Available online at www.sciencedirect.com
ScienceDirect
Molecular Phylogenetics and Evolution 44 (2007) 1333–1341
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Phylogeny of coral-inhabiting barnacles (Cirripedia; Thoracica; Pyrgomatidae) based on 12S, 16S and 18S rDNA analysis

N. Simon-Blecher [a], D. Huchon [b], Y. Achituv [a,*]

[a] *The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel*
[b] *Department of Zoology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel*

Received 2 November 2006; revised 8 March 2007; accepted 29 March 2007
Available online 6 May 2007

Abstract

The traditional phylogeny of the coral-inhabiting barnacles, the Pyrgomatidae, is based on morphological characteristics, mainly of the hard parts. It has been difficult to establish the phylogenetic relationships among Pyrgomatidae because of the apparent convergence of morphological characteristics, and due to the use of non-cladistic systematics, which emphasize ancestor-descendant relationships rather than sister-clade relationships. We used partial sequences of two mithochondrial genes, 12S rDNA and 16S rDNA, and a nuclear gene, 18S rDNA, to infer the molecular phylogeny of the pyrgomatids. Our phylogenetic results allowed us to reject previous classifications of Pyrgomatidae based on morphological characteristics. Our results also suggested the possibility of paraphyly of the Pyrgomatidae. The hydrocoral barnacle *Wanella* is not found on the same clade as the other pyrgomatids, but rather, with the free-living balanids. The basal position of *Megatrema* and *Ceratoconcha* is supported. The archeaobalanid *Armatobalanus* is grouped with *Cantellius* at the base of the Indo-Pacific pyrgomatines. Fusion of the shell plate and modification of the opercular valves are homoplasious features that occurred more than three times on different clades. The monophyly of the "*Savignium*" group, comprising four nominal genera, is also not supported, and the different taxa are placed on different clades.

Keywords: Phylogeny; Coral-inhabiting barnacles; Pyrgomatidae; 12S rDNA; 16S rDNA; 18S rDNA

1. Introduction

The coral-inhabiting barnacles of the Pyrgomatidae family are obligatory symbionts of scleractinian corals, hydrozoans, and sponges. They are distinguished from coral-inhabiting barnacles of the genera *Armatobalanus* (Archaeobalanidea) and *Megabalanus* (Megabalanidae) by the presence of four or fewer calcareous shell plates, instead of six in other members of the Balanoidea. Pyrgomatid coral barnacles were first described in the early 19th century. These early studies identified eight pyrgomatid genera and described their taxonomy but did not deal with the phylogeny of these cirripedes (Leach, 1817; Gray, 1825 cited by Ross and Newman, 1973). Darwin (1854) grouped these genera together into a single genus, *Pyrgoma*, writing (p. 354): "I feel no hesitation in including the above genera in one genus". Within *Pyrgoma*, he also recognized the subgenus, *Creusia*. While studying the variation problems in barnacles, Darwin found many varieties within the different species of *Pyrgoma*, including 11 within one species, *Pyrgoma* (*Creusia*) *spinulosa*. In 1973, Ross and Newman resurrected five genera of coral-inhabiting barnacles and established three new genera, a trend continued by Anderson (1992). Over the years, new genera and valid species of coral barnacles have been recognized (Anderson, 1992, 1993; Ross and Newman, 2002). Recently, Ross and Newman (2002) listed 24 nominal genera and 102 nominal species of pyrgomatids, of which 67 are extant.

The phylogenetic relationships among Pyrgomatidae have been difficult to establish due to the use of non-cladis-

* Corresponding author. Fax: +972 3 7384058.
E-mail address: achity@mail.biu.ac.il (Y. Achituv).

1055-7903/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2007.03.026

tic systematics, which emphasize ancestor-descendant relationships and not sister-clade relationships, and because of the apparent convergence of morphological characteristics, e.g., fusion of opercular plates and wall-plates occurred several times during Pyrgomatidae evolution. The family Pyrgomatidae is currently divided into three subfamilies: Megatrematinae, Ceratoconchitinae, and Pyrgomatinae (Ross and Newman, 2002). The phylogenetic affinities of these subfamilies have not yet been unequivocally demonstrated. Using traditional, non-cladistic systematics, the Pyrgomatidae family was suggested to be either monophyletic (Anderson, 1992; Ross and Newman, 2002), diphyletic, with the Megatrematinae and Ceratoconchitinae forming one lineage and Pyrgomatinae the second (Galkin, 1986), or triphyletic, with each subfamily representing an independent lineage (Ross and Newman, 1973). Because the monophyly of Pyrgomatidae is not fully accepted, its phylogenetic position among Balanomorpha has never been resolved. However, it is generally accepted that the family Archaeobalanidea, and more particularly the genus *Armatobalanus* among Archaeobalanidae, should be the sister clade to all or some Pyrgomatidae. For example, ultrastructural analysis of sperm morphology (Healy and Anderson, 1990) and functional morphology of mainly cirral activity (Anderson, 1992), indicate synapomorphies between *Armatobalanus* and the Pyrgomatidae. Consequently, it was suggested (Anderson, 1992) that a coral-associated archaeobalanid, like *Armatobalanus*, is the most recent common ancestor of the Pyrgomatidae. Conversely, some authors (e.g., Ross and Newman, 1973) suggest that only Pyrgomatinae and perhaps Megatrematinae evolved independently from an *Armatobalanus* ancestor, with Ceratoconchitinae affinities being "too obscure to conjecture" (Ross and Newman, 1973).

