963; Stephen and Edmonds 1972; Datta-Gupta 1974), and the presence of mixed asymmetrical arrangements including pairs, clusters and single gonoducts within a single species (compare chapter 4.4.1). By using a gross-classification into paired (“segmental metanephridia”) and unpaired as done by Ruppert et al. (2004) possibly significant information whether paired groups, or pairs consisting of two gonoducts have developed, is not considered.
4.6. Phylogeny of Echiura

Until today, the interrelationships of the traditional echiuran subgroups Bonelliidae, Echiuridae, Ikedaidae, Thalassematidae and Urechidae remain unclear (Fig. 46C). No phylogenetic analyses are available for Echiura, neither based on morphological data nor based on molecular sequences. This is on the one hand due to the presence of a few apomorphic characters for nearly all echiuran subgroups (except for Thalassematidae and Echiuridae according to Ruppert et al 2004), and even for single species autapomorphic character states are described. In addition only very few echiuran DNA sequences are presently available (http://www.ncbi.nlm.nih.gov/genbank/). The reason for this may be that the majority of echiurans is hard to obtain, either due to their hidden habitat or their rare occurrence.

4.6.1 Cladistic analysis of morphological data

In this study 47 potentially informative morphological characters were compiled that permit the first cladistic analysis of echiuran relationships (for character matrix see Appendix 2). Analyses were conducted i) using equally weighted characters and ii) using implied weights under variable weighting strengths (concavity function k=1-6). The results of both analyses are shown in Fig. 45 and Fig. 46A. Most of the in-group relationships are poorly supported (bootstrap frequencies below 50%). The unweighted analysis retrieved 12 shortest cladograms with varying topologies regarding the relationships among the subgroups (Fig. 45A-D). The analysis under implied weights found only three shortest cladograms, all with a similar topology with respect to the relationships among the subgroups (Fig. 45E-F, Fig. 46A). The weighting strength (k= 1-6) in the weighted analysis has no influence on the topology of the shortest cladograms. All analyses retrieve a clade composed of traditional Echiuridae (sampled by Echiurus echiurus) and traditional Urechidae (sampled by Urechis caupo and Urechis unicinctus), a clade here referred to as the Urechis-group. The interrelationships between the bonelliid subtaxa and the two Ikeda species are variably resolved under equal weights; implied weights favour their resolution as a clade, here referred to as the Bonellia-group, with a monophyletic Ikedaidae nested within a paraphyletic Bonelliidae. The traditional “Thalassematidae” are never retrieved as a monophyletic group. They either cluster as a paraphyletic assemblage with the Urechis-group, or are resolved as a polyphyletic assemblage with Arhynchite pugettensis as sister group to remaining echiurans.
The resolution of the taxa within the subgroups is disregarded here, because it is dependent on the current taxon sampling, and this is rather limited in this study due to the restricted availability of species. The few taxa included represent traditional subgroups but not the variability inside these subgroups. This applies especially to the included thalassematid (Thalassematidae), bonelliid (Bonelliidae) and ikedid species (Ikedaidae).

4.6.1.1 Favourable morphological tree

The topology that is retrieved under both equal and implied weights is considered here as the favoured morphological tree (equal weight: single tree, implied weights: all trees, Fig. 46A). It discovers two monophyletic groups: the Bonellia-group comprising traditional bonelliid taxa and a monophyletic Ikedaidae, and the Urechis-group consisting of traditional Echiuridae and traditional urechid taxa. The traditional Thalassematidae are polyphyletic, with Arhynchite pugettensis as basalmost offshoot within Echiura and remaining thalassematids as paraphyletic assemblage with respect to the Urechis-group.

i) Monophyletic Bonellia-group comprising traditional bonelliid taxa and a monophyletic Ikedaidae (traditional Bonelliidae paraphyletic). Monophyly of the Bonellia-group is supported by the presence of anal sac tubules (long funnel stalks) (characters 17), dwarf males (characters 46), and unpaired gonoducts (characters 47). The presence of a terminal gonostome (character 33) optimizes as apomorphy of traditional Ikedaidae (Ikeda taienioides and Ikeda pirotransis) and Pseudoikedella achaeta (traditional Bonelliidae). Monophyly of Ikedaidae is supported by a unique proboscis colour pattern (character 42). Sister to the clade composed of ikedid and a bonelliid species is a subgroup of traditional bonelliids consisting of Metabonellia haswelli, Bonellia viridis and Hamingia arctica. This clade is supported by a bifid (forked) proboscis (character 41) and a homoplastic sac-like shape of the end sacs (character 13), which is shared by traditional Urechidae.

ii) Monophyletic Urechis-group consisting of traditional Echiuridae and traditional urechid taxa. Monophyly of the Urechis-group is supported by the presence of rings of anal chaetae (character 36). Monophyly of traditional Urechidae (Urechis caupo + Urechis unicinctus) is supported by several unambiguous apomorphic changes: a very short proboscis (character 40), a glandular girdle on the anterior trunk (mucous net production for filter feeding) (character 43), the absence of a hemal system (character 44) and an enlarged cloaca (“water lung”) (character 45) serving as an organ of respiration. Additional support provides a homoplastic sac-like shape of the end sacs (character 13), which is shared by a subgroup of the Bonelliidae (Metabonellia haswelli, Bonellia viridis and Hamingia arctica).
iii) Polyphyletic Thalassematidae with Arhynchite pugettensis as sister group to remaining echiurans (remaining thalassematids as paraphyletic assemblage with respect to the Urechis-group). The thalassematid *A. pugettensis* is resolved as basalmost offshoot within Echiura with a sister group relationship to the remaining echiurans. This sister group relationship lacks unambiguous support. The remaining thalassematids are resolved as paraphyletic assemblage with respect to the *Urechis*-group. The favoured topology optimizes the presence of two spermatozoal characters (*character 3*, electron dense material restricted to basal ring component in the acrosome; *character 6*, spherical nucleus) and sessile gonostomes (*character 31*) as support for this grade. The sister group relationship of this paraphyletic assemblage to the *Bonellia*-group lacks unambiguous support.

**Alternative topologies (under equal weights)**

Since the interrelationships of the echiuran subgroups do not differ in the three cladograms of the weightened analysis (Fig. 45E, F, Fig. 46A), exclusively the alternative topologies under equal weights are considered in the following (Fig. 45A-D).

**Traditional Bonelliidae**

i) *Polyphyletic Bonelliidae* (Fig. 45B). Some shortest cladograms under equal weights retrieve a polyphyletic Bonelliidae with respect to the basal branching of *Pseudoikedella achaeta* and the clustering of a subgroup of the Bonelliidae (*Metabonellia haswelli*, *Bonellia viridis* and *Hamingia arctica*) with thalassematids and the *Urechis*-group. The basal resolution of *P. achaeta* is based on the absence of anterior ventral chaetae (*character 34*) that is shared by the outgroup taxon. The clustering of *Metabonellia haswelli* + *Bonellia viridis* + *Hamingia arctica* with thalassematids and the *Urechis*-group is based on the presence of a basal gonostome (*character 33*), which is optimized as unambiguous apomorphic change (but transformed into state “near distal end” in *M. haswelli*, Fig. 31A).

ii) *Monophyletic Bonelliidae as sister group to remaining echiurans (with monophyletic Ikedaidae; Fig. 45C)*. Monophyly of traditional Bonelliidae is supported by the presence of dwarf males (*character 46*). Their sister group relationship to remaining echiurans lacks unambiguous support. Monophyletic Ikedaidae cluster with “Thalassematidae” + *Urechis*-group due to the homoplastic gain of anterior ventral chaetae (shared by the bonelliids *B. viridis* + *M. haswelli*).
iii) Monophyletic Bonelliidae as sister group to remaining echiurans (with Ikedaidae paraphyletic with respect to “Thalassematidae” + Urechis-group; Fig. 45D). Compare ii, respectively iii) under “Traditional Ikedaidae”.

Traditional Ikedaidae

i) Monophyletic Ikedaidae nested within polyphyletic Bonelliidae (Fig. 45B). Monophyly of Ikedaidae is supported by the conspicuous proboscis colour pattern (character 42). The sister group relationship of *P. achaeta* and the remaining echiurans in contrast lack unambiguous support. Presence of anterior ventral chaetae (character 34) optimizes as apomorphy of traditional Ikedaidae and remaining echiurans, but is reversed in the bonelliid *Hamingia arctica* (compare i) under “Traditional Bonelliidae”).

ii) Monophyletic Ikedaidae as sister group to “Thalassematidae” + Urechis-group (Fig. 45C). Under this resolution monophyly of Ikedaidae is supported by the conspicuous proboscis colour pattern (character 42) and a homoplastic elongation of the proboscis (character 40), which is shared by the bonelliid *B. viridis*. Synapomorphic between *Ikeda* species + the remaining echiurans is the presence of ventral chaetae which is homoplastic due to the additional presence in some bonelliids.

iii) Ikedaidae paraphyletic with respect to “Thalassematidae” and the Urechis-group (Fig. 45D). Under this resolution paraphyly of Ikedaidae is supported by the presence of paired gonoducts, which are presumably paired in *I. pirotansis*, but presumably unpaired in *I. taenioides* and some bonelliids (character 47; see chapter 4.6.4 “gonoducts” for a discussion of this problematic character).
Traditional “Thalassematidae”

Thalassematidae as paraphyletic assemblage with respect to the *Urechis*-group (Fig. 45B-D). Thalassematids and the *Urechis*-group uniquely share the absence of anal sac tubules (*character 17*). Some topologies optimize the presence of basal gonostomes (*character 33*) and paired gonoducts (*character 47*) as additional support for this clade, but these characters are variably shared by some bonelliids. The variable absence or presence of characters shared only by some thalassematids with the *Urechis*-group further support the resolution as a paraphyletic assemblage. This especially concerns the shape as well as the extension of the acrosome (elongate versus oblate, *character 2*), the presence of spirally coiled gonostomal lips (*character 32*), and transformations of the sperm nucleus (*character 6*).

4.6.2 Molecular tree

A total of 16 new gene sequences for all five traditional subgroups together with already published sequence data from GenBank (compare Tab. 1) permit the first molecular analysis of echiuran intrarelationships. The two analysed alternative datasets of the multigene ML-analysis (18S rDNA + 16S rRNA + MT-CO1) resolved each one best tree with congruent topologies and likelihood bootstrap support values that do not differ substantially in the majority of cases (Fig. 46B). Thus, slightly varying likelihood support values will not be discussed any further in the following, unless stated otherwise (compare *Urechis*-group, “Thalassematidae”). The molecular data confirm Echiura as a monophyletic group (Ax 1999; Harris and Jaccarini 1981). Most of the recent published molecular analyses retrieve Echiura as sister group of the Capitellidae (Bleidorn et al. 2003a, 2003b; Struck et al. 2007, 2008; Dunn et al. 2008; Zrzavy et al. 2009; Struck et al. 2011). Taking account of the small outgroup taxon sampling (Capitellidae, sampled by Notomastus latericeus + Dasybranchus sp.; Eunicidae, sampled by Eunice pennata; Trichobranchidae, sampled by Artacamella tribranchiata; Terebellidae, sampled by Thelepus cincinnatus) this study confirms this hypothesis with a reservation.

According to the multigene ML-analysis two major clades are recovered: a well supported group including all bonelliid taxa and *Ikeda* (*Bonellia*-group; LBS 89/92%); and a group consisting of members of the Echiuridae and Urechidae (*Urechis*-group; LBS 67/95%). A sister group relationship between the *Bonellia*-and *Urechis*-groups is well supported (LBS 86/93%). “Thalassematidae” are resolved as a basal grade with regard to the clade comprising the *Bonellia*- and *Urechis*-groups, but the basal nodes are only weakly supported.
4.6.3 Comparison of phylogenetic trees

The favoured morphological tree and the molecular tree are congruent in identifying a monophyletic *Bonellia*-group (including monophyletic Ikedaidae within paraphyletic Bonellidae), a monophyletic *Urechis*-group (traditional Echiuridae + Urechidae), and a basal resolution of some thalassematid taxa (i.e., *Arhynchite pugettensis*) (Fig. 46A, B). Main difference between the molecular and the morphological results concern the resolution of remaining thalassematids: they form a paraphyletic assemblage either to remaining echiurans (favoured by molecular sequences), or to the *Urechis*-group (favoured by morphology). These resolutions oppose to the traditional classification of Echiura by Stephen and Edmonds (1972) and also to the revised classification by Nishikawa (2002) as well as to the phylogeny proposed by Ruppert et al. (2004) (Fig. 46C).

