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

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ABSTRACT

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 Clistosaccidae 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 Clistosaccidae 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

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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 rhizocephalan 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 rhizocephalan 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 rhizocephalan species. They concluded that all Rhizocephala 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., *Clistosaccus paguri* and *Chthamalophilus delagei*, were not available for their analysis.

Clistosaccus paguri is the only akentrogonid that has a structure somewhat comparable to the paired receptacles in kentrogonid species. Høeg (1982, 1985) showed that it functions to receive males cells that are implanted into the female parasite by so-called antennular penetration, a mechanism where the cyprid uses this appendage as a syringe to inject the male material into the virgin female parasite. Such a metamorphosis was unknown elsewhere in the Cirripedia, except for a single species of the rhizocephalan family Thompsoniidae (Yanagimachi and Fujimaki, 1967). In revising the Rhizocephala Akentrogonida, Høeg and Rybakov (1992) remarked on the striking similarity between the cyprids of *C. paguri* (Clistosaccidae) and *Sylon hippolytes* (Sylonidae), both monotypic families (Høeg, 1982; Lützen, 1981). Based exclusively on these larval characters they transferred *Sylon* to the Clistosaccidae. The adult parasites exhibit no similarities at all, and, as an example, a receptacle is absent from *Sylon*. The two species therefore offer a fascinating testing ground for the value of larval characters in rhizocephalan systematics and to which extent such traits are correlated with specializations in the reproductive system.

Chthamalophilus delagei was originally the species for which the Akentrogonida was erected. Having a very unique morphology with peculiar organization of the male part of the reproductive system and without the branching rootlets found in other rhizocephalans, *C. delagei* was claimed to represent the most primitive of all Rhizocephala and therefore a key to understanding the evolution of this taxon (Bocquet-Védrine, 1961; Newman et al., 1969).

In this study we use molecular methods to reanalyze the phylogeny of the Rhizocephala 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 rhizocephalan barnacles.

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

Species	Host	Collection locality and date	GenBank Accession[1]	Voucher[2]
Sacculina carcini Thompson	*Carcinus maenas* (L.)	Gullmar Fjord, W. Sweden (July 2000)	AY265366	ZMUC CRU-3867
Sacculina confragosa Boschma	*Pachygrapsus crassipes* Randall	Shirama, Chiba Prefecture, Japan (October 1999)	AY265363 GU190706	ZMUC CRU-3868
Polyascus gregaria Okada & Miyashita	*Eriocheir japonica* (de Haan)	Maruyama River, Hyogo, Japan (December 1999)	AY265365 GU190705	ZMUC CRU-3869
Sacculina leptodiae Guérin-Ganivet	*Leptodius exaratus* (M.-Edwards)	Labrador Beach, Singapore (Oct 1999)	AY265367	ZMUC CRU-3870
Sacculina oblonga Lützen & Yamaguchi	*Cyclograpsus intermedius* Ortmann	Tomioka, Kyushu, Japan (October 1998)	AY265368 GU190699	ZMUC CRU-3871
Polyascus plana Boschma	*Grapsus albolineatus* Lamarck	Kenting National Park, S. Taiwan (January 1999)	AY265362 GU190698	ZMUC CRU-3872
Polyascus polygenea Lützen & Takahashi	*Hemigrapsus sanguineus* (de Haan)	Ôyano Island, Kyushu, Japan (October 2000)	AY265360 GU190704	ZMUC CRU-3873
Sacculina sinensis Boschma	*Leptodius exaratus* (M.-Edwards)	Hong Kong (October 2001)	AY265359 GU190707	ZMUC CRU-3874
Parthenopea subterranea Kossmann	*Callianassa tyrrhena* (Petagna)	Adria, Volosko, Croatia	DQ826566 GU190703	ZMUC CRU-9903
Pottsia serenei Lützen & Du	*Squilla sp.*	Vietnam	DQ826567 GU190702	ZMUC CRU-9908
Tompsonia littoralis Kossmann	*Leptodius exaratus* (M.-Edwards)	Labrador Beach, Singapore (October 1999)	DQ826573	ZMUC CRU-9905
Diplothylacus sinensis (Keppen)	*Portunus pelagicus* (L.)	Singapore (October 1999)	DQ826568 GU190707	ZMUC CRU-9907
Polysaccus japonicus Høeg & Lützen	*Callianassa japonica* (Ortmann)	Tomioka, Japan	DQ826565 GU190708	ZMUC CRU-9906
Clistosaccus paguri Lilljeborg, 1861		Kristineberg, Sweden (2006)	GU190697 GU190709	
Sylon Hippolytes Sars	*Pandalus sp.*	Vancouver Island, Canada	DQ826564 GU190700	ZMUC CRU-9902
Boschamaella japonica Deichmann & Høeg	*Chthamalus challengeri* Hoek	Jôgashima, Kanagawa Prefecture, Japan (October 1999)	AY265369 GU190701	ZMUC CRU-3877
Chthamalophilus delagei (Bocquet-Védrine (1967)	*Balanus improvisus* (Darwin)	Roscoff, France	GU190696 GU190710	

