The monophyletic origin of a remarkable sexual system in akentrogonid rhizocephalan parasites: A molecular and larval structural study

Henrik Glenner a, *, Jens T. Høeg b, Jesper Stenderup c, Alexey V. Rybakov d

a Marin Biodiversity, Department of Biology, University of Bergen, Box 7800, N-5020 Bergen, Norway
b Comparative Zoology, Department of Biology, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen, Denmark
c Centre for GeoGenetics, Natural History Museums of Denmark, 5-7 Øster Voldgade, DK-1350 Copenhagen K, Denmark
d Institute of Marine Biology, Laboratory of Chorology, Russian Academy of Sciences, 690041 Vladivostok, Russia

A R T I C L E   I N F O

Article history:
Received 17 April 2009
Received in revised form 15 September 2009
Accepted 18 September 2009
Available online 26 September 2009

Keywords:
Parasitology
Comparative morphology
Molecular phylogenetics
Crustacea
Rhizocephala

A B S T R A C T

We use sequences from the nuclear ribosomal genes, 18S and 28S to analyze the phylogeny of the Rhizocephala Akentrogonida including two species, Clistosaccus paguri and Chthamalophilus delagei, that are critical for understanding rhizocephalan evolution but have not previously been part of a molecularly based study. In addition we use light and scanning electron microscopy to compare the cypris larvae of C. paguri, Sylon hippolytes and two species of the family Thompsoniidae, since this larval stage offers a suite of characters for analyzing the evolution of these otherwise highly reduced parasites. The Rhizocephala Akentrogonida form a monophyletic group nested within a paraphyletic “Kentrogonida”. C. paguri and S. hippolytes are sistergroups confirming the monophyly of the Chthamalophilidae that was originally based on similarities in the cypris larvae. We find numerous LM and SEM level similarities between the two species, many of which appear to be correlated with their specialized sexual system, where male cyprids use an antennule to implant cells into the virgin female parasite. Some of these traits are also found in cyprids of the thompsoniid species. We conclude that the special cypris morphology and the implantation of males by antennular penetration was present in the stem species to the Thompsoniidae and the Chthamalophilidae and emphasize the power of larval characters in rhizocephalan systematics. C. delagei is a sister group to Boschmaella balani and the two are nested deep within the Akentrogonida. This confirms the monophyly of the Chthamalophilidae and falsifies the theory that C. delagei should represent the most primitive extant rhizocephalan. Instead, chthamalophilid rhizocephalans represent some of the most highly advanced members of the parasitic barnacles.

1. Introduction

The 250+ species of Rhizocephala comprises a quarter of all barnacle species, and they therefore represent a major part of the radiation of the Cirripedia. Since (Darwin, 1851) cirripedes have been important models for testing theories on the evolution of complicated life cycles and reproductive systems (Charnov, 1979, 1987; Høeg et al., 2005; Høeg and Lützen, 1993). The Rhizocephala sport some of the most extreme variations, and it is therefore unfortunate that our understanding of the phylogeny of these parasites have until recently been virtually non existent.

The Rhizocephala is traditionally divided into two suborders based on whether a so-called kentrogon stage is involved in the infestation of the host crab (Høeg and Lützen, 1995). Species of the suborder Kentrogonida exemplify the “classical” life cycle illustrated in most modern invertebrate and parasitology text books (Brusca and Brusca, 2002; Høeg et al., 2005; Ruppert et al., 2004). A key event is the implantation into the juvenile female parasite of males that are housed in a pair of so-called receptacles (Høeg, 1987; Yanagimachi, 1961). All kentrogonid species infest decapod Crustacea and, despite comprising the majority of rhizocephalan species, there is little variation in the morphology of the parasite and details of the life cycle (Glenner, 2001).