The Megatrematinae and Ceratoconchitinae are distributed in the Atlantic Ocean except for three Megatrematinae species that are located in the West Pacific. They posses unmodified opercular valves and a four-plated wall (Ceratoconchitinae) or a partially fused plated wall (Megatrematinae). In contrast, the Pyrgomatinae are Indo-Pacific and possess a wide range of characteristics, varying from a four-plated wall and four opercular valves to a single plated shell and fused scuta and terga. The Archaeobalanidae and the Balanidae possess a six-plated wall and four opercular valves; it is therefore generally agreed that fused wall-plates and fused opercular valves represent derived character states (Ross and Newman, 2002).

Ogawa and Matsuzaki (1992) suggested that a single fused shell plate is the plesiomorphic condition. Their view is based on the assumption that barnacles that evolved earlier inhabit a greater number of host corals. This notion has never been accepted, and has received little attention by most researchers studying coral-inhabiting barnacles.

Ross and Newman (1973) presented a non-cladistic phylogenetic tree of pyrgomatines, in which *Cantellius* is the common ancestor of all other pyrgomatines (Fig. 1a). Two lineages evolve from a *Cantellius* ancestor, which possesses plesiomorphic characters: four-plated shells and four opercular valves. The first lineage possesses fused shells and four opercular valves, and includes *Savignium* as a common ancestor. From a *Savignium* ancestor, two lineages, *Pyrgopsella* and the Hoekinii tribe, are then derived. It is worth noting that *Savignium*, *sens.*, Ross and Newman (1973), includes not only *Savignium*, but also the genera *Wanella, Trevathana*, and *Neotrevathana* (cf., Anderson, 1992, 1993). The second main pyrgomatine lineage, with four shell plates and four moderately modified opercular valves, includes *Hiroa* as common ancestor. From the *Hiroa* ancestor, three lineages are then independently derived. The first one includes *Creusia* and *Utinomia*, with four shell plates and fused opercular valves. The second contains *Nobia* and *Darwiniella*, with fused shell plates and fused opercular valves. The third one includes *Pyrgoma*, with a fused shell and four highly modified opercular valves.

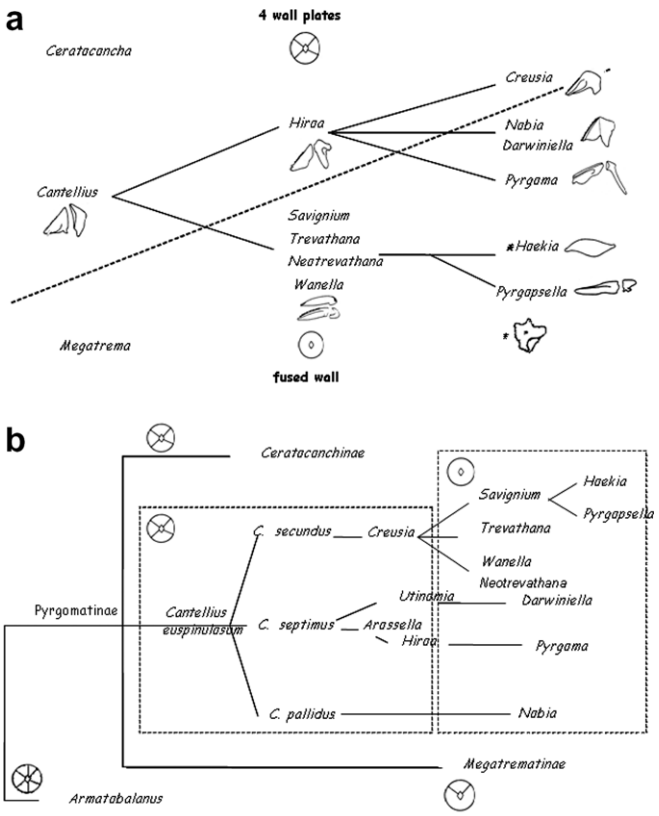

Fig. 1. Phylogenetic hypotheses for Pyrgomatidae previously suggested based on morphological characteristics. (a) Scheme based on phylogenetic tree as suggested by Ross and Newman (1973). Upper section (above diagonal) includes barnacles with four shell plates; lower section (below diagonal) comprises organisms with fused shell plates. The shape of the opercular valves are drawn at the right, or below, the name; note the irregularly lobate shell of *Hoekia*. (b) Phylogenetic tree modified from Anderson (1992). The three sub families Ceratoconchinae, Megatrematinae and Pyrgomatines share a common ancestor. Pyrgomatines with four shell plates are grouped in the left rectangle; those with fused shell plate are found in the right rectangle.