**Figure 46:** Phylogeny of Echiura. **A:** Presently favoured morphological tree retrieved from MP-Analysis of 47 morphological characters. Numbers at nodes refer to bootstrap support values above 50% under equal and implied weights. **B:** Molecular tree inferred from ML-Analysis of the current multigene dataset (MT-CO1 + 16S rRNA + 18S rDNA). Nodal support values represent bootstrap frequencies of two differently tested datasets. **C:** Phylogenetic hypothesis proposed by Ruppert et al. (2004). Apomorph characters for Echiuridae and Thalassematidae remain unknown. BON Bonellidae, ECH Echiuridae, IKE Ikedaidae, THA Thalassematidae, URE Urechidae.
Contrary to the results of the cladistic analyses Thalassematidae were reasoned by Ruppert et al. (2004) as a monophylum and as sister group to a clade comprising Bonelliidae and Ikedaidae in a sister group relationship. Present cladistic analyses instead suggest that Bonelliidae is not monophyletic but include Ikedaidae within a monophyletic clade, the Bonellia-group. Furthermore, Ruppert et al. (2004) considered traditional Echiuridae and Urechidae as basal groups within Echiura. Present results support a sister group relationship between Echiuridae and Urechidae (Urechis-group) but resolve this clade within the top of the echiuran tree.

4.6.4 Character evolution

Character evolution of the four investigated character complexes (spermatozoa, anal sacs, larval protonephridia and gonoducts) plus additional characters compiled from the literature, i.e. differentiations of the chaetae as well as probosces was reconstructed using WinClada 10.00.08 (© Nixon 2002; see character coding, Appendix 3). In a first step exclusively the unambiguous states were optimized onto the molecular phylogeny (Fig. 47). In a second step the character diagnoser was used to reconstruct the evolution of all character states. The ML-Analysis of the multigene dataset was chosen because it displays predominantly better support values compared to the favoured morphological tree. Outgroup comparison for morphological characters refer to Capitella teleta (Annelida, Capitellidae).

According to the character optimization procedure of Winclada the following non-homoplastic changes, i.e. apomorphous character states for higher taxonomic entities, were unambiguously identified (Fig. 47):

(i) *Urechis*-group: posterior rings of anal chaetae (in congruence with morphological tree),

(ii) *Bonellia*-group: pronounced sexual dimorphism with dwarf males (in congruence with morphological tree),

(iii)Subgroup within *Urechis*-group: traditional Urechidae: "short" proboscis (Ruppert et al. 2004), a glandular girdle on the anterior trunk (mucous net production for filter feeding), the absence of a hemal system and an enlarged cloaca (“water lung”) (all in congruence with morphological tree; optimizations of homoplastic changes differ among trees).
Species specific autapomorphies refer to (Fig. 47):

(i) *Metabonellia haswelli* (traditional Bonelliidae): gonostome near distal end (within proximal most third of gonoduct) (in congruence with morphological tree),

(ii) *Ikeda* sp. (traditional Ikedaidae): gonostome terminal (synapomorph for the clade *P. achaeta*, traditional Bonelliidae + *Ikeda* species in morphological tree) and proboscis colour pattern (synapomorph for *I. pirotansis* + *I. taenioides* in morphological tree; optimizations of homoplastic changes differ among trees, Fig. 47, Fig. 45E)
(iii) *Echiurus echiurus* (traditional Echiuridae): membrane bound subacrosomal vesicle, post-pharyngeal diaphragm (both in congruence with morphological tree),

(iv) *Urechis caupo* (traditional Urechidae): anal sac funnels increase from proximal to distal (in congruence with morphological tree),

(v) *Thalassema thalassemum* (traditional Thalassematidae): terminal structure of larval head kidney is composed of one cell only, filter structure of larval head kidney is composed of two to three layers of elongate microvilli emerging from the terminal cell (all in congruence with morphological tree; homoplastic changes in congruence with morphological tree, too, Fig. 45E).

The following character states were unambiguously identified as homoplastic changes (evolved convergently) (Fig. 47):

(i) fins on sperm flagellum (in congruence with morphological tree in Echiuridae: *E. echiurus* and Thalassematidae: *T. thalassemum*), possibly due to lack of data in the remaining taxa,

(ii) Sac-like end sacs (anal sacs) (in congruence with morphological tree in traditional Urechidae and bonelliid subgroup comprising *M. haswelli*, *A. belyaevi* and *B. viridis*),

(iii) spirally coiled gonostomal lips (traditional Urechidae, Thalassematidae: *O. erythrogrammon* and composite taxon *Anelassorhynchus*; not in congruence with morphological tree: synapomorphy of “Thalassematidae” (*T. thalassemum*, *A. pugettensis* excluded) + *Urechis*-group,

(iv) absence of anterior ventral chaetae: secondary loss in Bonelliidae: *A. belyaevi*, not present in outgroup taxon (in congruence with morphological tree, but *P. achaeta* with secondary loss, too),

(v) “very long” probosces (Ruppert et al. 2004) in Ikedaidae: *Ikeda* sp. and Bonelliidae: *B. viridis* (in congruence with morphological tree)
4.6.4.1 Spermatozoa

According to Schmidt-Rheasa (2007) spermatozoal characters can be used as phylogenetic characters because they proved to be informative in several cases (e.g. Ferraguti 1984, Ferraguti and Erseus 1999, Cardini and Ferraguti 2004). For Echiura, Lehrke and Bartolomaeus (2009) have shown that there are several characters and character states that seem to be phylogenetic informative, partly at species level and partly for higher taxonomic entities. This applies especially to the variety of the acrosomes, but not to the number and shape of mitochondria, because they proved to underlay individual variation (shown for Echiurus echiurus, Thalassema thalassemum and Bonellia viridis). Thus, these findings question the view of Franzen and Ferraguti (1992) that the number and shape of mitochondria are phylogenetic informative.

Within the phylogenetic analyses conducted in this study a total of nine spermatozoal characters were included; five of which refer to specifications of the acrosome (Appendix 3). Although all included spermatozoal characters turned out to be phylogenetically informative, many uncertain states in the outgroup taxon Capitella teleta (Capitellidae, Polychaeta) and echiuran taxa still hamper the reconstruction of ancestral states and a polarization of the spermatozoal character states (Fig. 48). Thus, the direction of change within character evolution is not definitely reconstructable for the shape of the spermatozoon (character 1), the shape of the acrosome (character 2), the distribution of electron dense material in the acrosome (character 3), the acrosomal rod within subacrosomal space (character 4), the membrane-bound subacrosomal vesicle (character 5), the shape of the nuclei (character 6), the relative position of the centrioles (character 8) and fins on the sperm flagellum (character 9).

Ground pattern reconstructions are presently uncertain for the structural variety of the acrosomes (shape and distribution of electron dense material within the acrosome, character 2, 3), and the shape of the nuclei (character 6), primarily due to a lack of additional structural data in the majority of echiuran species. However, despite the lack of sperm data in the majority of echiuran taxa, some limited statements and cautious suggestions are possible; they are made in the following paragraphs.

Shape of the spermatozoon (character 1). Regarding the shape of the spermatozoon the molecular tree suggests that the ancestral echiuran had a straight basal body axis that is in line with the ciliary axoneme. Due to the present data, exclusively bonelliids show an unusual oblique shape, i.e. a head and midpiece that is curved (shown for Bonellia viridis and Hamingia arctica in Franzen and Ferraguti 1992). Due to the lack of data for additional Bonellia-group members the ground pattern condition regarding this character state remains ambiguous at present. Nevertheless, it seems highly probable
that additional bonelliid and *Ikeda* species have similar aberrant filiform spermatozoa. Until today no additional data on the sperm morphology in *Ikeda* species are available. Basically, a polarization of the shape of the spermatozoon remains unclear, because this character was coded as uncertain for the outgroup taxon *Capitella teleta* on the basis of contradictory data. Franzen (1956, 1982 for *Capitella capitata*) reports at least a somewhat asymmetrically disposed acrosome on the nucleus (Fig. 10 in Jamieson and Rouse 1989) whereas Eckelbarger and Grassle (1987 for *Capitella* sp. I; Fig. 11 in Jamieson and Rouse 1989) illustrate a rather straight acroso-me-basal body-axis, a state found in the majority of echiurans (Fig. 33). Assumed *C. teleta* is synonymizable with *C. capitata* and *Capitella* sp. I, (Blake et al. 2009), this would indicate a high intraspecific variability regarding the general shape of the spermatozoon, or assumed closely related *Capitella* species are existent, this would indicate that *Capitella* species differ in their general shape. Both hypotheses, however, show that it is impossible to characterize the spermatozoon in *C. teleta* sufficiently. Subsequently, neither a polarization, nor a final statement on the phylogenetic significance of the shape of the spermatozoon is possible at the moment.

**Shape of acrosome (character 2).** Ground pattern reconstructions are presently uncertain for the shape of the acrosomes (Fig. 48). However, character mapping onto the molecular phylogeny presently suggests a multiple evolution of oblate acrosomes in Echiura: Once within *O. erythrogrammon* (presumably synonym to *O. caudex*, Stephen and Edmonds 1972), or within the highly supported clade of *O. erythrogrammon* + the composite taxon *Anelassorhynchus*, and once within the *Urechis*-group (Fig. 48; for alternative character evolution on basis of favoured morphological tree see Fig. 45F: single origin of oblate acrosomes, despite the lack of data in some of the included species). Elongate acrosomes as present in the outgroup taxon *C. teleta* and the thalassematid *T. thalassemum* presumably may be the plesiomorphic condition in Echiura or may have evolved convergently. According to the comparison of the longitudinal-transversal ratios of the acrosomes (compare Lehrke and Bartolomaeus 2009, Tab. 2) an additional thalassematid, *Listriolobus pelodes*, shares a similar ratio with *T. thalassemum*, respectively an elongate acrosome. Unfortunately, this species was not included into the molecular analysis. Nevertheless, the cladistic analysis does not indicate a closer relationship between *T. thalassemum* and *Listriolobus pelodes*, despite the similar acrosomes (Fig. 33, Fig. 45F). Since fertilization is exclusively internal in all traditional Bonelliidae (Schmidt-Rheasa 2007), it can be assumed that filiform acrosomes are apomorphic for the *Bonellia*-group despite the lack of sperm data in additional bonelliids and *Ikeda* sp. (traditional Ikedaidae). The non-discovery of males in the latter likely suggests that *Ikeda* species have tiny dwarf males along with internal fertilization, too. In addition, Franzen (1956) showed that mode of reproduction and sperm structure is generally correlated, this may also apply for the shape of the acrosomes, though oocytic external coats may also play a role (Lehrke and Bartolomaeus 2009). Surprisingly, the polychaete *C. teleta* does not
have filiform acrosomes (but elongate), although fertilization is internal, i.e. special genital tubes of the males transfer the gametes into the female (Eisig 1887, Franzen 1956).

**Figure 48:** Transformations of spermatozoal characters in Echiura (shape of acrosome, membrane bound subacrosomal vesicle, fins on flagellum) based on ML analysis of the molecular dataset. Asterisk marks composite taxon. Black squares are non-homoplastic changes, white squares homoplastic changes. At present structural data are missing in the majority of echiuran species (*dotted line*), and many character states are uncertain in the outgroup taxon. Thus, reconstruction of ancestral states and a polarization of many spermatozoal character states is not possible so far. Unambiguous apomorphic character states were exclusively optimized for *T. thalassemum* ("Thalassematidae") and *E. echiurus* (traditional Echiuridae, *Urechis*-group). Character 5: membrane bound subacrosomal vesicle; character 9: fins on flagellum. Additional spermatozoal characters were not unambiguously optimized onto the tree. All sperm type schemes are modified from the literature (*Thalassema thalassemum* and *Echiurus echiurus* modified from Lehrke and Bartolomaeus (2009). Scheme of *O. caudex* modified from Biseswar (1991); *B. viridis* modified from Franzén and Ferraguti (1992); *Urechis caupo* modified from Tyler (1965); Cross (1984); Cross et al. (1985). Figures not to scale).
Distribution of electron dense material in the acrosome (character 3). Ground pattern reconstructions and the direction of change are presently ambiguous for this character. Similar as in the outgroup Capitella teleta (compare Eckelbarger and Grassle 1987, Franzen 1982), the entire acrosomal vesicle is filled with electron-dense material in some members of the Bonellia-group (B. viridis and H. arctica in Franzén and Ferraguti 1992). This is in contrast to the state in the thalassematid T. thalassemum and some members of the Urechis-group where the electron dense material is restricted to the basal ring component. Presumably, the condition present in the outgroup taxon and the bonelliids has evolved on the basis of convergent transformations. The molecular tree presently implies a multiple origin of the state found in T. thalassemum and some members of the Urechis-group, whereas on the basis of the favoured morphological tree a single origin is indicated, despite the lack of data in some of the included species (Fig. 45F).