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

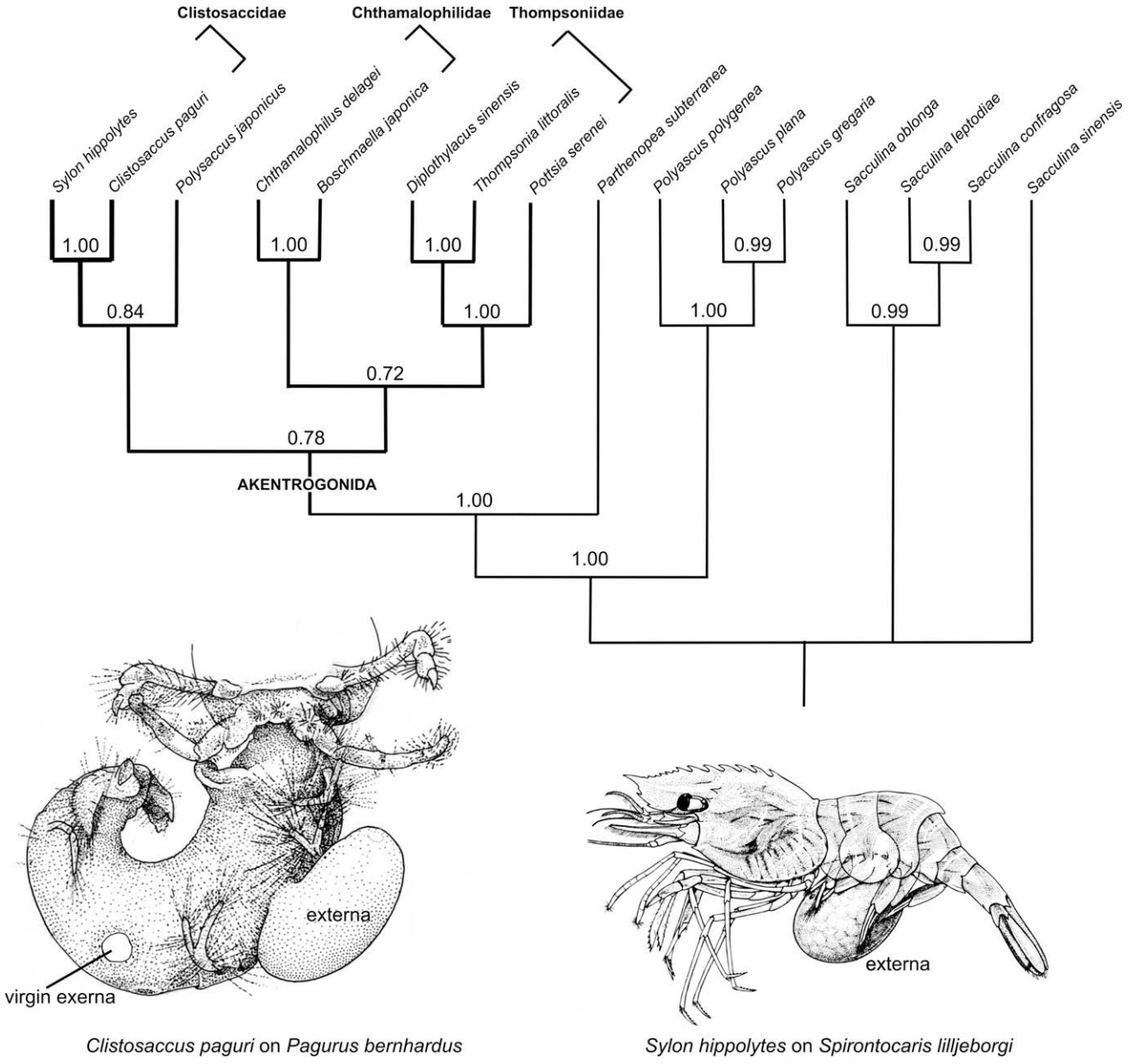

Fig. 1. Phylogenetic reconstruction of akentrogonid rhizocephalans and a few selected "kentrogonid" species. The reconstruction is based on Bayesian Inference of 18s and 28s rDNA data using *Sacculina sinensis* as outgroup. Fifty percent majority rule consensus showing posterior probability values at 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. littoralis*) nucleotides (nt). The 28S rRNA locus was amplified using primers from Stenderup et al., (2006) yielding 682 (*Polyascus polygenea*) to 739 (*S. 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 (GTR + I + Γ) 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-