In marked contrast, species of the Rhizocephala Akentrogonida exhibit a remarkable variation at all levels such as the morphology of the parasite and its larvae, the sexual system and the choice of host animals. Only a single species has a structure that may be compared to the kentrogonid receptacles. In other akentrogonids, spermatogenesis proceeds in many different ways such as in the connective tissue of the mantle enveloping the brood chamber, in the ovary or, as a remarkable extreme, in epithelium lined bodies that float freely among the eggs spawned into the mantle cavity (Høeg, 1990). Furthermore, akentrogonid species are not confined to decapods but infest a range of other Crustacea including...
peracarids, stomatopods and even other barnacles (Høeg et al., 2005; Høeg and Lützen, 1995). This disparity in morphology and biology begs the questions, whether the Akentrogonida forms a monophyletic unit, and, additionally, which if any akentrogonids are closely related with the species of the Kentrogonida (Glenner and Høeg, 1994).

The adult rhizoechal parasite has lost almost every character normally found in crustaceans or even arthropods in general, and it therefore offers few if any characters of value for reconstructing phylogeny (Høeg and Lützen, 1993, 1995; Anderson, 1994). In rhizoechal barnacles the cypris larva, the settlement stage common to all cirripedes, offers many potentially important characters that also are closely linked to variations in the sexual system (Glenner et al., 1989; Høeg, 1990). But use of these traits has been difficult, because it requires either laborious laboratory culture or the chance finding of mature cyprids in the mantle cavity of the parasite externa. Recently, Glenner and Hebsgaard (2006) used molecular methods to present a comprehensive phylogenetic analysis of both kentrogonid and akentrogonid rhizoechal barnacles. They concluded that all Rhizoechalae form a monophyletic group within which a monophyletic Akentrogonida is nested within a paraphyletic “Kentrogonida”. Unfortunately, two species, holding important keys to understanding akentrogonid evolution, viz., Chlostosaccus paguri and Chthamalophilus delagei, were not available for their analysis.

Chlostosaccus paguri is the only akentrogonid that has a structure somewhat comparable to the paired receptacles in kentrogonid barnacles. In this study we use molecular methods to reanalyze the phylogeny of the Rhizoechalae Akentrogonida including both C. paguri and C. delagei. In addition, we employ SEM to compare cypris morphology between C. paguri, S. hippolytes and representatives of the family Thompsoniidae. On this platform we discuss the evolution of the male part of the sexual system in rhizoechal barnacles.

Table 1
List of taxa included in the study, their host species, sample locations, GenBank accession numbers, and voucher numbers.