Acrosomal rod within subacrosomal space (character 4). On the basis of the molecular phylogeny the ancestral echiuran lacks an acrosomal rod within the subacrosomal space. Since there are no data available for the outgroup taxon C. teleta, the direction of change regarding character evolution remains unknown. At present the presence of such an acrosomal rod is apomorphic for some members of the Bonellia-group (B. viridis and Hamingia arctica, Fig. 33), but ancestral state reconstruction for the stem lineage of the Bonellia-group is presently not possible due to the lack of data.

Membrane bound subacrosomal vesicle (character 5). The molecular tree implies that the ancestral echiurid had a subacrosomal space that was devoid of a membrane-bound subacrosomal vesicle. Since there are no data available for the outgroup taxon C. teleta, the direction of change regarding character evolution remains unknown. Thus far, the presence of a subacrosomal vesicle is apomorphic for Echiurus echiurus (traditional Echiuridae) (Fig. 47).

Shape of nucleus (character 6). Although there are at least five different nuclei shapes, ground pattern reconstructions are still uncertain (compare above, character 1). Contrary to the molecular phylogeny that suggests a multiple origin of spherical nuclei as detected in T. thalassemum and the E. echiurus (Fig. 48) the character evolution on the basis of the favoured morphological tree implies a single origin of spherical nuclei (Fig. 45F). Autapomorphic transformations in O. erythrogrammon (respectively O. caudex: “barrel-shaped”, Bisewar 1991) as well as in U. caupo (spherical + indended apical) are indicated on the basis of the molecular phylogeny. However, referring to the morphological taxon sampling, additional autapomorphic nuclei shapes were identified in the morphological trees for the thalassematids Ikedosoma gogoshimense (ovoid, Sawada et al. 1975) and Listriolobus pelodes (ellipsoid, Pilger 1993), which were both not available for molecular analyses.
"Kern-Mantel" (electron-dense material forming a cylinder around the nucleus, character 7). On the basis of the molecular phylogeny the ancestral echiurid lacks a "Kern-Mantel" (sensu Leutert 1974 and Franzén and Ferraguti 1992). At the same time, the absence is a plesiomorphic character state because as some of the included non-bonelliiid species the outgroup taxon C. teleta is devoid of a “Kern-Mantel”, too. The presence of such an electron-dense cylinder is apomorphic for B. viridis and H. arctica. Ancestral state reconstruction for the stem lineage of the Bonellia-group is presently not possible due to the lack of data for additional bonelliiid and ikedid species.

Relative position of the proximal and distal centriole to each other (character 8). The molecular tree implies that the ancestral echiurid had spermatozoa with centrioles in a co-axial position to each other (rectangular and aligned; distal centriole in one axis with the nucleus). Whether this state is the plesiomorphic condition in Echiura presently remains uncertain, because there are no data available for the outgroup taxon C. teleta. A laterally displaced position (rectangular, but proximal centriole lateral displaced proportional to the basal-body, distal centriole not in one axis with the nucleus) is so far apomorphic for members of the Bonellia-group (B. viridis and H. arctica). However, due to the lack of data in additional bonelliiid and ikedid species, ancestral state reconstruction is not possible for the stemline of the Bonellia-group.

Flagellum (character 9). According to the molecular phylogeny the ancestral echiurid had a flagellum that lacks fin-like extensions of the plasma membrane. These “fins” evolved convergently: once in Thalassema thalassemum (“Thalassematidae”) and once in Echiurus echiurus (traditional Echiuridae, Urechis-group). Since there are no data available for the outgroup taxon C. teleta, the direction of change regarding character evolution remains unknown.

Character trait reconstruction on the basis of the current molecular phylogeny showed that final conclusions on the evolution of echiuran spermatozoa need to be based on more detailed (non-ambiguous) data for the present outgroup taxon Capitella teleta (Annelida, Capitellidae), and the inclusion of a broader outgroup comparison (within Capitellidae and beyond). In order to elucidate the evolution of echiuran spermatozoa it is also essential to include additional sperm data including members of all subgroups, especially from “Thalassematidae” and the Bonellia-group.
4.6.4.2 Anal sacs

Composition (character 11). The majority of known echiuran taxa develop end sacs, the most demonstrative element of the excretory anal sacs with respect to size. On the basis of the current molecular phylogeny the ancestral echiuran had an end sac that is anteriorly directed within the coelom, and is significantly larger in diameter than the funnel or its stalk (tubule) at its broadest diameter (Fig. 49). Species that seem to lack a uniting end sac are uniquely observed in a few bonelliids that were not available for phylogenetic analyses (compare character description). In these species the anal sacs are rather short, look tuft-like and are exclusively composed of ciliated funnels sitting atop their tubules. Thus, an end sac is not visible from the exterior. Character trait reconstruction on the basis of the molecular phylogeny considering the position of traditional bonelliid taxa within the tree presently implies a secondary loss or at least a reduction of the end sacs in these bonelliid species (Fig. 36D, E). This is also supported by the cladistic analyses despite the more basal position of the Bonellia-group therein. However, since the decision regarding the presence or absence of an end sac is made on external characters alone, it remains to be investigated to what extend the end sac is actually reduced in the relevant species. It is also unresolved at present whether a single origin of this secondary character loss has occurred, or whether these forms have evolved convergently within the Bonellia-group. Therefore, it is highly recommended to include the relevant species within morphological and molecular studies. Species presumably lacking end sacs are assigned to various genera (e.g. Acanthobonellia, Alomasoma, Ikedella), but not all species included into these genera lack end sacs (compare character description). The given characterization of the anal sac substructures (chapter 4.2.2) is intended to help future workers to better distinguish between end sac and tubule tissue.

Characteristics of end sacs (characters 12 to 16). Character trait reconstruction on the basis of the current molecular phylogeny indicates the ancestral echiuran had a tubular end sac that was connected to the hindgut via two pores (Fig. 49). The muscle fibers within the end sac were single, building a fine meshed muscle net. Ground pattern reconstructions are uncertain for the shape of the mesenteries due to a lack of data. At present, rope-like mesenteries are apomorphic for traditional Urechidae; laminar mesenteries are apomorphic for Bonellia viridis, though not unambiguously indicated by the character optimization. The opening mode of the end sacs into the hindgut via one pore presents the derived condition in the relevant thalassematid and bonelliid species, but seem to have evolved independently within the thalassematid and bonelliid stem lineage. Character mapping furthermore suggests a multiple evolution of sac-like end sacs, which are unambiguously indicated as homoplastic character change in traditional Urechidae and some bonelliid species (Fig. 49). Regarding the
musculature within the end sacs very little comparable information is available. The urechid *Urechis caupo* (this study) and the ikedid *Ikeda pirotansis* (Datta-Gupta and Singh 1976) show grouped muscle fibers within the end sacs. Thus, character distribution on the molecular tree indicates that this state presents the derived condition and presumably evolved independently in traditional Urechidae and Ikedaidae.

**Figure 49:** Transformations of the anal sacs (composition, general shape, tubules) based on ML analysis of the molecular dataset. Ambiguous states indicated by dotted line and question marks. Asterisk marks composite taxon. Inset shows additional anal sac morphologies in traditional Bonellidae (A: *Torbenwolffia galatheae* with short tubules; B: Funnel stalks of unknown identity in *Jakobia densopapillata*; C-D: Tuft-like anal sacs presumably lacking an end sac (C: *Alomasoma nordpacificum*, D: *Ikedella misakiensis*). The current molecular phylogeny implies the ancestral echidran had tubular end sacs covered uniformly with sessile or short-stalked funnels. Long funnel stalks (tubules) have evolved within members of the *Bonellia*-group; ground-pattern reconstruction for the *Bonellia*-group remains unknown. White squares indicate unambiguous convergent transformation of sac-like end sacs (character 13) in traditional Urechidae and a bonelliid subgroup. *Alomasoma nordpacificum* modified from Zenkevitch 1958; *Arhynchite pugettensis* based on remarks of Fisher 1949; *Echiurus echiurus* modified from Baltzer 1931; *Ikeda sp.* adopted as for *Ikeda taenioides* in Ikeda 1904; *Ikedella misakiensis* modified from Stephen and Edmonds 1972; *Maxmuelleria lankesteri* modified from Bock 1942; *Jakobia densopapillata* modified from Biseswar 2006; *Ochetostoma erythrogrammon* modified from Stephen and Edmonds 1972; *Torbenwolffia galatheae* modified from Biseswar 2010 and Zenkevitch 1966. Figures not to scale.)
Tubules (character 17). Due to ambiguous data for the basalmost offshoot of the Bonellia-group (Protobonellia sp.), the current molecular phylogeny implies that tubules (long funnel stalks) are apomorphic for a clade within the Bonellia-group (exclusive of Protobonellia sp., Fig. 49). However, this finding seems to be caused by the lack of data in Protobonellia sp. respectively the small taxon sampling in the molecular analysis. Since tubules have developed in the majority of traditional Bonelliidae (Bock 1942, Menon et al. 1964, Datta-Gupta and Menon 1976) and all known Ikedaidae, it seems highly probable that tubules have evolved within the stem lineage of the Bonellia-group, and are apomorphic for the latter. Tubules are lacking in traditional Thalassematidae (Lehrke and Bartolomaeus 2011), Urechidae (Stephen and Edmonds 1972) and putatively Echiuridae (Stephen and Edmonds 1972; Datta-Gupta 1976; Datta-Gupta and Menon 1976). The structure of the funnel stalks in Echiurus species remains arguable, because different funnel stalk lengths are indicated for Echiurus echiurus individuals, which are presently difficult to classify (compare character description). Anyhow, following the hypothesis that tubules have evolved within the stem lineage of all members of the Bonellia-group, this would imply that shorter tubules in some bonelliiids (deviating from the usually observed so called long tubules) have reduced their length secondarily (Fig. 49A, B). Regarding bonelliiid species showing funnel stalks that externally resemble short-stalked funnels (Fig. 49B), it is adopted here that these are extremely reduced tubules. But due to a lack of histological and ultrastructural data in bonelliiid species lacking a clearly discernable tubule, the true identity of these stalks still remains arguable.

Generally, more metrical data as well as developmental studies are needed prior to phylogenetic inferences regarding tubule length or branching pattern. The latter has been critically discussed in the chapter 4.2.2 “problematic characters”). The phylogenetic significance of the tubule length remains ambiguous. Conclusions made above suppose future studies will confirm that the varying tubule lengths are consistent character states. Baltzer (1931) and Fisher (1946) hypothesize the number of tubules generally increases with age, but this assumption lacks any reliable documented ontogenetic study, and it remains unclear whether one can adopt this hypothesis for tubule length. Furthermore, intra-specific variation may also play a role, at least to a certain degree.