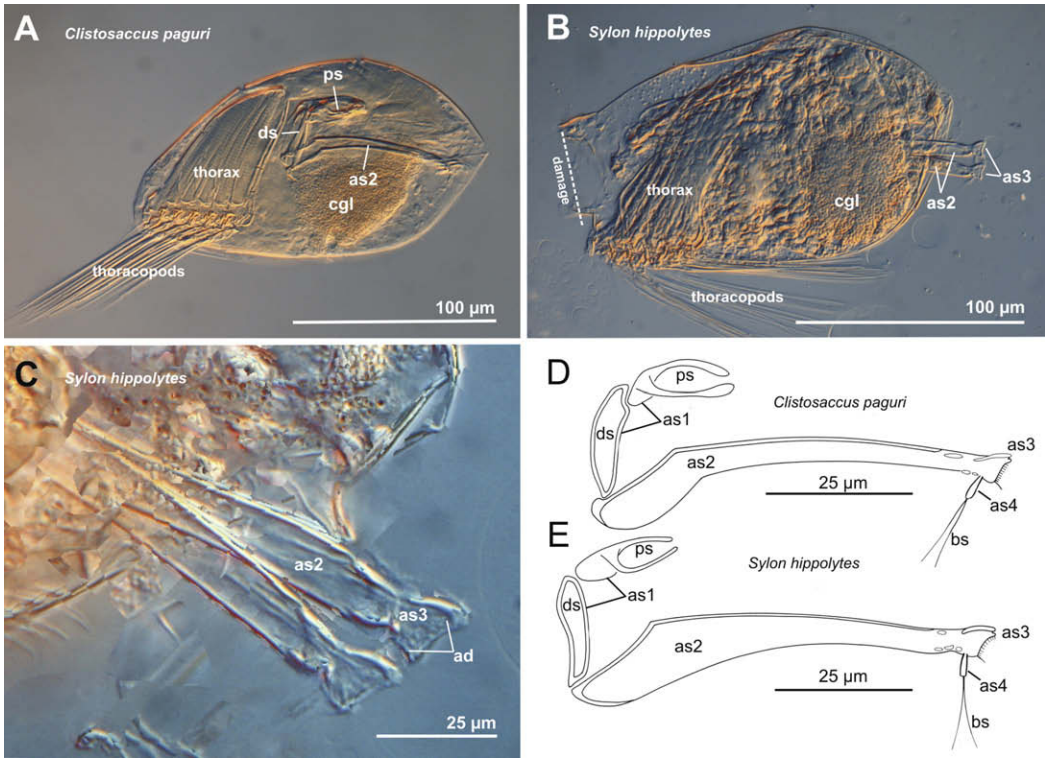

Fig. 2. Cyprids of *Clistosaccus paguri* and *Sylon hippolytes*. (A and B) Enhanced focus depth (EDF) composite of a stack of DIC optics light micrographs; the *S. hippolytes* specimen damaged posteriorly during preparation. (C) *S. hippolytes* cyprid. EDF stack of both antennules. (D and E) Outlines of the antennules drawn by camera lucida from whole mount preparations. Note the near identical shape of both the body outline and the antennules in the two species. Ad, attachment disc; an, antennule; as1–4, antennular segments 1–4; bs, bifid seta; cgl, cement gland; ds, distal sclerite of first antennular segment; ps; proximal sclerite of first antennular segment.