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Collection locality and date</th>
<th>GenBank Accession1</th>
<th>Voucher2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sacculina carcini</td>
<td>Carcinus maenas</td>
<td>Gullmar Fjord, W. Sweden (July 2000)</td>
<td>AY265366</td>
<td>ZMUC CRU-3867</td>
</tr>
<tr>
<td>Sacculina confroga</td>
<td>Pachygrapsus crassipes</td>
<td>Shirama, Chiba Prefecture, Japan (October 1999)</td>
<td>AY265363</td>
<td>ZMUC CRU-3868</td>
</tr>
<tr>
<td>Boschma</td>
<td>Eriocheir japonica</td>
<td>Maruyama River, Hyogo, Japan (December 1999)</td>
<td>AY265365</td>
<td>ZMUC CRU-3869</td>
</tr>
<tr>
<td>Polysaccus gregaria</td>
<td>Leptodius exaratus</td>
<td>Labrador Beach, Singapore (Oct 1999)</td>
<td>AY265367</td>
<td>ZMUC CRU-3870</td>
</tr>
<tr>
<td>Guérin-Canivet</td>
<td>Cyclograpsus intermedius</td>
<td>Tomioka, Kyushu, Japan (October 1998)</td>
<td>AY265368</td>
<td>ZMUC CRU-3871</td>
</tr>
<tr>
<td>Sacculina oblonga</td>
<td>(M.-Edwards) Ortmann</td>
<td>Kenting National Park, S. Taiwan (January 1999)</td>
<td>AY265362</td>
<td>ZMUC CRU-3872</td>
</tr>
<tr>
<td>Boschma</td>
<td>Hemigrapsus sanguineus</td>
<td>Óyano Island, Kyushu, Japan (October 2000)</td>
<td>AY265360</td>
<td>ZMUC CRU-3873</td>
</tr>
<tr>
<td>Lützen &amp; Yamaguchi</td>
<td>(de Haan)</td>
<td>Guangdong, China (1999)</td>
<td>AY265359</td>
<td>ZMUC CRU-3874</td>
</tr>
<tr>
<td>Polysaccus plana</td>
<td>Leptodius exaratus</td>
<td>Hong Kong (October 2001)</td>
<td>AY265358</td>
<td>ZMUC CRU-3875</td>
</tr>
<tr>
<td>Boschma</td>
<td>(M.-Edwards) Lamarcq</td>
<td>Adria, Volosko, Croatia</td>
<td>DQ826566</td>
<td>ZMUC CRU-9903</td>
</tr>
<tr>
<td>Polysaccus polygenea</td>
<td>Squilla sp.</td>
<td>Vietnam</td>
<td>DQ826567</td>
<td>ZMUC CRU-9904</td>
</tr>
<tr>
<td>Lützen &amp; Takahashi</td>
<td>Leptodius exaratus</td>
<td>Labrador Beach, Singapore (October 1999)</td>
<td>DQ826573</td>
<td>ZMUC CRU-9905</td>
</tr>
<tr>
<td>Sacculina sinensis</td>
<td>(M.-Edwards) Ortmann</td>
<td>Guangdong, China (2000)</td>
<td>AY265370</td>
<td>ZMUC CRU-9906</td>
</tr>
<tr>
<td>Parthenopea subterranea</td>
<td>Callianassa tyrrenhena</td>
<td>Adria, Volosko, Croatia</td>
<td>DQ826568</td>
<td>ZMUC CRU-9907</td>
</tr>
<tr>
<td>Kossmann</td>
<td>(Petagna)</td>
<td>Vietnam</td>
<td>DQ826569</td>
<td>ZMUC CRU-9908</td>
</tr>
<tr>
<td>Potzisa sereni</td>
<td>Squilla sp.</td>
<td>Guangdong, China (2000)</td>
<td>AY265359</td>
<td>ZMUC CRU-9909</td>
</tr>
<tr>
<td>Lützen &amp; Du</td>
<td>Leptodius exaratus</td>
<td>Labrador Beach, Singapore (October 1999)</td>
<td>DQ826573</td>
<td>ZMUC CRU-9910</td>
</tr>
<tr>
<td>Tompsonia littoralis</td>
<td>(M.-Edwards) Lamarcq</td>
<td>Guangdong, China (2000)</td>
<td>AY265370</td>
<td>ZMUC CRU-9911</td>
</tr>
<tr>
<td>Diphyllobothacus sinensis</td>
<td>Portunus pelagicus</td>
<td>Singapore (October 1999)</td>
<td>DQ826568</td>
<td>ZMUC CRU-9912</td>
</tr>
<tr>
<td>Kossmann</td>
<td>Callianassa japonica</td>
<td>Tomioka, Japan</td>
<td>DQ826565</td>
<td>ZMUC CRU-9913</td>
</tr>
<tr>
<td>Heeg &amp; Lützen</td>
<td>(Ortmann)</td>
<td>Kristineberg, Sweden (2006)</td>
<td>DQ826564</td>
<td>ZMUC CRU-9914</td>
</tr>
<tr>
<td>Clitosaccus paguri</td>
<td>Leptodius exaratus</td>
<td>Labrador Beach, Singapore (October 1999)</td>
<td>DQ826573</td>
<td>ZMUC CRU-9915</td>
</tr>
<tr>
<td>Lützen &amp; Du</td>
<td>Squilla sp.</td>
<td>Vancouver Island, Canada</td>
<td>AY265369</td>
<td>ZMUC CRU-9916</td>
</tr>
<tr>
<td>Sylon Hippolytes</td>
<td>Balanus improvisus</td>
<td>Roscoff, France</td>
<td>AY265369</td>
<td>ZMUC CRU-9917</td>
</tr>
<tr>
<td>Deichmann &amp; Haeg</td>
<td>Chthamalus challengeri</td>
<td>Darwin</td>
<td>AY265369</td>
<td>ZMUC CRU-9918</td>
</tr>
<tr>
<td>Boschmaella japonica</td>
<td>(Bocquet-Védrine, 1961)</td>
<td>Darwin</td>
<td>AY265369</td>
<td>ZMUC CRU-9919</td>
</tr>
</tbody>
</table>