Funnels (characters 18 – 21). Except for the studied species, no comparable information on the funnel structure is available for the majority of echirurans. Thus, the following conclusions should be viewed with caution regarding phylogenetic significance. Some limited statements are included anyhow, due to reasons of comprehensiveness and to reflect the current state of the scientific knowledge in the evolution of anal sac funnels in Echiura. Character trait reconstruction on the basis of the current molecular phylogeny indicates the ancestral echiruran had end sacs covered uniformly with conical ciliated funnels (Fig. 49) (also supported by favoured morphological tree). These funnels had a neck
region, but further specification of the neck region (i.e. inconspicuous or distinct) remains ambiguous due to a lack of comparable data (compare character 20). Reliable data are exclusively available for Anelassorhynchus adelaidensis and Thalassemata thalassemum (“Thalassematidae”). The neck region may be short giving the funnel a sessile appearance (e.g. T. thalassemum, Lehrke and Bartolomaeus 2011, Fig. 37A), or it may be distinct, giving the funnel a short-stalked appearance (e.g. Lissomyema mellita, Anelassorhynchus adelaidensis; Fig. 37B). Although not unambiguously indicated by character optimization, a funnel polymorphism (i.e. slender conical + slender cylindrical funnels simultaneously developed on end sac), is hitherto apomorphic for traditional Urechidae. The same holds true for the absence of a neck region which has been exclusively observed in U. caupo and U. unicinctus. In these species the funnel is composed of a slender conical or slender cylindrical segment which lacks an externally assignable neck segment (Fig. 37C-D). Character mapping on the current molecular tree suggests that deviations from the plesiomorphic uniform arrangement of funnel are derived with respect to the basal grades of thalassematid taxa in the molecular tree (or with respect to the phylogenetic position of Urechidae in the favoured morphological tree). A decrease of funnels from proximal to distal seems to have evolved within the stem lineage of the Urechis-group (present in E.echiurus and Urechis unicinctus). However, in Urechis caupo, this state has transformed into an increase from proximal to distal.

4.6.4.3 Larval protonephridia

By providing ultrastructural data for Thalassemata thalassemum (“Thalassematidae”) our study in collaboration with Kato et al. (2011) provides the first ultrastructural data for larval protonephridia (head kidneys) in Echiura. Hitherto, only light microscopical observations were conducted (Baltzer 1914, 1917; Goodrich 1910, 1945; Dawydoff 1959). Light microscopical studies comprise one species of traditional Bonelliidae (Bonellia sp.) and one species of traditional Echiuridae (Echiurus sp.). Thus, the following conclusions should be viewed with caution. They are included anyhow, due to reasons of comprehensiveness and to reflect the current state of the scientific knowledge in the evolution of larval protonephridia in Echiura.

On the basis of the molecular phylogeny the ancestral echiuran had a head kidney that was tubular and the terminal structure was composed of several multiciliated cells (Fig. 50). Whether the head kidney had characteristics of a so-called solenocyte (sensu Goodrich 1910, 1945) remains unclear, because more details on the head kidneys in E. echiurus and the outgroup taxon Capitella teleta are unknown. The filter structure was built up by a perforated cytoplasm (sensu Kato et al. 2011), which has
transformed into layers of elongate microvilli in *T. thalassemum*. The duct cell of the ancestral echiuran head kidney lacked microvilli and was multiciliated. Ground pattern reconstructions are presently uncertain for the presence of circumciliary microvilli within terminal structure (present in *T. thalassemum* and *B. viridis*), the number of cells involved into the composition of the duct and the structure of the nephridiopore.

**Figure 50:** Transformations of the larval protonephridia (head kidneys) in Echiura (general shape, filter, number of cells of the terminal structure) based on ML analysis of the molecular dataset. Asterisk marks composite taxon. Black squares are non-homoplastic changes. Although structural data are missing in the majority of species (*dotted line*), the current molecular phylogeny implies to include a filter structure built up by a perforated cytoplasm (sensu Kato et al. 2011) into the echiuran ground pattern. Due to the lack of comparable data unambiguous apomorphic character states were optimized for the thalassematid *T. thalassemum* (character 23: number of cells of the terminal structure; character 24: cilia per terminal cell; character 26: filter structure). All schemes are modified from the literature (*Bonellia* sp. modified from Dawydoff 1959; *Echiurus* sp. modified from Goodrich 1910, 1945; *T. thalassemum* modified from Kato et al. 2011); Figures not to scale.

On the basis of the molecular phylogeny the structural correspondences in *B. viridis* and *T. thalassemum* (i.e. tubular shape, multicilarity of terminal cells, absence of circumciliary microvilli in
terminal cells) are not based on convergent evolution; they had already developed in the stem lineage of Echiura (tubular shape, multicilarity of terminal cells), or from *T. thalassemum* downwards (absence of circumciliary microvilli in terminal cells). Due to the lack of comparable data *T. thalassemum* shows unambiguous apomorphic character states (Fig. 47; Fig. 50). These character states are: composition of the terminal structure is via one cell only and composition of the filter structure by two to three layers of elongate microvilli emerging from the terminal cell.

Althought not unambiguously indicated by character optimization (Fig. 47; Fig. 50), presently apomorphic for *Echiurus echiurus* and rather derived within echiurans are the general shape (branched) and the monociliarity of the terminal cells. This is in contrast to previous hypotheses that the larval proctonephridium in *E. echiurus* generally represents the plesiomorphic condition (Kato et al. 2011). Based on the data available, only the shared presence of several terminal cells and an assumed similar filter as in *Capitella teleta* (perforated cytoplasm sensu Kato et al. 2011; pers. comm. B. Quast) is revealed as a plesiomorphic character state.

### 4.6.4.4 Gonoducts

Within the scope of ancestral character trait reconstruction taxonomically relevant characters and character states exclusively referring to the general appearance (character 31), shape (character 32) and position of the gonostome (character 33) were mapped onto the molecular tree, for reasons discussed elsewhere (4.4.1 “Comparison within Echiura”). In addition the arrangement of the gonoducts (character 47) was included in order to test Ruppert et al. (2004) hypothesis of being a phylogenetic significant character for traditional Bonelliidae + Ikedaidae.

**General appearance of gonostome (character 31).** On the basis of the molecular phylogeny and with respect to the limited taxon sampling the ancestral echiuran had a sessile gonostome (Fig. 51). Sessile gonostomes are plesiomorphic for all included thalassematid taxa (except for *Arhynchite pugettensis*) and the *Urechis*-group. Although not unambiguously indicated by the character optimizations, character mapping furthermore suggests a multiple evolution of stalked gonostomes in Echiura. Stalked gonostomes are present in the thalassematid *Arhynchite pugettensis* and all bonelliids included into the molecular analysis. It is not clear whether *A. pugettensis* is the only thalassematid developing stalked gonostomes, but it appears like the majority of traditional Thalassematidae has sessile gonostomes (Stephen and Edmonds 1972). Due to a lack of data on the gonostome structure in many bonelliids (compare character description) it remains unknown whether a stalked gonostome was present in the stem lineage of the *Bonellia*-group as it is indicated by the restricted (bonelliid) taxon
sampling in the current molecular phylogeny (Fig. 51). Character polarization is not possible, due to ambiguous data for the outgroup *Capitella teleta* (Annelida, Capitellidae).

Figure 51: Transformations of the gonostomes in Echiura (general appearance, position and shape of lips) based on ML analysis of the molecular dataset. Asterisk marks composite taxon. Black squares are non-homoplastic changes, white squares homoplastic changes. The molecular tree implies the ancestral echiurid had a sessile gonostome lacking spirally coiled (filamentous) lips. Unambiguous apomorphic character states were exclusively optimized for the thalassematids *O. erythrogrammon* + the composite taxon *Anelassorhynchus* and traditional Urechidae within the *Urechis*-group. Both clades display spirally coiled lips (character 32), which is indicated as homoplastic change. *Ikeda sp.* proved to be unique (within this taxon sampling) in featuring a terminal gonostome. Apomorphic for *M. haswelli* is a gonostome near the distal end (character 33). All gonostome type schemes are modified from the literature except for *M. haswelli* and *T. thalassemum* (*A. pugettensis* and *U. caupo* modified from Fisher 1946; *B. viridis* modified from Greef 1879); Figures not to scale.

Shape of gonostomal lips (character 32). Regarding the shape of the gonostomal lips (spirally coiled or not), the molecular tree suggests that the ancestral echiuran had a gonostome lacking spirally coiled (filamentous) lips, but the further specification of the structure of these lips remains unclear (compare chapter 4.4.1 “problematic characters”). However, gonostomal lips that are not elongated into spirally
coiled filaments have to be interpreted as plesiomorphic character state on the basis of the molecular phylogeny and outgroup comparison. This is in contrast to Bock (1942), who hypothetized spirally coiled lips “represent a very old feature”. Based on the variability of the gonostomal lips in the bonelliid *Maxmuelleria lankesteri* (Fig. 42A-C) he suggested that their shape is probably a case of reduction. According to Bock (1942) the ancestral state was a gonostome with a bi-lipped structure, which was first spirally coiled, than simple, followed by forms lacking a bi-lipped structure, but featuring a funnel with different borders (primary simple border, more derived: frilled border or even a petaloid funnel). Character distribution on the molecular tree indicates that this hypothesis is controversial, mainly due to the small taxon sampling, and the shape of lips in the thalassematid *Arhynchite pugettensis* respectively its position within the molecular tree. The lips of the latter species are not bi-lipped, but are rather leaf-like with a frilled border (Fig. 51). This would hence be a derived state according to Bock’s (1942) evolutionary chain of transformations, which is not supported by the molecular tree (Fig. 51). Instead species belonging to the *Bonellia*-group show derived states, anyhow with stalked gonostomes and lips that are frilled. Nevertheless, the basalmost offshoot in the molecular tree is a thalassematid clade (*Anelassorhynchus adelaidensis* and *Ochetostoma erythrogrammon*) that develops spirally coiled lips. But since these are also present in traditional Urechidae spirally coiled lips are unambiguously identified as a homoplastic character state by the phylogenetic analysis (Fig. 51). Besides the latter two thalassematid genera, spirally coiled lips also develop in the thalassematid genera *Ikedosoma* and *Listriolobus* (Stephen and Edmonds 1972). Since these taxa were included into the cladistic analysis of the morphological data, the favoured morphological tree in contrast implies that spirally coiled lips are apomorphic for a clade comprising some thalassematid taxa (*Anelassorhynchus adelaidensis*, *Listriolobus pelodes*, *Ochetostoma caudex*, *Ikedosoma gogoshimense*) + the *Urechis*-group (Fig. 45F). Within the *Urechis*-group exclusively *E. echiurus* develops not spirally coiled lips, which is unambiguously indicated as a homoplastic transformation.

Position of the gonostome (character 33). Regarding the position of the gonostome, the molecular tree suggests that basal gonostomes belong to the echiuran ground pattern, which is in accordance with Fisher (1946), but conflicts with Ruppert et al. (2004). Terminal gonostomes (at distal tip of gonoduct) represent a rather derived state on the basis of the molecular data; they are unambiguously identified as apomorphic for certain members of the *Bonellia*-group (Fig. 45F; Fig. 51) (all Ikeda species and some traditional bonelliids, e.g. *Pseudoikedella achaeta*, compare character description). This is in contrast to Bock (1942) and Ruppert et al. (2004), who stated that the ancestral echiuran had a terminal gonostome. However, a position near the distal end (within proximal most third of gonoduct) as present in *Metabonellia haswelli* is also indicated as apomorphic and a rather derived state within the *Bonellia*-group. Such a position is hitherto only known from the latter species and the bonelliid, *Pseudobonellia biuterina*. 

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Arrangement of gonoducts (character 47). Ruppert et al. (2004) use the arrangement of gonoducts in their hypothesis on the phylogeny of Echiura. Although their hypothesis is not based on a phylogenetic analysis, they include conclusions regarding the arrangement of gonoducts by implementing “segmental metanephridia” for the echiuran ground pattern and an “unpaired metanephridium” as a synapomorphy for a clade comprising traditional Bonelliidae and Ikedaidae in a sister group relationship (Fig. 46C). It is assumed here that Ruppert et al. (2004) thought of paired gonoducts (as referred to in Stephen and Edmonds 1972, Datta-Gupta 1974, Pilger 1993) by using the term “segmental metanephridia” and unpaired gonoducts as abrasively defined in Datta-Gupta (1974). Arguments that question the view of being a consistent phylogenetic informative character in the relevant subgroups have been already discussed (chapter 4.4.2, 4.5 (i); character description). In order to test Ruppert et al. (2004) hypothesis, character evolution of this arguable character is reconstructed anyhow on the base of the phylogenetic analyses (Fig. 52). According to the molecular phylogeny the ancestral echiuran had paired gonoducts, with one member on each side of the ventral nerve cord, notwithstanding there are polymorphisms included into the present matrix (Fig. 52). Unpaired gonoducts are plesiomorphic for all included thalassematid taxa and the Urechis-group. The unpaired ground pattern condition is in accordance with Ruppert et al. (2004). But on the basis of the phylogenetic analyses (morphological + molecular) it remains highly questionable whether changes
from the echiuran ground pattern towards unpaired gonoducts can be interpreted as a synapomorphy for Ikedaidae + Bonelliidae as assumed by Ruppert et al. (2004).