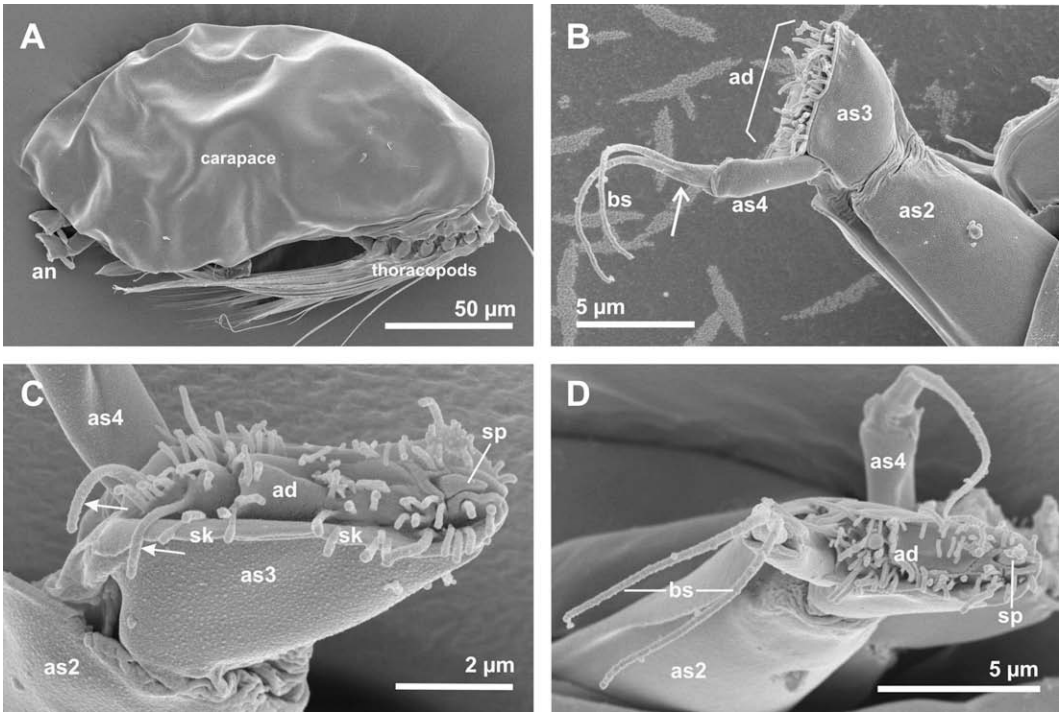

Fig. 3. *Clistosaccus paguri*, SEM of cyprids. (A) Whole cyprid. (B) Distal part of antennule. (C) Medial view of the third segment. (D) Oblique ventral view showing both the attachment disc of the third segment and the fourth segment; the two projections indicated by arrows might be true setae, but more likely they are just exceptionally long cuticular villi. In C and D parts of the antennule also visible. Ad, attachment disc; an, antennule; as31–4, antennular segments 3–4; bs, bifid seta; sk, skirt; sp, spinous process.