Missing accession numbers will be released upon publication.

1. GenBank accession numbers for 18S rDNA and 28S rDNA respectively.

2. Collection numbers from the Zoological Museum, University of Copenhagen, Denmark.
2. Material and methods

2.1. Taxon sampling and molecular procedure

We sampled a total of 16 taxa belonging to the Rhizocephala. Of these we sequenced 14 taxa for the 28S rRNA and 2 for the 18S rRNA gene. The remaining data were downloaded from GenBank (http://www.ncbi.nlm.nih.gov). We were unable to obtain sequence data from 28S rRNA for 2 taxa (Thompsonia littoralis and Diplothylacus sinensis). For the Akentrogonida, the sample includes all species for which sequence data is presently available and all were sampled by us. Selected “kentrogonid” rhizocephalan species were included in the sample based on their position in the analysis of Glenner and Hebsgaard (2006). Table 1 shows origin and availability of all species sequenced and analysed in this study as well as GenBank accession numbers, and museum voucher numbers. All material were preserved in 96% (or higher) ethanol and stored refrigerated or frozen from the time of collection and identification to processing.

2.2. Extraction and amplification

DNA was extracted from alcohol-preserved specimens using the QIAGEN DNeasy Tissue Kit (Qiagen Inc., USA). The 18S rRNA locus was amplified using polymerase chain reaction (PCR) and primers from Abele et al. (1992) yielding 1815 (Sacculina confragosa) to

Fig. 1. Phylogenetic reconstruction of akentrogonid rhizocephalans and a few selected “kentrogonid” species. The reconstruction is based on Bayesian Inference of 18s and 28s rDNA data using Sacculina sinensis as outgroup. Fifty percent majority rule consensus showing posterior probability values at supported bisections. Outgroup choice based on Glenner and Hebsgaard (2006), which featured a much broader taxon sampling of rhizocephalan species. The Akentrogonida (thick lines) are monophyletic and nested within a paraphyletic “Kentrogonida”. The Clistosaccidae (double thick lines), Chthamalophilidae and Thompsoniidae are monophyletic families within the Akentrogonida. Vignettes shows two Clistosaccus paguri externae on the abdomen of a Pagurus bernhardus hermit crab and a Sylon hippolytes externa on the abdomen of the caridean prawn Spirontocaris lilljeborgi. The hermit crab carries one almost mature externa and a very recently emerged externa at the stage when it becomes invaded by males. Drawing of S. hippolytes from Lützen (1981). Drawing of C. paguri from Lützen.
2220 \((T. \text{littoralis})\) nucleotides (nt). The 28S rRNA locus was amplified using primers from Stenderup et al., (2006) yielding 682 \((\text{Poly}

yascus polygenea)\) to 739 \((S. \text{hippolytes})\) nt. PCR products were purified using MSB Spin PCRapace (Invitek) and sequenced using Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, CA). Sequences were run in an ABI Prism 3100 Analyser (Applied Biosystems).

2.3. Sequence alignment

Sequences were assembled using Sequencher 3.1.1 (Gene Codes Corporation, Michigan, USA) and aligned in CLUSTAL X (Thompson et al., 1997) using default settings (multiple alignment option; gap opening penalty 10 gap; extension penalty 0.20; sequences with >30% divergence delayed; and DNA Transition Weight 0.50). The analyses were performed including and excluding all gaps in the alignment.