First of all, the term unpaired is very imprecise still it is used by Ruppert et al. (2004) without giving a definition of this character. While the term “paired” is rather clear regarding its meaning (one member on each side of the ventral nerve cord, compare Datta-Gupta 1974, Pilger 1993), the term “unpaired” includes variable information. According to Stephen and Edmonds (1972) and Datta-Gupta (1974) the term “unpaired” does include single gonoducts or clusters of gonoducts (= group of gonoducts on the same side of the ventral nerve cord, Datta-Gupta 1974). Ruppert et al. (2004), however, do not differentiate between these two states. Thus, it is completely unclear what kind of transformations should be assumed for unpaired gonoducts, i.e. single ones or clusters.

Secondly, the resolution within the Bonellia-group still has to be treated with reservation, mainly because of the small taxon sampling. Tree topology and respectively evolutionary implications for character trait reconstruction may change with the inclusion of additional taxa. Ancestral trait reconstruction for the Bonellia-group is highly dependent on the inclusion of the paired condition. So, on the basis of the molecular phylogeny the ground pattern for the Bonellia-group remains ambiguous regarding the arrangement of gonoducts (Fig. 52). Unlike the favoured morphological tree (Fig. 45F) the current molecular phylogeny indicates a multiple origin of unpaired gonoducts (Fig. 52). But as said above, this should be interpreted with caution in light of the small taxon sampling. Unpaired gonoducts are exclusively optimized as apomorphy for the Bonellia-group assuming fast optimization of evolution (reversals allowed, early development of character state but later transformed).

Thirdly, it is ambiguous which state should be adopted for Ikeda sp. (molecular taxon sampling), because the trunks of all specimens were damaged (Edmonds 1987, this study). In addition, there is varying information on the arrangement in the better known Ikeda species, I. pirotansis and I. taenioides (Ikeda 1904; Stephen and Edmonds 1972; Datta-Gupta and Menon 1976; Saiz-Salinaz 1996; chapter 4.5 “Additional characters”, i); Fig. 52). On the assumption that Ikeda sp. has unpaired gonoducts, this state is optimized as apomorph for the Bonellia-group (just as in the favoured morphological tree with I. pirotansis and I. taenioides), but this would also imply that reversals occur: two within the molecular taxon sampling (Maxmuelleria lankesteri and Alomasoma belyaevi: secondary paired gonoducts as convergent transformations) and one within the favoured morphological tree (I. pirotansis). Under the assumption Ikeda sp. has paired gonoducts it remains still unclear which state should be included into the stem lineage of the Bonellia-group.

Forthly, the arrangement of gonoducts is basically related to the number of gonoducts (Fig. 52), which is often affected by a high intra-specific variability (Lacaze-Duthiers 1858; Stewart 1900; Ikeda 1904,
1907; Baltzer 1931; Bock 1942; Edmonds 1963; Stephen and Edmonds 1972; Datta-Gupta 1974). For example, there are reports of mixed asymmetrical arrangements including pairs, clusters and single gonoducts even within a single species (chapter 4.4.1 “Comparison of gonoducts within Echiura”). On the other hand, possibly significant information, e.g. whether paired groups, or pairs consisting of only two gonoducts have developed, is not yet considered.

However, the current molecular tree topology implies that several gonoducts (exact number remains ambiguous) belong to the echiuran ground pattern, which is in accordance with Bock (1942). A single gonoduct in contrast has to be interpreted as resulting from secondary loss, which was already suggested by Bock (1942) and Datta-Gupta and Menon (1976). Assuming *Ikeda taenioides* would be included into the current molecular tree topology with a corresponding position as *Ikeda* sp. (presumably as sister to *Ikeda* sp.) the extremely large number of gonoducts would have to be interpreted as secondarily increased, which is in accordance with Bock (1942) and Ruppert et al. (2004). But as already discussed before (chapter 4.5 “Additional characters”, i), the extremely high number of gonoducts in *I. taenioides* seems to be apomorphic for the species alone, and not characteristic for traditional Ikedaidae as assumed by Ruppert et al. (2004). Furthermore, both, molecular and favoured morphological tree, suggest that the large number is not an ancestral character (Ikeda 1904) although the phylogenetic position of the *Bonellia*-group is more basal in the favoured morphological tree (Fig. 46A).

4.6.4.5 Chaetae

Ax (1999) and Ruppert et al. (2004) assume that one pair of anterior ventral chaetae and two posterior rings of anal chaetae are plesiomorphic characters being part of the echiuran ground pattern. Thereby, they postulate that annelidan segmental chaetae are directly homologue to the echiuran ventral chaetae and the rings of anal chaetae. Consequently, Ruppert et al. (2004) regard traditional Echiuridae with two rings of anal chaetae as “primitive” group bearing the remnants of originally three segments. Taxa that lack the chaetal rings, i.e. traditional Thalassematidae, Bonelliidae and Ikedaidae are interpreted as evolutionary derived by these authors (secondary loss of anal chaetae). But contrary to Ruppert et al. (2004) and Ax (1999) the phylogenetic analyses (both morphological and molecular) identified the presence of rings of anal chaetae (character 36) unambiguously as apomorphic character for the *Urechis*-group (synapomorphic for traditional Echiuridae and traditional Urechidae) (Fig. 45A, F; Fig. 47). Two rings are apomorphic for basal branching *E. echiurus* (respectively all *Echiurus* species), one
ring for all *Urechis* species. Thus, traditional echiurids and urechids are not basal taxa, but rather derived with respect to “Thalassematidae”, which is in contrast to Ruppert et al. (2004).

On the basis of the molecular phylogeny and with respect to the limited taxon sampling the ancestral echiuran had one pair of ventral chaetae (*character 34*), which is in congruence with Fisher (1949), Ax (1999) and Ruppert et al. (2004). Following Ax (1999) it is assumed here that the anterior ventral chaetae with its typical arrangement are apomorphic for Echiura, because they are not known from any polychaete, though they are secondarily reduced in some echiuran species (compare last two paragraphs of this section). But contrary to the rings of posterior anal chaetae, the anterior ventral chaetae can be easily inferred from the chaetal formation in annelids, respectively polychaetes (Schweigkofler et al 1998 for *Capitella capitata*; Hausen 2005) within a hypothetical evolutionary scenario (Fig. 53).

Considering this scenario, it seems highly probable that the ventral chaetae correspond to the neuropodial chaetae of annelids regarding their position and formation. This requires that the notochaetae dropped out over time and that the neurochaetae shifted then from medial to ventral. Contrary to the state in echiuran ventral chaetae, the posterior rings of anal chaetae cannot be easily deduced from the expected pattern of chaetal formation in annelids, respectively polychaetes. Based on personal observations as indicated for *Echiurus echiurus* in Greet (1879, Fig. 25), each adult anal chaeta possesses its own formation side (with a young developing chaeta laterally), so that one can

**Figure 53:** Simplified scheme of chaetal formation in Echiura inferred from chaetogenesis in Annelida featuring chaetal rings, serial chaetae or fields of chaetae. Contrary to the anterior ventral chaetae, the posterior rings of anal chaetae in *Echiurus echiurus*, traditional Echiuridae (and traditional Urechidae) cannot be deduced from the expected pattern in annelids. *d* dorsal side, *v* ventral side.
find as many formative sides (chaetal sacs) as adult chaetae are present. This assembly is untypical for annelids featuring chaetal rings, serial chaetae or fields of chaetae (compare Hausen 2005, Fig. 8). Thus, it can be concluded that the anal rings are not (directly) homologue to the chaetal rings, serial chaetae or fields of chaetae in annelids as suggested by Ruppert et al. (2004). Although data on the formation of the anal chaetae in *Urechis* species are presently not available, it is assumed, that these species have a similar formation pattern as indicated for the posterior rings of anal chaetae in *E. echiurus* (Greef 1879; Fig. 25).

Character trait reconstruction on the basis of the molecular phylogeny regarding the absence-presence of ventral chaetae implies a secondary loss of such chaetae in the bonelliid *Alomasoma belyaevi*. A compilation of literature data furthermore indicates that the secondary loss of ventral chaetae is not uncommon in traditional Bonelliidae as it occurs in additional species (compare character 34). But the question whether an increased taxon sampling of bonelliid species in future may detect a single origin of the character loss in bonelliids or not remains open. A multiple origin seems also likely, with respect to the account for the putative lack of ventral chaetae in the thalassematid *Ochetostoma senegalense* (see Stephen and Edmonds 1972). However, the latter finding is exclusively based on the holotype (Stephen and Edmonds 1972) and therefore remains problematic. Nevertheless, the lack of ventral chaetae as a derived character state, at least in traditional Bonelliidae, was already suggested by Fisher (1949). The molecular phylogeny also implies that additional variation from the ordinary paired occurrence of two ventral chaetae (character 35), i.e. one or several ventral chaetae in traditional Bonelliidae are derived states with respect to the remaining echiurans (Bock 1942). The single finding of several ventral chaetae (eight “spinlets”, Stephen and Edmonds 1972) in one thalassematid species, *Anelassorhynchus chaetiferus*, suggests an independent development of this increased number of chaetae, but this requires a careful re-investigation of the species with respect to character consistency prior to a final phylogenetic conclusion.

### 4.6.4.6 Proboscises

*Length (character 40).* At present, it is merely possible to differentiate easily between “short” (a few centimetres, Stephen and Edmonds 1972; Ruppert et al. 2004) and "very long" (0.75-2.0, Ikeda 1904; Baltzer 1931; Hughes and Crisp 1976; Ruppert et al. 2004) (compare character description). Species developing proboscises with rather moderate lengths that lie between these ranges are referred to as “elongate” (Ruppert et al. 2004). On the basis of the molecular phylogeny and with respect to the limited taxon sampling the ancestral echiuran had such an “elongate” proboscis (Fig. 54), which is in
accordance with Ruppert et al. (2004). Due to the lack of data the exact range for an “elongate” proboscis remains ambiguous. A short proboscis in contrast is unambiguously apomorphic for traditional Urechidae, which is indicated by both, cladistic analysis and molecular phylogeny (Fig. 45F, Fig. 47). Character mapping onto the molecular tree implies, furthermore, that these have be interpreted as secondarily reduced for adaptation to the specific filter feeding process in this subgroup (Bock 1942; Stephen and Edmonds 1972; Ruppert et al. 2004). In contrast to Ruppert et al. (2004) who proposed “very long” proboscises as a synapomorphy for a clade comprising traditional Bonelliidae and Ikedaidae in a sister group relationship, the current molecular phylogeny implies “very long” proboscises have evolved on the basis of convergent transformations (Fig. 47, Fig. 54). Hitherto “very long” proboscises are present exclusively in very few members of the Bonellia-group (Bonellia viridis and Ikeda sp. as well as Ikeda pirotansis, Ikeda taenioides). But presently, it cannot be excluded that a single origin may be detected by future studies, provided that the taxon sampling is enlarged, especially for traditional Bonelliidae.

Shape (character 41). The molecular phylogeny implies that the ancestral echiuran had a simple tongue-like (not bifid) proboscis (Fig. 54) which is in accordance to Fisher (1946) and Ruppert et al. (2004). Bifid (forked) proboscises have evolved convergently within some members of the Bonellia-group according to the current molecular tree, but within the slightly different taxon sampling for cladistic analysis bifid proboscises are unambiguously apomorphic for a small clade within the Bonellia-group (Metabonellia haswelli, Bonellia viridis, Hamingia arctica) (Fig. 45F). Ruppert et al. (2004) hypothesis that a forked proboscis is apomorphic for traditional Bonelliidae is problematic, because there are also many bonelliid species that develop tongue-like proboscises. Bifid proboscises presently seem to be apomorphic for a certain subgroup within traditional Bonelliidae. The enlargement of the molecular taxon sampling upon a simultaneous increase of structural information by future studies will clarify this issue also with respect to unusual differentiations of the lateral edges present in a few traditional Thalassematidae and specifications that surround the mouth not only in some deep-sea bonelliids (compare character description).