Based on the opercular valve structure and functional morphology, mainly cirral activity, Anderson (1992) proposed a different phylogeny for Pyrgomatinae (Fig. 1b). The Pyrgomatinae are divided into three groups that derived from a *Cantellius euspinulosum* ancestor. There are three lines of divergence represented by three species of *Cantellius*, namely "*pallidus*", "*septimus*" and "*secundus*". These three lineages display parallel character evolution, and include derived species with fused shell plates and fused opercular valves. The "*pallidus*" group is poorly diversified and isolates the genus *Nobia* from other Pyrgomatins. The "*septimus*" group includes two lineages. The first "*septimus"* lineage includes the *Utinomia* ancestor from which evolved *Darwiniella*, the most derived taxa of this lineage. It is worth noting that unlike Newman et al. (1976), Anderson considered *Nobia* to be polyphyletic. He thus erected the clade of *Darwiniella* and *Utinomia* from *Nobia* and placed them in a distinct lineage. The second "*septimus*" lineage is derived from an *Arossella* ancestor and includes *Hiroa* and *Pyrgoma* as the most derived taxa. Finally, the "*secundus*" lineage includes two lineages deriving from a *Creusia* ancestor. The first lineage is based on *Savignum* from which *Pyrgopsella* and *Hoekia* diverged; the second lineage includes *Trevathana* and its sister clades, *Neotrevathana* and *Wanella*.

Despite the considerable debate concerning phylogenetic relationships of Pyrgomatidae, a molecular based phylogenetic analysis of these organisms has never been conducted. In the present study, we applied, for the first time, molecular tools to examine the relationship within the Pyrgomatidae and the position of this taxon within the Balanoidea. To establish phylogenetic relationships, we used partial sequences of two mitochondrial genes, 12S rDNA and 16S rDNA, and the nuclear 18S rDNA gene.

2. Materials and methods

2.1. Species sampling

Sixteen species of coral-inhabiting barnacles were included in the analyses; these represent most of the nominal genera of the recent pyrgomatids. The list of species used in the analyses, their host coral, and collection sites are given in Table 1. The species *Hoekia* has been divided into several new genera (Ross and Newman, 1995, 2002); however we were unable to identify our "*Hoekia*" specimen to the genus level, and we regarded it as a representative of the tribe, Hoekiini. We included in our analysis two non-pyrgomatid coral-inhabiting barnacles, *Megabalanus stultus*, which is found on the Caribbean *Millepora*, and *Armatobalanus allium*, from *Montastrea curta* from Indonesia, which is regarded as the most recent ancestor of the Pyrgomatidae. The animals were dissected immediately after collection, fixed and preserved in 95% ethanol, and kept at −20 °C until extraction of DNA. We also sequenced the archaeobalanid *Semibalanus balanoides* from Plymouth, UK. We added to our analysis sequences of other balanomorphoids available from GenBank (Table 2). Vouchers preserved in 95% ethanol are housed in the Zoological Museum Tel Aviv University, Israel. Catalogue numbers are given in Table 1

2.2. DNA extraction, amplification, and sequencing

DNA was extracted from the alcohol-preserved specimens using high pure PCR template kit (Roche; Germany). ReadyMix kit (Sigma–Aldrich, St. Louis, MO) was used for amplification by the polymerase-chain-reaction (PCR) (Saiki et al., 1988) with 50 ng DNA per reaction. PCR primers are presented in Table 3. The primer set of Kocher et al. (1989) as modified by Mokady et al. (1999) was used for amplification and sequencing of the 12S subunit of mitochondrial rDNA. Primers 16SAR and 16SBR of Palumbi (1996) were used for amplification and sequencing of 16S rDNA gene fragments. Forward and reverse primers of Spears et al. (1994) were used for amplification of a 1.9 kbp fragment that included the entire 18S rDNA ribosomal gene. Internal primers designed by Mizrahi et al. (1998) were used for complete sequencing of the 1.9 kbp fragment. Amplification was carried out in a personal combi-thermocycler (Biometra, Germany). The 12S rDNA was amplified by performing 40 cycles of 30 s at 94 °C, 45 s at 47 °C and 15 s at 72 °C, followed by a final extension of 7 min at 72 °C. The 16S rDNA was amplified by performing 40 cycles of 25 s at 92 °C, 90 s at 50 °C and 25 s at 72 °C, followed by a final extension of 7 min at 72 °C. The 18S rDNA was amplified by 35 cycles of 70 s at 92 °C, 90 s at 54 °C and 50 s at 72 °C, followed by a final extension of 7 min at 72 °C. PCR products were purified by centrifugation through a high pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany).