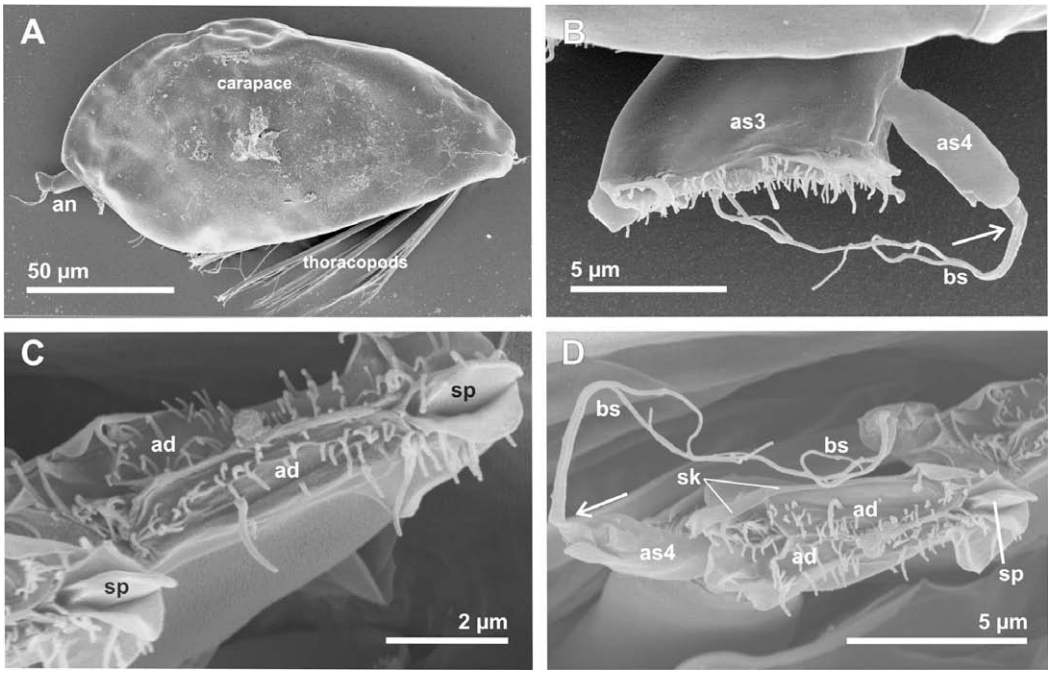

Fig. 4. *Sylon hippolytes*, SEM of cyprids. (A) Whole cyprid. (B) Segments 3 and 4. (C) Oblique ventral view of the attachment disc of the third segment. (D) Oblique ventral view of the third and fourth segments. Ad, attachment disc; an, antennule; as1–4, antennular segments 1–4; bs, bifid seta; sk, skirt; sp, spinous process.

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 CO_2, 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