Basically, shape and length of the proboscises seem to be related to some extent to the feeding process, as shown in the example of the short stout-like proboscis in traditional Urechidae. However, the precise constraints of natural selection in other echiurans presently remain unclear. It may be that some differentiations of the lateral edges in thalassematids and the bifurcation in bonelliids are also linked to sensory perception. Jameson (1899) and Baltzer (1931) have shown that sensory cells concentrate at the proboscis margin (shown for Thalassema neptuni) and the dorsal side of the fork (shown for Bonellia viridis).
Figure 54: Transformations of proboscis characters (length, shape and colour pattern) based on ML analysis of the molecular dataset. Ambiguous states indicated by dotted line. Asterisk marks composite taxon. The current tree topology implies the ancestral echiuran had a simple tongue-like proboscis of moderate length (elongate). Short proboscis are apomorphic for traditional Urechidae. Bifid proboscis have evolved convergently within some members of the Bonellia-group. The apomorphic colour pattern of Ikeda species is shown at the top right hand side (LM, in vivo; A: *I. taenioides* [http://suityusukima.sakura.ne.jp/hiroshimawan/sanadayumushi-hiro.html]; B: *Ikeda* sp. (kindly provided by G. Rouse)). All schemes are compiled from the literature except for *Protobonellia* sp. and *U. unicinctus* which are both based LM micrographs (*Protobonellia* sp. see Fig. 3E; *U. unicinctus* deduced from http://www.dvoutput.com/Image/2010031515571614089). *O. erythrogrammon* modified from Stephen and Edmonds (1972); *T. thalassemum* compiled from Baltzer (1931); *E. echiurus* modified from Greef (1880); *Urechis caupo* modified from Fisher (1946); *M. lankesteri* compiled from Bock (1942); *Ikeda* sp. modified from Ikeda (1904); *M. haswelli* modified from Edmonds (2000); *A. belyaevi* compiled from Saiz-Salinas et al. (2000); *B. viridis* modified from Ruppert et al. (2004). The scheme presented for the composite taxon Anellassorhynchus *refers to A. adelaidensis* (modified from Edmonds 2000). Figures not to scale.

**Colour pattern (character 42)**. The molecular phylogeny and the morphological analyses indicate the conspicuous colour pattern observed in *Ikeda* sp. and the remaining *Ikeda* species has exclusively evolved in traditional Ikedaidae within the Bonellia-group. The conspicuous colour pattern is apomorphic for *Ikeda* sp. (respectively *I. pirotaensis* + *I. taenioides*, Fig. 45F) and includes numerous dark brownish-black spots or transversal stripes on pale grayish-white dorsal subsurface (Fig. 54A, B). Character distribution on the molecular tree implies that the ancestral echiuran lacks such a
conspicuous colour pattern (pattern other than in *Ikeda* species or single-coloured, compare character description). The significance of these characteristic spots and stripes as well as other colour patterns, mainly observed in traditional Bonelliidae presently remains unclear.

4.6.5 Stem species reconstruction and concluding remarks on traditional “Thalassematidae”

For stem species reconstruction unambiguous character changes consistently implied by both, favoured morphological and molecular tree, are considered.

4.6.5.1 Echiura

Some information on the ground pattern of Echiura can presently be found in Ax (1999) and Ruppert et al. (2004). On the basis of this study, the postulated character states are reviewed and expanded with additional characteristics, especially the spermatozoa, the anal sacs, the larval protonephridia and gonoducts.

Despite the enlargement of the data set for echiuran spermatozoa (Lehrke and Bartolomaeus 2009, Tab. 1), little can be said regarding unambiguous character states of the spermatozoa in the ancestral echiuran. Thus far, the stem species had spermatozoa that lacked a membrane-bound subacrosomal vesicle (sensu Lehrke and Bartolomaeus 2009), a “Kern-Mantel” (sensu Leutert 1974, Franzén and Ferraguti 1992) and fins on the flagellum (Fig. 48).

The anal sacs were composed of a tubular end sac that was connected to the hindgut via two pores (Fig. 49). The muscle fibers within the end sac were single, building a fine meshed muscle net. The structure of the mesenteries emanating from the end sacs is unknown. Tubules (long funnel stalks) had not developed. All ciliated funnels of the anal sacs had the same shape (funnel polymorphism absent) and were equipped with a neck region. But a specification of the neck region (sessile appearance or short-stalked appearance of funnel) remains ambiguous. The arrangement of funnels upon the end sac was uniform.

The general shape of the ancestral larval protonephridium (head kidney) was tubular (unbranched) and the terminal structure was composed of several multiciliated cells (Fig. 50). The filter structure was
built up by a perforated cytoplasm (sensu Kato et al. 2011). The duct cell was multiciliated and lacked microvilli. Several states remain unknown (the presence of circumciliary microvilli within terminal structure, the number of cells involved into the composition of the duct and the structure of the nephridiopore).

The arrangement of gonducts was paired, one member on each side of the ventral nerve cord (sensu Datta-Gupta 1974, Pilger 1993), which is in accordance with Ruppert et al. (2004) (Fig. 52). Unlike the latter authors who assumed a terminal gonostome, the current phylogenetic analyses imply a gonostome with a basal position and gonostomal lips that were not spirally coiled (i.e. not filamentous) (Fig. 51). Any further specification of the shape of these lips is not possible at present (see chapter 4.4.1 “problematic characters” and 4.4.2).

The ancestral echiuran was further characterized by males and females that looked similar. The stem species had two anterior ventral chaetae, but posterior rings of anal chaetae had not developed, the latter is in contrast to Ax (1999) and Ruppert et al. (2004). A post-pharyngeal diaphragm was also not present. The proboscis was tongue-like (not bifid) and of moderate length (“elongate”). Furthermore, the stem species lacked a colour pattern on the proboscis as it is characteristic for *Ikeda* species (Fig. 54). So it is assumed that it was single-coloured or showed a pattern other than in *Ikeda* species. A hemal system had developed (Ruppert et al. 2004). The ancestral echiuran lacked an enlarged cloaca (“water lung”), as well as a glandular girdle on the anterior trunk.

### 4.6.5.2 *Bonellia*-group

The *Bonellia*-group comprises monophyletic traditional Ikedaidae nested within a paraphyletic traditional Bonelliidae. This opposes to the traditional classification of Echiura by Stephen and Edmonds (1972) and also to the revised classification by Nishikawa (2002) as well as to the phylogeny proposed by Ruppert et al. (2004) (Fig. 46C). Monophyly of the group is well supported on the basis of the molecular data (LBS 89/92%), but weakly supported by the bootstrap support of the cladistic analysis (50%). Monophyly of the group is based unambiguously on the presence of dwarf (paedomorphic) males by both, favoured morphological and molecular tree (Fig. 45F, Fig. 47). Contrary to Ruppert et al. 2004 who assume dwarf males to be apomorphic for traditional Bonelliidae, this study supports the hypothesis *Ikeda* species are characterized by dwarf males along with internal fertilization, too. Additional support comes from the fact that male specimens never have been found so far in *Ikeda* species (Ikeda 1904; Datta-Gupta and Menon 1976; Hughes and Crisp 1976; Nishikawa
Besides a pronounced sexual dimorphism the stem species of the *Bonellia*-group showed the following unambiguous character states.

Due to the current taxon sampling, little can be said regarding unambiguous character states of the spermatozoa in the ancestral bonelliid. It lacked a subacrosomal vesicle and fins on the flagellum (sensu Lehrke and Bartolomaeus 2009). Additional states such as general shape and characteristics of the acrosome remain ambiguous (Fig. 48; see chapter 4.6.4 “Character evolution-Spermatozoa”).

The anal sacs were composed of a tubular end sac that was connected to the hindgut via two pores (Fig. 49). The muscle fibers within the end sac were single, building a fine meshed muscle net. Additional states respectively the evolution of the diversity of tubule lengths and branching remains ambiguous (compare chapter 4.6.4 “Character evolution-Anal sacs”).

The characterization of the ancestral bonelliid head kidney should be viewed with reservation because only some limited data for one member (*Bonellia* sp.) are available at present. The stem species of the *Bonellia*-group had a tubular (unbranched) shape (Fig. 50). The terminal structure was composed of several multiciliated cells lacking circumciliary microvilli. The filter structure was built up by a perforated cytoplasm (sensu Kato et al. 2011). The duct cell was multiciliated and lacked microvilli. Stem species reconstruction remains unknown for the number of cells involved into the composition of the duct and the structure of the nephridiopore.

In addition the stem species had a stalked gonostome with a basal position and gonostomal lips that were not spirally coiled (Fig. 51). The shape of these lips is unknown to date (compare character description and 4.6.4 “Character evolution-Gonducts”). The ancestral bonelliid was further characterized by two anterior ventral chaetae, a hemal system, a tongue-like (not bifid) proboscis, which had a single-colour, or a colour pattern other than in *Ikeda* species.

Contradictory implications with respect to the *Bonellia*-group stem species are based on the different positions of the group within favoured morphological, respectively molecular tree, and the slightly varying taxon sampling applied therein. These conditions hamper presently a final statement on the relevant character states. Inconsistent reconstructions of the ground pattern refer to few spermatozoal characters (distribution of electron dense material in the acrosome, shape of nucleus), the arrangement of gonoducts, the length of probosces and the presence of anal sac tubules.

Contrary to the molecular phylogeny, the favoured morphological tree implies the spermatozoon of the stem species had an acrosome filling the entire acrosomal vesicle and a sausage-shaped nucleus (Fig. 45F). On the basis of the molecular tree, these structural correspondences with the outgroup taxon *Capitella teleta* suggest a homoplastic origin instead (Fig. 48).
According to the molecular analysis the arrangement of the gonducts is presently not inferable (Fig. 52). This is in contrast to the favoured morphological tree that implies unpaired gonducts have evolved within the stem lineage of the *Bonellia*-group (Fig. 45F). To include unpaired gonducts within the ground pattern of the *Bonellia*-group, or the stem lineage of a clade consisting of monophyletic Bonelliidae + monophyletic Ikedaidae (Ruppert et al. 2004) is very arguable (see chapter 4.6.4 “Character evolution-Gonoducts”).

The length of the proboscis in the stem species remains ambiguous on the basis of the favoured morphological tree, but is elongate on the basis of the molecular tree. Assuming such an elongate proboscis of moderate length to be present in the *Bonellia*-group stem lineage, implies very long probosces have evolved on the basis of convergent transformations within some members of the group (*Ikeda* species, *Bonellia viridis*, Fig. 47, Fig. 54). This is in contrast to Ruppert et al. (2004) who state that the stem species of Ikedaidae + Bonelliidae was equipped with a very long proboscis.

Unlike the implications from the molecular tree or the assumptions of Ruppert et al. (2004), anal sac tubules are apomorphic for traditional Bonelliidae, it is assumed here, that tubules have evolved within the stem lineage of the *Bonellia*-group. This is supported by the favoured morphological tree and all cladograms of the weighted analysis (Fig. 45E, F). The fact that tubules have developed in the majority of traditional Bonelliidae (Bock 1942, Menon et al. 1964, Datta-Gupta and Menon 1976) and all known Ikedaidae (Ikeda 1904, Stephen and Edmonds 1972; Datta-Gupta and Menon 1976; Nishikawa 2002) supports this assumption, too.

### 4.6.5.3 *Urechis*-group

The *Urechis*-group is composed of two traditional urechid taxa (*Urechis caupo*, *Urechis unicinctus*) that share a sister group relationship and *Echiurus echiurus*, a member of traditional Echiuridae, which is resolved as basal grade. Monophyly is highly supported by the molecular data (“original dataset”: LBS 95%), as well as by all cladograms of the cladistic analyses (98-99%). Monophyly of the group is based unambiguously on the presence of rings of anal chaetae (Fig. 45A, E, F; Fig. 47). Although the *Urechis*-group is retrieved differently within the phylogenetic trees, both, molecular and favoured morphological tree, are congruent in identifying the *Urechis*-group as a rather derived group with respect to “Thalassematidae” (molecular tree), respectively the remaining echiumans (favoured morphological tree). This is in contrast to Ruppert et al. (2004), who assume traditional Urechidae and Echiuridae as basal groups with their interrelationships unresolved (Fig. 46C).
Spermatozoa of the stem species were characterized by a straight basal body axis that is in line with the ciliary axoneme. The acrosome was oblate and the electron dense material in the acrosome was restricted to the basal ring component in the basal part of the acrosomal vesicle. The subacrosomal space was not membrane bound and lacked an acrosomal rod. The centrioles were co-axial (rectangular and aligned; distal centriole in one axis with the nucleus). Furthermore, spermatozoa of the stem species lacked a “Kern-Mantel” (sensu Leutert 1974 and Franzén and Ferraguti 1992) and fins on the flagellum.