2.4. Phylogenetic analyses

Bayesian Inference of Phylogeny. We found the appropriate substitution models using MrModeltest version 2.2 (Nylander, 2004) for the Bayesian analyses. This application offers likelihood ratio test and Akaike Information Criterion to test for statistically significant differences in model fit for models with increasing complexity. Both methods suggested the general time-reversible model with a proportion of invariant sites and gamma-distributed rates \((\text{GTR} + \Gamma)\) for the 18S RNA and the 28S RNA genes. The two datasets were therefore combined in one. Bayesian posterior probabilities (bpp) were estimated as the proportion of trees sampled after burn-in that contained each of the observed bipartitions (Larget and Simon, 1999). The default four Markov Chains were used (on multiple chains, see e.g. Wilcox et al. (2002). The Monte Carlo Markov chain (MCMC) length was 5,000,000 generations, and we sampled the chains every 500 generations yielding 10,000 trees. Log-likelihood values for sampled trees were visualized and had stabilized after 500,000 generations. Therefore, we used the last 9000 sampled trees to estimate Bayesian posterior probabilities (bpp). The analyses were repeated three times starting from independent, randomly chosen trees producing an identical 50% majority rule consensus tree with almost identical posterior probabilities. Sacculina sinensis was used as outgroup due to its (sistergroup) position in the Glenner and Hebsgaard (2006) analysis that included a much broader taxon sampling.

2.5. Light and scanning electron microscopy

The larval material derived from the samples studied in Glenner et al. (1989). Cyprids of C. paguri and of S. hippolytes had their provenance as for the molecular samples. The cyprids of two unidentified species of the Thompsoniidae (hosted on prawns and hermit crabs) were originally sampled from the San Juan Straits, Washington, USA as described in Glenner et al. (1989). For LM, larvae were dehydrated and infiltrated with TAAB 812 “Epon” epoxy resin. The infiltrated larvae were placed under a coverslip in a small drop of resin on a microscopic slide and allowed to polymerize at room temperature. This method produces an almost indefinitely lasting preparation that allows use of oil immersion objectives without risking damage to the larva when the slide is cleaned. Whole mounted larvae were examined in a Leica DM-RXA with DIC optics and a motorized z-drive. The soft-
ware package ImagePro 6© with the module SCOPE were used to acquire images at multiple Z-planes through the specimens, which were fused into enhanced depth of focus (EDF) pictures by the inherent software procedure. For SEM larvae were dehydrated in alcohol and acetone, critical point dried in CO₂, mounted on stubs and coated with platinum. Examination was in a JEOL JSM-6335 FEG-gun SEM.

3. Results

3.1. Phylogenetic analysis

The combined 18s and 28s tree is presented in Fig. 1. When analysing the two genes separately the topology of the 18S tree was not affected by the in- or exclusion of gaps in the dataset.

Fig. 3. Clisosoacus paguri, SEM of cyprids. (A) Whole cyprid. (B) Distal part of antennule. (C) Medial view of the third segment. (D) Oblique ventral view showing both the attachment disc of the third segment and the fourth segment; the two projections indicated by arrows might be true setae, but more likely they are just exceptionally long cuticular villi. In C and D parts of the antennule also visible. Ad, attachment disc; an, antennule; as3–4, antennular segments 3–4; bs, bifid seta; sk, skirt; sp, spinous process.

Fig. 4. Sylon hippolytes, SEM of cyprids. (A) Whole cyprid. (B) Segments 3 and 4. (C) Oblique ventral view of the attachment disc of the third segment. (D) Oblique ventral view of the third and fourth segments. Ad, attachment disc; an, antennule; as1–4, antennular segments 1–4; bs, bifid seta; sk, skirt; sp, spinous process.
(2000 bp/1400 bp, respectively), and identical to the combined tree (Fig. 1). Inclusion of gaps in the 28S dataset (800 bp) produced a slightly better resolved topology than if gaps were excluded (600 bp) by placing Parthenopea subterranea as sister taxon to an akentrogonid clade. Otherwise the results of the two analyses were identical.