PCR products were sequenced on both strands using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) at Tel Aviv University or by Macrogene Inc., Seoul (Korea). Sequences were then subsequently manually inspected and edited using the BioEdit program (Hall, 1999). All sequences have been deposited in GenBank, and the accession numbers are given in Table 2.

2.3. Sequence alignments

Sequences were aligned using PROBCONS (Do et al., 2005) with three consistency steps and 500 iterative refinement repetitions. The alignments were then corrected by hand and gaps present in more than 25% of the taxa were removed from the analyses. The corrected alignment for the 12S rRNA included 337 characters, of which 114 were parsimony informative. The corrected alignment for the 16S rRNA included 498 characters, of which 129 were parsimony informative. Finally, the corrected alignment for the 18S rRNA included 1787 characters, of which 60 where parsimony informative. A 5% χ-square test for unequal base composition was performed using the command

Table 1
Material used for DNA extraction and sequencings

Barnacle species	Host coral	Location	Catalogue number (Zoological Museum, Tel Aviv University)
Semibalanus balanoides	Free-living barnacle	Plymouth, UK English Channel, Atlantic Ocean	TAU Ar27833
Megabalanus stultus	*Millepora complanata*	Pampatar, Margarita Is. Venezuela, Caribbean	TAU Ar27834
Armatobalanus allium	*Montastrea curta*	Sulawesi, Indonesia, , Indian Ocean	TAU Ar27835
Cantellius palidus	*Porites* sp.	Phuket Is., Thailand, Andaman Sea, Indian Ocean	TAU Ar27836
Ceratoconcha domingensis	*Porites* sp.	Bermuda, Atlantic Ocean	TAU Ar27837
Creusia indica	*Favites* sp.	Eilat, Red Sea	TAU Ar27838
''Hoekia''	*Hydnophora exesa*	Sulawesi, Indonesia, Indian Ocean	Lost
Darwiniella conjugatum	*Galaxea* sp.	Okinawa, Japan	TAU Ar27839
Hiroa stubbingsi	*Astreopora miryophtalma*	Phuket Is., Thailand, Andaman Sea, Indian Ocean	TAU Ar27840
Megatrema anglicum	*Occulina patagonica*	Portman , Spain, Mediterranean	TAU Ar27841
Neotrevathana elongatum	*Echinopora* sp.	Eilat, Red Sea	Lost
Nobia grandis	*Galaxea fascicularis*	Eilat, Red Sea	TAU Ar27843
Pyrgoma cancellatum	*Turbinaria* sp.	Phuket Is., Thailand, Andaman Sea, Indian Ocean	TAU Ar27844
Pyrgopsella youngi	*Symphyllia radians*	Sulawesi, Indonesia, Indian Ocean	TAU Ar27804
Savignium crenatum	*Acantasra* sp.	Eilat, Red Sea	TAU Ar27845
Trevathana dentata	*Favites abdita*	Eilat, Red Sea	TAU Ar27846
Wanella milleporae	*Millepora dichotoma*	Eilat, Red Sea	TAU Ar27847

Table 2
Taxonomy and GenBank accession numbers for each sequences used in this study

Superfamily/family	Subfamily		12S rDNA	16S rDNA	18S rDNA
Balanoidea					
Archaeobalanidea	Archaeobalaninea	*Armatobalanus allium*	AM497878*	AM497877*	AM497876*
	Elminiinea	*Elminius kingi*	AY520670	AY520738	AY520636
		Elminius modestus	AY 520669	AY520737	AY520635
	Semibalaninea	*Semibalanus balanoides*	AM497884*	AM497883*	AM497882*
		Semibalanus cariosus	AY520661	AY520729	AY520627
Balanidae	Balaninae	*Balanus balanus*	AY520662	AY520730	AY520628
		Balanus crenatus	AY520658	AY520726	AY520624
		Balanus glandula	AY520659	AY520727	AY520625
		Balanus perforatus	AY520663	AY520731	AY520629
	Concaviinae	*Menesiniella aquila*	AY520664	AY520732	AY520630
	Megabalaninae	*Austramegabalanus psittacus*	AY520668	AY520736	AY520634
		Megabalanus californicsus	AY520666	AY520734	AY520632
		Megabalanus spinosus	AY520667	AY520735	AY520633
		Megabalanus tintinabulum	AY520665	AY520733	AY520631
		Megabalanus stultus	AM497926*	AM497925*	AM497924*
Pyrgomatidae	Ceratoconchitinae	*Ceratoconcha domingensis*	AM497887*	AM497886*	AM497885*
	Megatrematinae	*Megatrema anglicum*	AM497890*	AM497889*	AM497888*
	Pyrgomatinae	*Cantellius palidus*	AM497881*	AM497880*	AM497879*
		Creusia indica	AM497893*	AM497892*	AM497891*
		Darwiniella conjugatum	AM497902*	AM497901*	AM497900*
		''Hoekia''	AM497923*	AM497922*	AM497921*
		Hiroa stubbingsi	AM497896*	AM497895*	AM497894*
		Neotrevathana elongatum	AM497917*	AM497916*	AM497915*
		Nobia grandis	AM497899*	AM497898*	AM497897*
		Pyrgoma cancellata	AM497905*	AM497904*	AM497903*
		Pyrgopsella youngi	AM497920*	AM497919*	AM497918*
		Savignium crenatum	AM497911*	AM497910*	AM497909*
		Trevathana dentata	AM497914*	AM497913*	AM497912*
		Wanella milleporae	AM497908*	AM497907*	AM497906*
Tetraclitoidea					
Tetraclitidae		*Tetraclita japonica*	AY520674	AY520741	AY520640
		Tetraclita squamosa	AY520673	AY520740	AY520639