The anal sacs were composed of a tubular end sac that was connected to the hindgut via two pores. Details of the muscle fibers within the end sac remain ambiguous. The end sacs were covered with ciliated funnels of unknown structure, but they lack tubules (Fig. 49). The arrangement of funnels upon the end sac followed a certain pattern: decrease from proximal to distal.

The characterization of the ancestral urechid head kidney should be viewed with reservation because only some limited data for one member are presently published (Echiurus sp.) and there is some arguable information for another member (U. caupo). The general shape of the head kidney remains unknown. But there is an arguable cLSM micrograph in Hessling (2002, Fig. 1D, E), which suggests a branched general morphology of the head kidneys in U. caupo. But as the resolution is too low to allow a final unambiguous statement, this remains unclear at present. In case future studies will prove this to be true, branched head kidneys could be included into the ground pattern of the Urechis-group.

Unambiguous characteristics for the stem species are: a terminal structure composed of several cells, a filter built up by perforated cytoplasm (sensu Kato et al. 2011) (Fig. 39C, Fig. 50) and multiciliated duct cells that lacked microvilli. Besides the general shape several additional states remain ambiguous at present: the number of cilia per cell of the terminal structure, the presence of circumciliary microvilli within terminal structure, the number of cells involved into the composition of the duct and the structure of the nephridiopore.

Regarding the arrangement of the gonoducts it is unambiguously indicated that the stem species had paired gonoducts, i.e. one member on each side of the ventral nerve cord (Fig. 52). The gonostome was sessile and had a basal position. The gonostomal lips were not filamentous and not spirally coiled (Fig. 51). The shape of these lips remains unknown, because the shape in Echiurus echiurus (and additional non-spirally coiled taxa) remains ambiguous (see chapter 4.4.1 “problematic characters”, 4.4.2).

The stem species of the Urechis-group was further characterized by males and females that looked similar. Two anterior ventral chaetae and one or two rings of posterior anal chaetae (number remains ambiguous) had developed. In addition, the stem species lacked a post-pharyngeal diaphragm, a
glandular girdle on anterior trunk and an enlarged cloaca ("water lung"). A hemal system had
developed. The proboscis was elongate (moderate length) and tongue-like; it had a single-coloured or
a pattern other than in Ikeda species.

Thus far, exclusively the shape of the sperm nucleus provides contradictory signal regarding stem
species reconstruction in the Urechis-group. Contrary to the molecular phylogeny the favoured
morphological tree implies the stem species of the Urechis-group had a spherical nucleus. On the basis
of the molecular tree the shape of the nucleus remains ambiguous.

4.6.5.4 “Thalassematidae”

In accordance with Ruppert et al. (2004) both, molecular and cladistic analyses, show the lack of
apomorphic characters for traditional “Thalassematidae” thus far. But contrary to the latter authors
who reasoned “Thalassematidae” as a monophylum, the conducted analyses of this study imply that
“Thalassematidae” do not go back to one stem species that is exclusively shared by its own group
members, i.e. thalassematid taxa. The included thalassematid species either form (i) a paraphyletic
assemblage to the remaining echinurans (favoured by molecular sequences, Fig. 46B), or (ii) they are
polyphyletic, with Arhynchite pugettensis as basalmost offshoot within Echiura (favoured by
morphology). In the latter case the remaining thalassematids are resolved as paraphyletic assemblage
with respect to the Urechis-group (Fig. 45F, Fig. 46A).

Contrary to the basal resolution of thalassematid species within the molecular tree, the favoured
morphological tree implies thalassematid taxa (except A. pugettensis) and members of the Urechis-
group derived from a single stem species (Fig. 45F). The favoured topology optimizes the presence of
two spermatozoal characters (electron dense material restricted to basal ring component in the
acrosome; spherical nucleus) as well as sessile gonostomes as unambiguous apomorphies, i.e. support
for this clade. Due to a lack of apomorphic characters for traditional “Thalassematidae”, but the
presence of symplesiomorphic character states that have transformed to apomorphic states within the
stem species of the Urechis-group, the relevant thalassematid taxa turn out as paraphyletic assemblage
with respect to the Urechis-group. It is hypothesized that such symplesiomorphic characters are the
arrangement of funnels upon the end sac, the general shape of the head kidneys and the absence of
posterior rings of anal chaetae. Rings of anal chaetae were absent in the stem species of all echinurans;
they developed in the stem species of the Urechis-group. Though, especially the latter character
appears to be the most reliable symplesiomorphy, because the current taxon sampling considers
character distribution in all known echinurans. Thus, there is unambiguous information available for all
species included into the analyses. Anyhow, this is not the case for the other two potential symplesiomorphic characters. Regarding the arrangement of anal sac funnels it can be inferred from character mapping that it was “uniform” in the stem species of the clade (“Thalassematidae” + *Urechis*-group), but changed within the stem species of the *Urechis*-group into “decrease from proximal to distal”. This is based on the presence of data for two of totally five thalassematid species involved into the paraphyletic assemblage. Regarding the general shape of the head kidneys, the lack of data is even worse and a final evaluation remains highly arguable. But it seems likely that the larval head kidneys were tubular in the stem species of the clade (“Thalassematidae” + *Urechis*-group), but changed within the stem species of the *Urechis*-group into “branched”. However, this hypothesis is only verifiable provided that future studies will clarify the question of the shape of the head kidneys in additional thalassematid and urechid larva in support of branched head kidneys in the latter (compare previous chapter) and tubular head kidneys in additional thalassematid species.

Polyphyletic “Thalassematidae” with *Arhynchite pugettensis* as sister group to remaining echiurans lacks unambiguous support (Fig. 45F). The cladistic analysis of the morphological dataset has shown that the thalassematid *A. pugettensis* can neither be assigned to one of the monophyletic groups (*Bonellia*-, *Urechis*-group), nor the paraphyletic assemblage consisting of the remaining thalassematids. This is notwithstanding *A. pugettensis* is traditionally a member of “Thalassematidae” (Fisher 1949, Stephen and Edmonds 1972). Traditional characterization of “Thalassematidae” was mainly based on negative characters, i.e. lack of sexual dimorphism, lack of rings of anal chaetae, lack of a post-pharyngeal diaphragm, and unspecific plesiomorphic character states, i.e. proboscis not bifid, anal sacs not branched, paired gonoducts, two ventral chaetae (Stephen and Edmonds 1972). As the cladistic analysis has shown, these morphological data plus the newly included characters (compare Appendix 1, 2) are not yet sufficient and accordingly hitherto not phylogenetic informative to support “Thalassematidae” as a monophyletic group. On the one hand *A. pugettensis* shares a few character states with the outgroup taxon, some with the *Bonellia*-group. On the other hand it shares others with the remaining thalassematids + *Urechis*-group. On the basis of the favoured morphological tree congruences with the latter clade are based on convergent evolution.

The favoured morphological tree and the molecular tree are congruent in identifying a basal resolution of thalassematid taxa (i.e., *Arhynchite pugettensis*), which may indicate that thalassematids are similar to the ancestral echiuran. This is supported by the molecular tree topology with respect to the basal paraphyletic assemblage of all included thalassematids, however, the support is very low. With respect to the small taxon sampling and the inclusion of many uncertain character states, it is doubted that *A. pugettensis* is the basal most echiuran offshoot.
In conclusion, the comparison of the favoured morphological and molecular tree has shown that “Thalassematidae” presently lack any known apomorphic characters and character states. Therefore, “Thalassematidae” appear as a morphologically character-poor, and polyphyletic or paraphyletic group within the echiurans. For a significant assignment it is highly recommended to enlarge the database with respect to morphological as well as sequence data. The presented character matrices (Appendix 1, 2) may serve as a starting point for future analyses to fill the gaps of our knowledge, not only of thalassematid species.
5 Summary

Contrary to echiuran monophyly, which is unambiguously supported by several autapomorphies, echiuran phylogenetic intra-relationships are still unknown to date. This is due to the putative lack of structural phylogenetic informative data and a consequent systematical data acquisition across all echiuran subgroups. Consequently, morphological phylogenetic analyses have never been conducted, but even molecular analyses are missing so far. Phylogenetic analyses are essential to provide an independent assessment of the already known characters and their distribution. In order to contribute to a clarification of echiuran phylogeny this thesis investigates the phylogenetic relationships of all high-ranking subgroups using morphological and molecular cladistic analyses. Therefore, at first, two traditional diagnostic character complexes were studied comparatively on the basis of light and scanning electron microscopic as well as histological investigations (anal sacs: seven species, gonostomal lips: five species). For one species (*Thalassema thalassemum*) the ultrastructure of the anal sacs including their funnels was studied. In addition, the immunocytochemical staining of the anal sac musculature was tested successfully in the same species. With regard to newly studied character complexes the ultrastructure of spermatozoa was investigated in two species each assigned to one subgroup; the ultrastructure of the larval protonephridia was made accessible for *T. thalassemum*. After a critical evaluation of the relevant states, potentially phylogenetic informative characters were compiled within a data matrix. The data matrix was complemented by literature data for missing representatives of all subgroups and additional diagnostic characters from literature (specifications of the proboscis, chaetae, gonoducts, hemal system, cloaca, sexual dimorphism). In total, a cladistic analysis was conducted using 47 morphological characters and 15 terminal taxa. Moreover, for the first time, a molecular phylogeny was established on the basis of 16 new gene sequences together with already published data from Genbank. The multigene maximum likelihood analysis is based on the combination of two mitochondrial genes and a nuclear coded gene (18S rDNA + 16S rRNA + MT-CO1). The analysis comprises members of all traditional subgroups, altogether 14 terminal taxa were considered. In order to test the stability of the resulting topologies, two alternative datasets with varying contingents of aligned positions were analyzed. Regardless of the method used, all analyses recover a resolution that opposes to the traditional classification and to previous phylogenetic hypotheses. Favoured morphological and molecular tree are congruent in identifying two major clades, hitherto referred to as *Bonellia*-group and *Urechis*-group as well as a basal resolution of some thalassematid taxa (i.e., *Arhynchite pugettensis*). The *Bonellia*-group includes monophyletic Ikedaidae within paraphyletic Bonelliidae. Monophyly of the *Bonellia*-group is well supported by the molecular tree. The presence of a pronounced sexual dimorphism with dwarf males, anal sac tubuli and unpaired gonoducts are discussed as constitutive apomorphies for the group. Within the *Bonellia*-group the
conspicuous colour pattern on the dorsal side of the proboscis supports monophyletic Ikedaidae. Previous hypotheses that traditional Ikedaidae are based on a multiplication of gonoducts (200-400) are abolished and turned out to be an apomorphy for *Ikeda taenioides*. A further resolution within the *Bonellia*-group is achieved via the proboscis shape (bifid-not bifid) and the position of the gonostome (basal-terminal) in the favoured morphological tree. A terminal gonostome supports the sister group relationship of Ikedaidae and a bonelliid taxon. The *Urechis*-group is highly supported by both analyses and incorporates traditional Echiuridae + Urechidae. Rings of anal chaetae turn out to be apomorphic for the group. Traditional Urechidae are recovered as a clade supported by diagnostic characters, i.e. short proboscies, a glandular girdle on the anterior trunk, the loss of the hemal system and an enlarged cloaca serving as an organ of respiration. Due to the molecular tree a sister group relationship of *Bonellia* - and *Urechis*-group is well supported. The favoured morphological tree in contrast supports a sister group relationship of *Bonellia*-group and the remaining echiurans (*Arhynchite pugettensis* excluded). However, thus far no morphological characters are known that could sustain any of the two sister group hypotheses. The main difference between morphological and molecular tree concern the resolution of “Thalassematidae”: they either form a paraphyletic assemblage to the remaining echiurans (favoured by molecular sequences), or they are polyphyletic, with *Arhynchite pugettensis* as basalmost offshoot within Echiura (favoured by morphology). In the latter case the remaining thalassematids are resolved as paraphyletic assemblage with respect to the *Urechis*-group. Subsequently, a final conclusion is not possible so far, because neither the analyzed sequence data nor the enlarged morphological dataset provide an apomorphy for “Thalassematidae”.