The results of the phylogenetic analyses of both the 18S and the 28S genes were congruent for most clades. The only difference concerned the basal part of the tree where S. leptodiae grouped together with S. oblonga and S. confragosa in the 18S dataset, while the 28S dataset separated S. leptodiae from the S. oblonga and S. confragosa clade.

All analyses strongly support a monophyletic Akentrogonida nested within the kentrogonid taxa and with P. subterranea as the most closely related taxon. Akentrogonida is divided in two major branches. One clade contains D. sinensis, T. littoralis, Potssia serenaei, C. delageii, and Boschmaella japonica. The other clade contains S. hippolytes, C. paguri and Polysaccus japonicus. C. delageii and B. japonica, the only rhizocephalan species parasitizing other cirripeds, form a robust monophyletic group. Also strongly supported is the relationship between S. hippolytes and C. paguri. The datasets from the 28S gene alone supported the C. delageii/Boschmaella japonica and the S. hippolytes/C. paguri relationship, but failed to resolve the phylogeny between the remaining akentrogonid species.

3.2. Cyprids of C. paguri and S. hippolytes

The cypris larvae of C. paguri and S. hippolytes are remarkably similar. In light microscopic whole mounts, they have an almost identical outline of the body. The cyprids are short, high and with a characteristic blunt angle anteriorly (Fig. 1A and B). The head shield (carapace) has an almost identical setation pattern. Similarly, the five pairs of chemosensory lattice organs have an identical morphology in the two species (see Jensen et al., 1994, for details). The morphology of the antennules is also next to identical.
(Fig. 1D and E). In the first segment, the proximal and distal sclerites have very long, slender and inwardly curving arms (Fig. 2: ps and ds). This is a shape not seen anywhere else in rhizocephalans (Glenner et al., 1989). The second segment is characteristically curved and extremely long and slender (Fig. 2: 2as). The third segment (Fig. 2D and E: 3as) is hoof shaped rather than the more elongated hand shape found in most other rhizocephalan cyprids (Glenner et al., 1989).

These similarities in antennular morphology extend to the SEM level of analysis (Figs. 3 and 4). The attachment disc of the third segment has an unusual scarcity of cuticular villi, leaving large naked areas of disc cuticle exposed (ad in Fig. 3C, D and Fig. 4C, D). There is a spiny process at the distal end of the disc (sp in Fig. 3C, D and Fig. 4C, D), while a longitudinal furrow traverses the entire disc and most likely houses the exit pore of the cement gland at the bottom. The attachment disc carries no other conspicuous elements. In C. paguri a couple of longer projections, sited proximally close to the articulation to the second segment, might represent true setae (sensilla), but are more likely they are just exceptionally long cuticular villi (arrows in Fig. 3C). The small, cylindrical fourth segment sports only a single bifid seta, situated at the apex (bs in Figs. 2D, E, 3B and 4B, but it lacks the subterminally situated seta or setae that Høeg et al. (2004) found in most other cirripede cyprids. The bifid seta starts with a wider base (Figs. 3B and 4B) but soon splits into two very narrow and diverging projections that are also easily seen in the light microscope (Fig. 2D and E).

3.3. Cyprids of the Thompsoniidae

The cyprids of the two thompsoniid species share several of the characteristics seen in C. paguri and S. hippolytes, but can still be clearly distinguished from the latter. In one species the cyprid outline resembles that in C. paguri and S. hippolytes (Fig. 5A), but the other species have a more rounded anterior end (Fig. 5B). Both species have a very slender second antennular segment and the third segment takes the form of a hoof, but neither with an outline exactly as in the two clistosaccid species (Fig. 5C and D). The attachment disc is rather naked and with what appears to be a spinous process or “fold” near the distal end but the thin cuticular skirt encircling the disc is wider and thus much more conspicuous than in either C. paguri or S. hippolytes (Fig. 5D and E). Both thompsoniid species carry a bifid seta at the tip of the fourth segment (Fig. 5C, E and F), but in one species (Fig. 5F) the apex has a more complex shape and carries at least one additional, thin but undivided seta and a flap like structure.