Newly determined sequences are indicated by an asterisk (*).

Table 3
Primers used for amplification and sequencing of the different genes

Gene	Primers	Primer sequence	Source
12S rDNA	Forward	5′-GAAACCAGGATTAGATACC	Mokady et al., 1999
	Reverse	5′-TTTCCCGCGAGCGACGGGCG.	Mokady et al. (1999)
16S rDNA	Forward	5′-CGCCTGTTTAACAAAAACAT	Palumbi (1996)
	Reverse	5′-CCGGTTTGAACTCAGATCATGT	Palumbi (1996)
18S rDNA	Forward amplification	5′-TAATGATCCTTCCGCAGGTT	Spears et al. (1992)
	Reverse amplification	5′-CCTGGTTGATCCTGCCAG	Spears et al. (1992)
	Forward sequencing	5′-ACTTACCCACTCCCAGTTC	Mizrahi et al. (1998)
	Forward sequencing	5′-GTTCGAAGGCGATCAAATACC	Mizrahi et al. (1998)
	Forward sequencing	5′-TCCGATAACGAACGAGAC	Mizrahi et al. (1998)
	Reverse sequencing	5′-TCTAAGGGCATCACAGAC	Mizrahi et al. (1998)
	Reverse sequencing	5′-CGTTTCGCAGTAGTTCGTC	Mizrahi et al. (1998)
	Reverse sequencing	5′-TGCTGCCTTCCTTAGATG	Mizrahi et al. (1998)

BASEFREQS in PAUP* 4.0b1 (Swofford, 2000). This allowed verification of the absence of significant base composition heterogeneity in the datasets considered.

2.4. Phylogenetic reconstructions

Two tree reconstructions were conducted: a maximum likelihood (ML) analysis performed with the program PAUP*, and a Bayesian analysis with the program MrBayes3.1 (Ronquist and Huelsenbeck, 2003). For the ML analysis, the best probabilistic model of sequence evolution was determined with the program MODELTEST 3.07 (Posada and Crandall, 1998) using the Akaike information criterion (AIC). The parameters of the model were then determined in an iterative manner using PAUP*. First, a heuristic search was conducted using the best parameters identified with the program MODELTEST. This search was performed starting with a NJ tree and using TBR branch-swapping. The command LSCORES was then used to re-estimate the likelihood and the best parameters of the trees obtained in the previous search. The new parameters were then used to conduct a new heuristic search. These operations were repeated until convergence. To improve the chance of finding the best tree, the last step was conducted with 100 random sequence additions. Bootstrap percentages (BP) were computed using the best parameters found, as indicated above, after 500 replicates starting with an NJ tree and with TBR branch-swapping.

The Bayesian analysis was performed on partitioned data assuming each of the three genes evolving with independent model parameters. Each partition evolved under the GTR model of sequence evolution and a mixed distribution model of among-site rate variation (invariable sites plus gamma distribution). Two simultaneous independent runs were performed. For each run, four chains were sampled every 100 generations and each chain was run for 20,000,000 generations. The average standard deviation of split frequencies remained below 0.005 after 10,000,000 generations. Consequently, clade posterior probabilities (PP) were calculated after removal of the first 150,000 trees. The potential scale reduction factors of the parameters were close to 1 at the end of the run, which indicated that the run had most likely converged.