The new data regarding the structure of the spermatozoa, the anal sacs, the gonostome and the larval protonephridia have shown that Echiura is not a character-poor taxon. Based on own observations and comprehensive literature search several potentially informative characters have been acquired. In this context, further investigations on the morphology of the spermatozoa, the anal sac funnel stalks as well as the end sacs (mesenteries, musculature) and the larval protonephridia seem promising for additional members of Echiura, notably “Thalassematidae”. The arrangement of gonoducts as a consistent diagnostic character remains problematic due to the ambiguous discrimination of relevant states so far, and a dependency on the number of gonoducts, which is often affected by a high intraspecific variability. The phylogenetic relevance of the shape of the gonostomal lips is arguable, because the molecular phylogeny implies a convergent evolution of spirally coiled lips. The same applies for fins on the sperm flagellum, sac-like excretory organs and the development of very long proboscies. The lack of anterior ventral chaetae in members of the *Bonellia*-group is secondary. Additional hypotheses on the evolution of the considered characters are presented and discussed in this thesis.
6 Zusammenfassung


7 References


José, K. V. 1964. The morphology of *Acanthobonellia pirotanensis* sp. nov., a bonellid from the Gulf of Kutch. J. Morph. 115(1): 53-68.


Nixon, K., 2002. WinClada, Version 1.00.08. Published by the Author, Ithaca, NY.


Appendix 1

Complete data matrix representing all echiuran species considered in character discussion (chapter 4.1-4.5) to demonstrate the existing knowledge gaps in Echiura with respect to the 47 morphological characters (Appendix 3). Question marks (?) indicate missing data; dashes ( -) indicate inapplicable character states.

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<td>????1??-</td>
<td>-????????</td>
<td>100110-01?</td>
<td>1001011</td>
</tr>
<tr>
<td>Acanthobonellia pirotansis</td>
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Appendix 2

Reduced data matrix coded for cladistic analysis representing the taxon sampling and the 47 morphological characters (Appendix 3). Question marks (?) indicate missing data; dashes (-) indicate inapplicable character states. Character states of outgroup taxon *Capitella teleta* (Capitellidae, Annelida) according to Table 1 in Kato et al. (2011).

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Appendix 3

Character Coding

Characters and character states inferred from the available data. Many of them are presently uninformative, but have been included for reasons of comprehensiveness. Characters and character states refer to adult female echiurans, except for characters and their corresponding states of the larval protonephridia (head kidneys).

Sperm data

(compare Fig. 33; Lehrke & Bartolomaeus 2009, Fig. 1-3 unless stated differently)

1. Shape of the whole spermatozoon:
   0. longitudinal axis (acrosome-basal body-axis) in line with ciliary axoneme
   1. longitudinal axis oblique relative to ciliary axoneme axis, head and midpiece are curved

2. Acrosomal vesicle (=acrosome): (compare Lehrke & Bartolomaeus 2009, Table 2)
   0. acrosome wider than long (oblate), acrosomal ratio of longitudinal to transversal axis \( \leq 1 \)
   1. longer than wide (elongate), acrosomal ratio of longitudinal to transversal axis is 1-2
   2. extremely longer than wide (filiform, extremely elongate); acrosomal ratio of longitudinal to transversal axis many times higher than 2 (7 in the investigated species)

3. Distribution of electron dense material in the acrosome:
   0. restricted to basal ring component in the basal part of the acrosomal vesicle (Lehrke and Bartolomaeus 2009)
   1. overall, electron-dense material fills entire acrosomal vesicle (Franzén and Ferraguti 1992)

4. Acrosomal rod within subacrosomal space (= perforatorium sensu Franzén and Ferraguti 1992):
   0. absent
   1. present

5. Membrane bound subacrosomal vesicle (sensu Lehrke and Bartolomaeus 2009):
   0. absent
   1. present

6. Shape of nucleus:
   0. ovoid (Sawada et al. 1975)
1. ellipsoid (Pilger 1993)
2. barrel-shaped (Biseswar 1991)
3. spherical (Lehrke and Bartolomaeus 2009)
4. spherical, but indented apical (Cross 1984; Cross et al. 1985)
5. sausage-shaped (Franzén and Ferraguti 1992)

   0. absent
   1. present

8. Centrioles, relative position of the proximal and distal centriole to each other:
   0. co-axial (rectangular and aligned; distal centriole in one axis with the nucleus)
   1. laterally displaced (rectangular and proximal centriole lateral displaced proportional to the basal-body, distal centriole not in one axis with the nucleus)

9. Flagellum:
   0. fins absent
   1. fins present (fin-like extensions of the plasma membrane; they dispose an angle of approximately 90° to an analogical longitudinal axis through the cross-section of the flagellum; Lehrke and Bartolomaeus 2009)

Anal sac data

10. Anal sacs:
    0. absent
    1. present (Fig. 7A; Figs. 34, 35, 36)

11. Composition:
    0. end sac absent (anal sacs exclusively composed of ciliated funnels- sitting atop tubules (Figs. 36D, E)
    1. end sac present (anal sacs composed of uniting end sac and ciliated funnels that may or may not sit upon tubules) (Fig. 7A; Figs. 34, 35, 36A- C)

12. Connection between end sac and hindgut:
    0. via two pores (Fig. 34A)
    1. via one pore (Fig. 34B)

13. Shape of end sacs:
    0. sac-like (Figs. 35C- E)
    1. tubular (Fig. 35A, B; Figs. 36A- C)

14. Arrangement of muscle fibers within end sac:
    0. single (isolated) fibers (Figs. 9B, C; 15D; 17C)
1. fibers concentrated in groups (bundles) (Figs. 22B-C)

15. Texture of muscle net:
   0. fine meshed (Figs. 9B, C)
   1. wide-meshed (Figs. 22B, C)

16. Mesenteries:
   0. rope-like (Fig. 21)
   1. laminar (Fig. 13A)

17. Tubules (= long funnel stalks):
   0. absent (Figs. 35A-C; Figs. 37A-D)
   1. present (Figs. 35D, E; Figs. 36A, B, D, E; Fig. 37E)

18. Funnel polymorphism (slender conical + slender cylindrical funnels simultaneously present):
   0. absent
   1. present (Fig. 23; Figs. 37C-D)

19. Funnel structure:
   0. neck region present (Figs. 37A,B, E)
   1. neck region absent (Figs. 37C-D)

20. Specification of funnel neck region (only applicable to species lacking tubules):
   0. short/ inconspicuous (sessile appearance of funnel) (Fig. 37A)
   1. distinct (short-stalked appearance of funnel) (Fig. 37B)

21. Arrangement of funnels upon the end sac (only applicable to species lacking a tubele):
   0. mostly distal (Jones and Stephen 1955)
   1. mostly proximal (Prashad and Awati 1929)
   2. decrease from proximal to distal (Greef 1879, Spengel 1880, Baltzer; Fig. 20B)
   3. increase from proximal to distal (Fig. 20A)
   4. uniform, without any pattern (Fig. 35A)
   5. arranged in rows (Stephen and Edmonds 1972)

**Larval protonephridia**

(compare Table 1, Kato et al. (2011) unless stated differently)

22. General shape:
   0. branched (Fig. 39C; Hatschek 1880, Goodrich 1910, Baltzer 1917, Korn 1960)
   1. unbranched (tubular) (Fig. 25A, Figs. 39A, B)
23. Number of cells of the terminal structure:
   0. several
   1. one

24. Cilia per cell of the terminal structure:
   0. several (=multiciliated terminal cell)
   1. one (=monociliated terminal cell)

25. Circumciliary microvilli within terminal structure:
   0. absent (Figs. 25A, D)
   1. present

26. Filter structure:
   0. by perforated cytoplasm (sensu Kato et al. 2011)
   1. by two to three layers of elongate microvilli emerging from the terminal cell (Figs. 25A, D)

27. Number of cells involved into the composition of the duct:
   0. several
   1. one

28. Number of cilia per duct cell:
   0. several
   1. one

29. Microvilli emerging from the duct cells:
   0. absent
   1. present

30. Nephridiopore:
   0. via a specialized nephropore cell
   1. nephropore cell absent (Figs. 25A, I)

Gonoduct data

31. General appearance of gonostome:
   0. sessile (Figs. 27, 40, 41)
   1. stalked (Figs. 31, 42)

32. Shape of gonostomal lips:
0. not spirally coiled (not filamentous)
1. spirally coiled (filamentous) (Figs. 28, 30, 40)

33. Position of gonostome:

0. basal (near the genital pore, within basal most third of gonoduct: e.g. Stephen & Edmonds 1972)
1. central (Stephen & Edmonds 1972, Edmonds 1987)
2. near distal end (within proximal most third of gonoduct: Fig. 31A; Fig. 2 in Edmonds 1987)
3. terminal (at distal tip: e.g. Ikeda 1904; Stephen & Edmonds 1972, Fig. 46D; Datta-Gupta & Menon 1976; Biseswar 2006)

Additional characters

34. Anterior ventral chaetae (Ruppert et al. 2004):

0. absent
1. present (Fig. 43)

35. Number of anterior ventral chaetae (Ruppert et al. 2004):

0. two (Figs. 43A, B)
1. more than two (Stephen & Edmonds 1972)
2. one (single) (Stephen & Edmonds 1972)

36. Posterior rings of anal chaetae (Ruppert et al. 2004):

0. absent
1. present (Fig. 44)

37. Number of posterior rings of anal chaetae (Ruppert et al. 2004):

0. one (Stephen & Edmonds 1972, Fig. 59B)
1. two (Fig. 44)

38. Post-pharyngeal diaphragm (Stephen & Edmonds 1972, Fig. 52E):

0. absent
1. present

39. Proboscis (Ruppert et al. 2004):

0. absent
1. present

40. Proboscis length (relaxed condition, living specimen) (Ruppert et al. 2004): (Figs. 3, 54)

0. "short" (Stephen & Edmonds 1972; Ruppert et al. 2004), a few centimeters
1. "elongate" (Ruppert et al. 2004), moderate length
2. "very long" (Ruppert et. al 2004); 0.75- 2.0 (shown for *I. pirotansis*, Hughes & Crisp 1976; 1.50 m shown for *B. viridis* and *I. taenioides*, Baltzer 1931, Ikeda 1904).

41. Proboscis shape (Stephen and Edmonds 1972; Ruppert et al. 2004): (Fig. 54)
   0. tongue-like (not bifid: Figs. 3A- C, E-F)
   1. forked, bifid (Ruppert et al. 2004: e.g. Fig. 3D)

42. Proboscis colour pattern (living animal): numerous dark brownish- black spots or transversial stripes on pale grayish- white dorsal subsurface:
   0. absent (single-coloured or pattern other than in *Ikeda* species; Figs. 3A-E)
   1. present (Fig. 3F; Figs. 54A, B)

43. Glandular girdle on anterior trunc (mucous net production for filter feeding; Ruppert et al. 2004):
   0. absent
   1. present

44. Hemal system (Ruppert et al. 2004):
   0. absent
   1. present

45. Enlarged cloaca ("water lung") serving as an organ of respiration (Stephen & Edmonds 1972, Ruppert et al. 2004):
   0. absent
   1. present

46. Sexual dimorphism (dwarf males) (Ruppert et al. 2004):
   0. absent
   1. present

47. Arrangement of gonoduct (Ruppert et al. 2004):
   0. paired (one member on each side of the ventral nerve cord sensu Datta-Gupta 1974, Pilger 1993)
   1. unpaired (Ruppert et al. 2004) (in case that more than one gonoduct is present: arrangement in "clusters"- not in pairs sensu Datta-Gupta 1974)
Appendix 4

Complete list of echiuran species referred to in this thesis (in alphabetical order) with information on the first authors. Traditional high-ranking taxa according to Dawydoff (1959). BON Bonelliidae, ECH Echiuridae, IKE Ikedaidae, THA Thalassematidae, URE Urechidae.

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