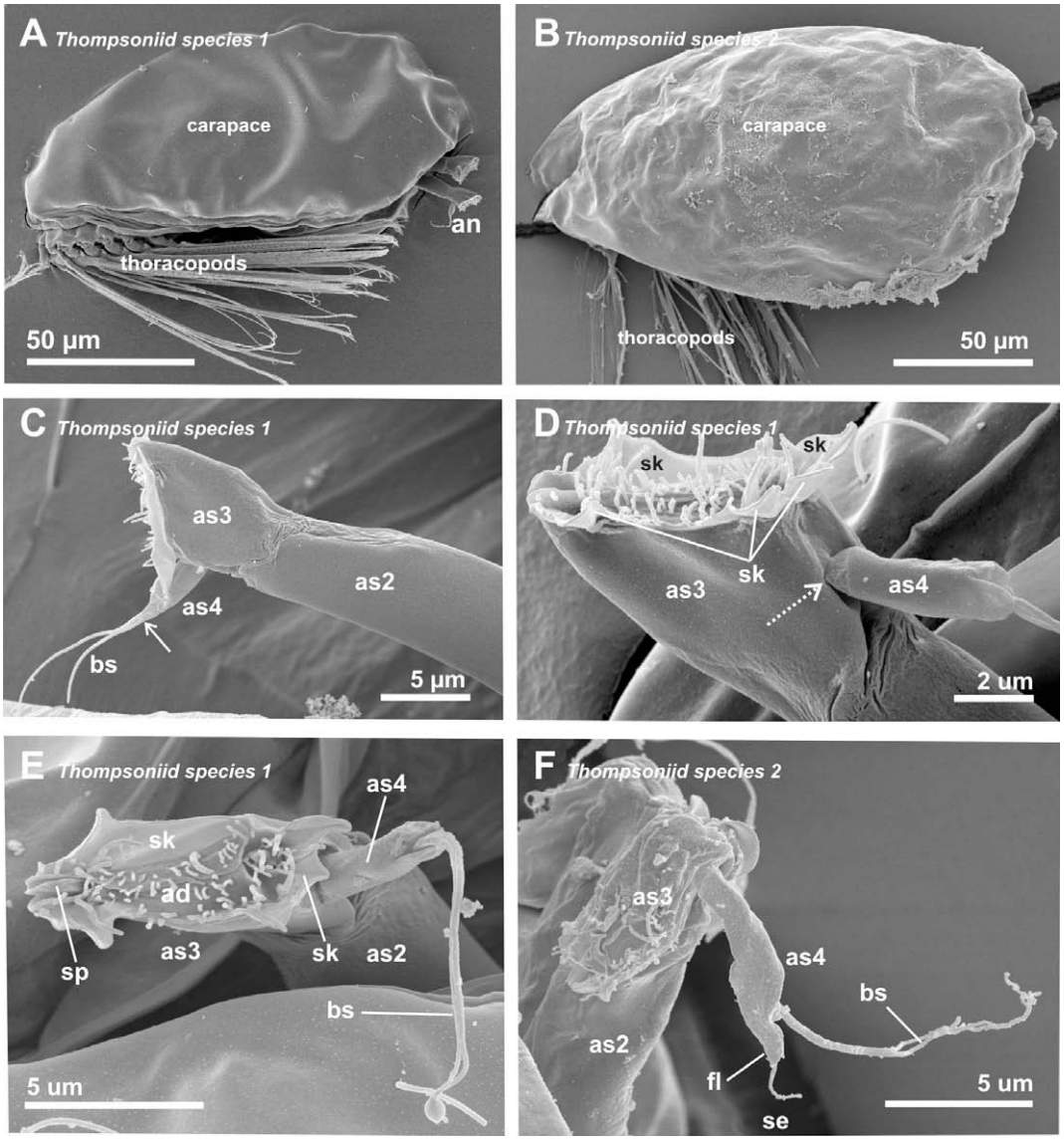

Fig. 5. Thompsoniidae; cyprids of two unidentified species. (A and C–E) species 1. (B and F) species 2. (A and B) Whole cyprid, note the angled outline of the anterior end in (A) (species 1) and the rounded anterior end in (B) (species 2). (C) Distal end of antennule. (D) Medio-lateral view of the third and fourth segments. (E) Oblique ventral view of the third and fourth segments. (F) Oblique view of the third and fourth segments. Note in (D) how the fourth segments attaches lower down on the third segment than in *Clistosaccus paguri* and *Sylon hippolytes* (Figs. 3 and 4). Note in (F) the presence apically on segment 4 of a bifid seta, a simple seta and a flap like structure. Ad, attachment disc; an, antennule; as1–4, antennular segments 1–4; bs, bifid seta; fl, flap distally on segment 4; se, simple seta; sk, skirt.

(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 supports 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*, *Pottsia serenei*, *C. delagei*, and *Boschmaella japonica.* The other clade contains *S. hippolytes*, *C. paguri* and *Polysaccus japonicus*. *C. delagei* 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. delagei*/*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 spinous 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*, *Diplothylacus*, *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 Hebsgaard (2006) and was already predicted by Glenner and Høeg (1994). But the taxon sample of Glenner and Hebsgaard (2006) 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-