2.5. Testing of alternative hypotheses

The best ML tree was compared to several constrained topologies using the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) as implemented by PAUP*. The tests were conducted with RELL optimization and 100,000 bootstrap replicates. Eight alternative topologies were considered: (1) the best topology based on Anderson's (1992) morphological tree; (2) the best topology based on Ross and Newman's (1973) morphological tree; (3) the best topology supporting *Armatobalanus* as the sister clade of *Cantellius* and its putative derivative taxa (i.e., *Nobia*, *Pyrgoma*, *Darwiniella*, *Hiroa*, *Creusia*, *Savignium*, *Hoekia*, *Trevathana*, *Neotrevathana*, *Pyrgopsella*; Ross and Newman, 1973); (4) the best topology that supports the paraphyly of a clade including all coral-inhabiting barnacles except *Wanella*; (5) the best topology supporting the monophyly of coral-inhabiting barnacles (i.e., Pyrgomatidae); (6) the best topology supporting the paraphyly of *Ceratoconcha* + *Megatrema*; (7) the best topology supporting the paraphyly of *Hiroa* + *Darwiniella*; (8) the best topology supporting the paraphyly of *Hoekia* + *Trevathana* + *Neotrevathana* + *Pyrgopsella*. These eight topologies were built using constrained ML heuristic searches. Each search was conducted starting with a NJ tree, the TBR branch-swapping option, and using the parameters of the best ML tree.

3. Results

3.1. Phylogenetic tree

We first identified the optimal phylogenetic model for coral-inhabiting barnacles based on sequence analysis of their 12S, 16S and 18S rDNA. The best model selected by AIC in Modeltest 3.5 for the combined dataset was TVM + I + G. The phylogenetic trees were rooted with two outgroups, *Tetraclita* and *Elminius*, according to

Pérez-Losada et al. (2004). In the ML tree, the Pyrgomatidae were divided among two clades suggesting the paraphyly of coral-inhabiting barnacles (Fig. 2). The Bayesian tree supports a different topology, in which the coral barnacles are also paraphyletic. Although the Bayesian and ML trees support slightly different topologies, those differences only involve weakly supported nodes (i.e., nodes with BP < 50% or PP < 0.80; data not shown).

In the ML tree, the groups in the first clade are the free-living genera *Menesiniella*, *Balanus*, *Semibalanus*, *Austromegabalanus*, and *Megabalanus*, including *M. stultus* from *Millepora*, together with the pyrgomatid *Wanella*. However, the bootstrap supports for the monophyly of this group and for the relationships within this group are very weak (BP ⩽ 51%; PP ⩽ 0.52). Among free-living barnacles, our tree agrees with the previous findings of Pérez-Losada et al. (2004), supporting the sister-clade relationship of *Austromegabalanus* and *Megabalanus*, as well as the paraphyly of the genera *Balanus* relative to *Semibalanus*.

The second clade groups the other pyrgomatids and *Armatobalanus*, with moderate support (BP = 87; PP = 1.0). Among coral-inhabiting barnacles, the first diverging clade group is *Cantellius* + *Armatobalanus* with *Ceratoconcha* + *Megatrema* (BP = 48; PP = 0.99). The grouping of *Ceratoconcha* + *Megatrema* within a clade is strongly supported (BP = 100; PP = 1.0). Other relationships among coral barnacles are weakly supported other than two exceptions which are strongly supported: the grouping of *Hiroa* with *Darwiniella* (BP = 95; PP = 1.0) and the grouping of *Hoekia* with *Trevathana*, *Neotrevathana, and Pyrgopsella* (BP = 100; PP = 1.0).