4. Discussion

4.1. The phylogeny of the Akentrogonida

The present molecularly based analysis suggests that the Akentrogonida represents a monophyletic taxon within which Clistosaccus and Sylon form a monophyletic Clistosaccidae and Boschmaella and Chthamalophilus a monophyletic Chthamalophilidae. Our study provides additional support for the Clistosaccidae in the several autapomorphies in cyprid morphology discussed below (Fig. 1). At present, the following akentrogonid families, erected or rediagnosed by Høeg and Rybakov (1992), have therefore received support by molecular data: The Clistosaccidae (Clistosaccus, Sylon), the Thompsoniidae (Thompsonia, Diplotylacus, Pottsia) and the Chthamalophilidae (Chthamalophilus, Boschmaella, Bocquetia). (Bocquetia is monotypic and should probably be subsumed in Boschmaella.) Concerning the remaining akentrogonid families, sequence data is not yet available for the Mycetomorphidae and Duplorbidae, while the status of the Polysaccidae is discussed below.

The present pattern with a monophyletic Akentrogonida nested within a paraphyletic “Kentrogonida” is in agreement with Glenner and Høeg (1994). But the taxon sample of Glenner and Høeg (1994) included neither C. paguri nor C. delagei. Both species are critical to understanding rhizocephalan evolution, because Chthamalophilus is the taxon on which the Akentrogonida was originally erected and claimed to represent the most “primitive” rhizo-
ceptosaccus (Bocquet-Védrine, 1961), while Clistosaccus remains the only rhizocephalan in which the absence of a kentrogon stage ("akentrogon condition") has actually been demonstrated experimentally (Høeg, 1990). We therefore conclude that our analysis settles the long standing debate concerning both the mutual relationship of the akentrogonid taxa and their position within the Cirripedia in general (Bocquet-Védrine, 1961; Newman et al., 1969; Høeg, 1982; Bocquet-Védrine and Bourdon, 1984; Glenner et al., 1989; Høeg and Rybakov, 1992; Anderson, 1994; Glenner and Høeg, 1994; Glenner and Hebsgaard, 2006).

4.2. Cypris morphology and phylogeny

Cyprids from a considerable number of rhizocephalan species, both akentrogonids and kentrogonids, have now been described in detail using scanning electron microscopy (Glenner et al., 1989; Høeg and Rybakov, 2007; Jensen et al., 1994; Rybakov et al., 2002, 2003). Several of the distinctive similarities between cyprids of Clistosaccus and Sylon are not known from elsewhere in the Rhizocephala, and we therefore designate them as autapomorphic character states for the Clistosaccidae. This concerns the identical outline of cyprid body, the almost identical setation pattern on the carapace, the elongated, hairpin-shape of lattice organs pair 2 and features in the identical morphology of the antennules (see also Jensen et al., 1994).