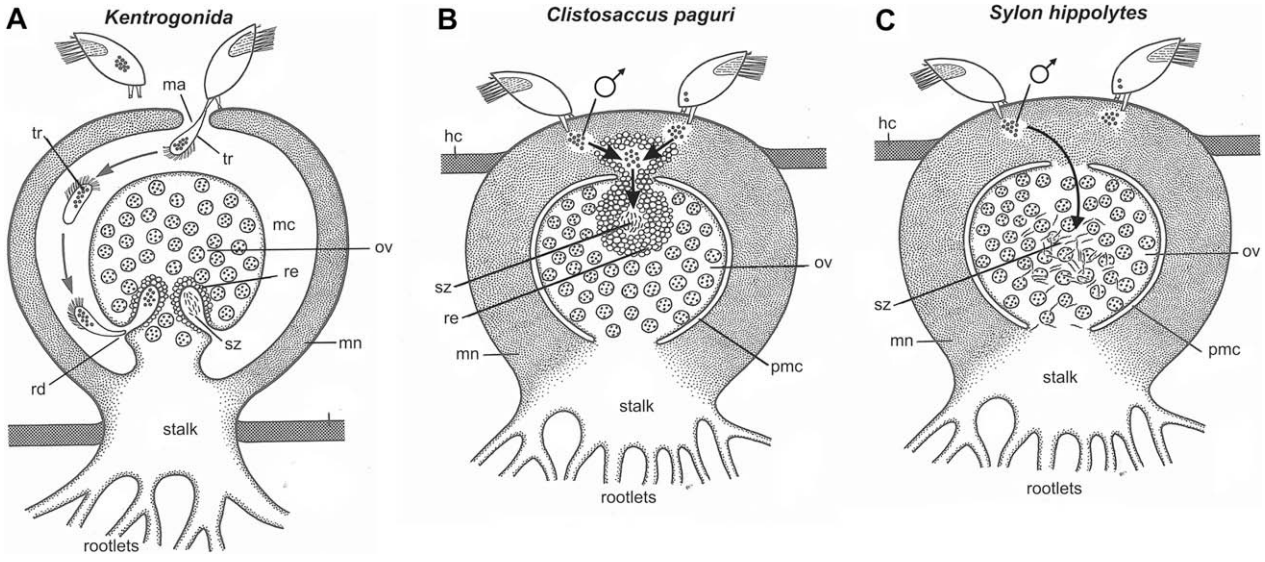

Fig. 6. Sexual systems in the Rhizocephala. (A) Kentrogonida. Male cyprids settle at the open mantle aperture of the virgin female parasite. They metamorphose into trichogons which are implanted into the two receptacles opening into the mantle cavity. Here they enter spermatogenesis. (B) *Clistosaccus paguri*. In the absence of a mantle aperture, male cyprids settle on the general surface of the virgin externa. Here they penetrate the integument with one of the antennules, which they use as a syringe to inject male cells. The implanted male travels through the connective tissue of the mantle and into the single and solid receptacle, where they start spermatogenesis. G. *Sylon hippolytes*. Settlement and implantation of males occur exactly as in *Clistosaccus*. But in the absence of a receptacle, the male cells end up in the ovary, where they commence spermatogenesis among the ovules. In both *Clistosaccus* and *Sylon* the mantle cavity does not open until the eggs are spawned. Hc, host cuticle; ma, mantle aperture; mc, mantle cavity; mn, mantle; ov, ovary; pmc, primordial mantle cavity; tr, trichogon; rd, receptacle duct; re, receptacle; sz, spermatozoa.

cephalan (Bocquet-Védrine, 1961), while *Clistosaccus* remains the only rhizocephalan in which the absence of a kentrogon stage (“akentrogonid 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 distinct 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 thompsoniid 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 thompsoniid 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 thompsoniids 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 thompsoniids have 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 *Polysaccus 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 setae 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 thompsoniid species. Høeg and Rybakov (2007) used SEM to study cyprids in *P. mediterraneus*, the only other species of the genus *Polysaccus*, 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 Polysaccidae.