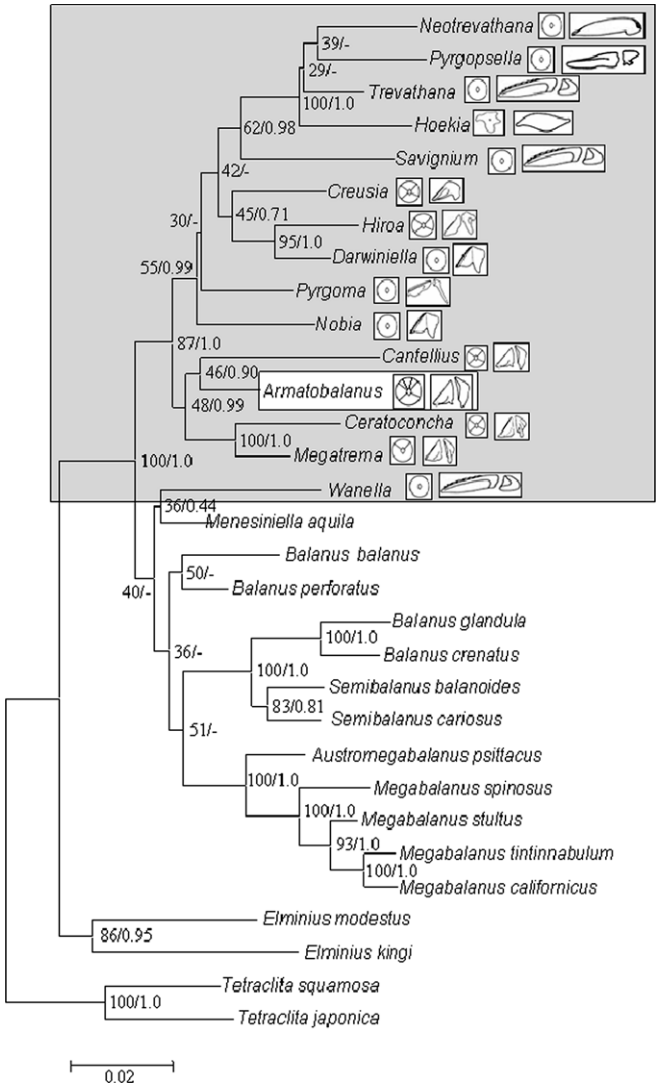

Fig. 2. Maximum likelihood (ML) tree based on concatenated 12S rRNA, 16S rRNA and 18S rRNA sequences of barnacles. For each node, the ML bootstrap percentage (BP) and the bayesian posterior probabilities (PP) are given at the right and left of the slash, respectively. Coral-inhabiting barnacles, of the family Pyrgomatidae, are indicated by grey background; note the position of *Wanella* and of the archaeobalanid, *Armatobalanus*. For each pyrgomatid genus, the number of the shell plates and the shape of the opercular valves are drawn at the right of the name. It is worth noting that the fusion of the four wall-plates and of the two opercular valves occurred several times independently during the evolution of coral-inhabiting barnacles.

3.2. Testing of alternative hypotheses

Despite the fact that many branches of our tree are weakly supported, the classical morphological hypotheses of Anderson (1992) and Ross and Newman (1973) are statistically rejected when compared to the best ML tree (Anderson: *P*-value SH < 0.00001; Ross and Newman: *P*-value SH = 0.01). However, none of the six other hypotheses tested appear to be significantly less likely than the best tree (Table 4).

4. Discussion

4.1. Balanoidea and Pyrgomatidae phylogeny

Our phylogenetic results complement the barnacle tree presented by Pérez-Losada et al. (2004), as our analysis includes Pyrgomatidae and the genera *Armatobalanus*. The perfect agreement between our tree and the one in Pérez-Losada et al. (2004) is not surprising since our analysis is based on three out of the six genes that had been used in their study. Our results confirm the polyphyly of the Archaeobalanidea, *Elminius* and *Semibalanus* as already shown by Pérez-Losada et al. (2004) and strengthen their results by suggesting that *Armatobalanus* form a third lineage not connected to the other two. Additionally, our analysis, surprisingly, suggests the paraphyly of Pyrgomatidae by placing *Wanella* among free-living balanids, and by nesting *Armatobalanus* within Pyrgomatidae. Our results also reject previous phylogenies based on morphological characteristics, as presented by Ross and Newman (1973), Newman and Ladd (1974) and by Anderson (1992).

Wanella was suggested to be a derived taxon that evolved from a *Savignium* ancestor (Anderson, 1992). Our molecular results instead place *Wanella* with the

Table 4
Shimodaira–Hasegawa test results

Topology	−ln L	Diff −ln L	*P*-values SH-test
Best ML tree	11112.88475	(best)	—
1. Anderson hypothesis	11250.72447	137.83972	0.000001*
2. Ross and Newman hypothesis	11170.42425	57.53949	0.010079*
3. *Armatobalanus* sister clade of "*Cantellius*-derived species"	11115.23326	2.34850	0.899317
4. Paraphyly of main coral barnacle clade	11138.75635	25.87160	0.277796
5. Monophyly of coral barnacles	11126.72002	13.83527	0.596553
6. Paraphyly of (*Ceratoconcha*, *Megatrema*)	11128.97020	16.08545	0.517946
7. Paraphyly of (*Hiroa*, *Darwiniella*)	11136.13248	23.24772	0.339342
8. Paraphyly of (*Hoekia*, *Trevathana, Neotrevathana*, *Pyrgopsella*)	11149.53817	36.65341	0.143891

* $P < 0.05$.

balanid *Menesiniella*, very near the dichotomy with the pyrgomatids. Support for this relationship, however, is very weak, and we cannot reject the possibility that *Wanella* could be the first diverging Pyrgomatidae (Table 4). Interestingly, *Wanella* does not inhabit a scleractinian coral; instead, it is hosted by the hydrocoral *Millepora*, like some *Megabalanus* species. The fact that *Wanella* does not live on stony coral strengthens the idea that this species is not closely related to the "*Savignium*" group.