Other similarities are also shared with one or both the thompsonioid species studied here. One of them has an outline resembling, although not identical to, cyprids of the Clistosaccidae, but the other species have a more rounded anterior end. Both thompsonioid cyprids also have a very slender second segment and the third segment has the form of a hoof, but, again, neither of them shaped exactly as in Clistosaccus and Sylon. One difference is the position of the fourth segment, which is at the rim of the attachment disc in the Clistosaccidae but lower down on the side of the third segment in the Thompsoniidae. The third segment is also “higher” in the Thompsoniidae than in Clistosaccus and Sylon, and the thin cuticular skirt encircling the attachment disc is much wider in thompsonioids than in the two clistosaccid species. It is interesting that cyprids of both families have a bifid seta apically on the fourth segment, but whilst this is the only seta in the clistosaccid species, one of the thompsonioids has at least one extra seta in this position, and the apex is not a flat platform but takes a more complicated shape with a flap like structure. We therefore suggest that a very slender second antennular segment, a hoof shaped third segment and a bifid seta on the fourth segment and absence of any subterminally sited setae are apomorphies for a clade that at least comprises the Thompsoniidae, the Clistosaccidae and Polyascus japonicus. Cyprids of P. japonicus have only been studied by light microscopy and were never illustrated by micrographs. But the drawing in Lützen and Takahashi (1996) clearly depicts a bifid seta apically on the fourth segment but also an additional seta-like structure that might be homologous with the one faintly seen in our SEM micrographs of one thompsonioid species. Høeg and Rybakov (2007) used SEM to study cyprids in P. mediterraneus, the only other species of the genus Polyascus, and found that the morphology showed no resemblance at all to that seen in P. japonicus. P. mediterraneus is not available for molecular analysis, but without any autapomorphies in cypris morphology there is now little if any support for the family Polyascidae.

4.3. Cypris morphology and the sexual system

The almost identical cyprids found in Sylon and Clistosaccus, and the many similarities they share with thompsonioid cyprids, take special interest when discussing the sexual systems of these akentrogonid rhizocephalans. In all species of the Rhizocephala “Ken-
cently, Bresciani and Haeg (2001) used TEM to show that the nutritive bladder of *C. delagei* does penetrate the host integument, and this is similarly so for the ramifying root system found in the con-familiar genera *Boschmaella* and *Bocquetia* (Haeg et al., 1990). The original case for a plesiomorphic morphology in *C. delagei* is therefore no longer present, and our phylogeny shows that the Chthamalophiidae are instead nested deeply within the otherwise highly specialized Akentrogonida. The Chthamalophiidae therefore represents a pinnacle in rhizocephalan evolution. This is evident both in their sexual system (Haeg et al., 1990; Haeg and Lützen, 1995) and in their cypris larvae which, unique among rhizocephalans, lack a thorax and therefore cannot swim but disperse only by walking on the antennules (Haeg and Möller, 2006).

5. Conclusion

The Akentrogonida is a monophyletic group within the Cirripedia Rhizocephala, within which C. poguari and S. hippolytes form a monophyletic Clistosaccidae. The Clistosaccidae are characterized by unique similarities in their cypris larvae correlated with their highly advanced sexual system, but elements of which are also shared with the Thompsoniidae. *C. delagei*, originally claimed as the most primitive rhizocephalan, is member of a monophyletic Cthamalophiidae, tested deeply within the akentrogonid Rhizocephala and representing a pinnacle of specialization.

Acknowledgements

This study received financial support from to The Villum Kann Rasmussen foundation (HG and JTH), the Carlsberg Foundation (HG and JTH) and the Danish natural Science Research Council (SNF, FNU) to JTH. We also acknowledge support from the European Union COBICE and SYNTHESYS programs to JTH. We are greatly indebted to Dr. J. Shields for most kindly collecting specimens of *Chthamalophilus delagei* at our request. Studies of Clistosac-cus poguari over several years were possible only because of the skill and dedication of skipper Sylve Robertson, Christer Gren and Berne Pettersson at the Kristineberg Marine Research Center, Swe-den and the hospitality and never ceasing assistance of its former director Prof. Jarl-Ove Strömberg. We all salute our friend, prof. Jørgen Lützen as a pioneer in unraveling the biology and life cycle of rhizocephalan barnacles. Finally, we take this opportunity to mourn the loss of Prof. V.L. Kasyanov, an eminent marine ecologist and a key contributor to our understanding of the biology and life cycle of rhizocephalans. We also acknowledge support from the Euro-america 76, 2480–2484.


Lützen, J., 1981. Observations on the rhizocephalan barnacle *Lilljeborgia brenti* (Crustacea, Cirripedia) and the hermaphroditiccty of its cypris larvae, injected in the antennules (Høeg and Møller, 2006).