4.3. Cypris morphology and the sexual system

The almost identical cyprids found in *Sylon* and *Clistosaccus*, and the many similarities they share with thompsoniids cyprids, take special interest when discussing the sexual systems of these akentrogonid rhizocephalans. In all species of the Rhizocephala “Kentrogonida” the male cyprids settle at the open mantle aperture of the virgin female parasite (externa) recently emerged from the interior of the host animal (Fig. 6A). Here they metamorphose into slug-shaped trichogon stages that subsequently migrate short distances through the mantle cavity and into the tubular ducts leading to the paired male cells receptacles. Once arrived in the receptacles, the implanted trichogons differentiate into sperm producing dwarf males (Høeg, 1987; Høeg and Lützen, 1995). In contrast, all akentrogonid species lack a mantle aperture in the juvenile parasite, whence the male must gain access to the female by another mechanism. (Høeg, 1985) showed that in *C. paguri* the male cyprids settle on the general surface of the juvenile female. Once attached, they use one of their slender antennules as syringe to piece the integument and transfer male material into the tissue of the female (Fig. 6B). The implanted male migrate through the connective tissue and into the single and solid receptacle found in *Clistosaccus*, where they commence the process of spermatogenesis (Høeg, 1982). In a brief note, Bower and Boutillier (1990) found that cyprids of *Sylon* employ exactly the same mechanism for male cell implantation (Fig. 6C). The male cells take roughly the same pathway as in *Clistosaccus*, but, in the absence of a receptacle, they end up in the ovary where spermatogenesis proceeds freely between among ovules (Lützen, 1981). Finally, Yanagimachi and Fujimaki (1967) and Lützen et al., (1996) demonstrated that such antennular implantation of males also occurs in species of the Thompsoniidae.

Our cladogram supports that the similarities in cyprid morphology and the antennular implantation of males must be homologous between the Clistosaccidae and the Thompsoniidae. The cladogram (Fig. 1) furthermore suggests that this mechanism for implanting males, although as yet unobserved, is present also in *Polysaccus japonicus* and the Chthamalophilidae. The mechanism of male implantation is also uncertain for the remaining akentrogonid taxa (*Pirusaccus*, Duplorbidae and Mycetomorphidae). Høeg and Rybakov (1996) provided strong indirect evidence for the presence of antennular penetration in *Mycetomorpha vancouverensis* (not available for molecular analysis). As previously suggested (Høeg, 1990; Glenner and Høeg, 1994), this indicates that male implantation by antennular penetration evolved at the base of the Rhizocephala Akentrogonida.

The detailed similarities between the larvae of *Clistosaccus* and *Sylon* are remarkable, since otherwise the two species differ extensively in both morphology and biology. There is no apparent similarity between the externae, and *Clistosaccus* parasitizes hermit crabs (Paguridae) while *Sylon* infests prawns (Caridea). Thus, the claim of Glenner et al. (1989) that SEM based studies of cyprid morphology will prove highly informative in reconstructing rhizocephalan evolution and phylogeny has received much support from the present study.

4.4. The case of C. delagei

Members of the family Chthamalophilidae, consisting of four species in three genera, are remarkable in infesting other cirripeds, viz. balanomorphan barnacles. The taxon Akentrogonida was first created to accommodate *C. delagei*, which Bocquet-Védrine (1961) considered as the most primitive rhizocephalan and thus a model for the origin of this taxon. She based this on the absence of a ramifying root system in this species, where the externa possesses only a bladder-like extension into the host animal. Claiming that this nutritive organ did not penetrate the integument of the host barnacle, she claimed that *C. delagei* is an ectoparasite that develops *in situ* at the site of cyprid settlement. This would differ from all other rhizocephalans, where an endoparasitic phase is always intercalated into the life cycle and the externa normally develops far from the site of infection (Høeg and Lützen, 1995). Re-

cently, Bresciani and Høeg (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 confamiliar genera *Boschmaella* and *Bocquetia* (Høeg et al., 1990). The original case for a plesiomorphic morphology in *C. delagei* is therefore no longer present, and our phylogeny shows that the Chthamalophilidae are instead nested deeply within the otherwise highly specialized Akentrogonida. The Chthamalophilidae therefore represents a pinnacle in rhizocephalan evolution. This is evident both in their sexual system (Høeg et al., 1990; Høeg 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 (Høeg and Møller, 2006).

5. Conclusion

The Akentrogonida is a monophyletic group within the Cirripedia Rhizocephala, within which *C. paguri* 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 Chthamalophilidae, tested deeply within the akentrogonid Rhizocephala and representing a pinnacle of specialization.

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