It is generally accepted that the Pyrgomatidae have been derived from a six-plated balanoid ancestor (Anderson, 1992; Ross and Newman, 1973, 2002; Newman and Ladd, 1974). This assumption is based on morphological characteristics, the mode of interlock of the rostrum with the latera, the "balanoid" opercular valves (Ross and Newman, 1973, 2002), their growth pattern (Ross and Newman, 1973, 2002), cirral activity (Anderson, 1992) and sperm ultrastructure (Healy and Anderson, 1990). Within the balanoids, it is assumed that the archaeobalanid is the stem from which the Pyrgomatidae evolved (Ross and Newman, 1973; Anderson, 1992). The solid basis and the pyrgomatids opercular valves resemble those of *Armatobalanus*. *Armatobalanus* is found in the Atlantic and the Indo-Pacific; some species occur exclusively on corals. Surprisingly, our phylogenetic results did not support the hypothesis that the Archaeobalanidae served as a stem of the Pyrgomatidae. Instead they cluster *Armatobalanus* with *Cantellius* and the two 'Atlantic' coral-inhabiting barnacles, *Ceratoconcha* and *Megatrema*. This clade is located on a basal node of the pyrgomatids, forming a sister group to all other pyrgomatids, except *Wanella*.

The basal position of the clade grouping *Cantellius, Armatobalanus*, *Ceratoconcha*, and *Megatrema* allows us to refute the hypothesis of Ogawa and Matsuzaki (1992) that the Pyrgomatids evolved from a barnacle with a single shell plate to the four shell plate *Creusia*. Indeed, none of these taxa possess completely fused wall-plates.

Armatobalanus and *Cantellius* are located at the basis of other Indo-Pacific Pyrgomatidae. *Cantellius*, the genus with the highest number of species within the Pyrgomatidae, has rather plesiomorphic characteristics. The shell of *Cantellius* is made of four plates, the opercular valves have a 'Balanid' shape comprised of four separate plates, the scuta are usually triangular, and the terga show an articular ledge.

In classic systematics, the two 'Atlantic' genera form two different subfamilies. From a morphological point of view, *Ceratoconcha* shows the most plesiomorphic characters, four shell plates and balanomorph opercular valves. In *Megatrema*, the shell is partly fused but the opercular valves are of the balanoid type. Based on our phylogenetic tree, we suggest that these two subfamilies share the same phylogenetic line as suggested by Galkin (1986). However, here again, the alternative hypothesis suggesting the paraphyly of the 'Atlantic' genera cannot be statistically rejected (Table 4).

Nobia and *Pyrgoma* have traditionally been placed in the same clade based on the presence of a fused shell and highly modified opercular valves (Ross and Newman, 1973; Anderson, 1992). In our tree, they are found on separate internal clades, suggesting that their characteristics are the result of convergent evolution. However, these separate internal clade relationships are not supported (BP < 50% PP < 0.5). Based on opercular valve morphology, Anderson (1992, 1993) erected the taxa *Darwiniella*, *Arossella* and *Utinomia* which had been formerly assigned to the genus *Nobia* (Ross and Newman, 1973). Anderson (1992) concluded that these four taxa do not belong to the same clade (Fig. 1b). We confirm the polyphyly of the former '*Nobia*' since, in our analyses, *Nobia* and *Darwiniella* are not sister clades.

Creusia and *Hiroa*, with intermediate morphological characteristics, four shell plates and fused or highly modified opercular valves, cluster together with *Darwiniella*, which possess apomorphic characteristics. This taxa forms a sister clade to a clade that encompass *Savignium*, *Tervathana*, *Neotrevathana*, *Hoekia* and *Pyrgopsella. Savignium*, *Tervaethana*, and *Neotrevathana* were formerly placed together with *Wanella*, under a single genus, *Savignium*. The inclusion of *Wanella* in the "*Savignium*" group (Ross and Newman, 1973) is not supported by our analysis. These five taxa share some morphological characteristics including fused shell and elongated scuta, and have been grouped together with *Wanella*, by Anderson (1992). In *Neotrevateha* and *Hoekia*, the scutum and tergum are fused and form a single opercular plate on each side of the barnacle. One of the features characteristic of "*Savignium*" is the tapering basis embedded deep in the coral skeleton. In *Pyrgopsella* there is only a rudimentary calcareous basis,

and the basis is reduced to a membranous one; in *Hoekia* the calcareous basis was lost.

4.2. Evolution of morphological characteristics in coral-inhabiting barnacles

Fusion of all shell plates, a unique feature of the Pyrgomatidae, is a homoplasious characteristic. Reduction of shell plates evolved more than once within the Pyrgomatidae. In the Pyrgomatidae, the basis is generally cup shaped or comprised of a deep cone embedded in the host coral skeleton. The shell plate reaches essentially its maximum diameter early in life, and growth occurs mainly between the shell perimeter and the basis. This is in contrast to free-living barnacles that exhibit the most growth in the sutures between radii and alae. As a result, basal height continues to increase in Pyrgomatidae, forming the characteristic cone shape of the basis, and in some cases forming chimneys over the coral surface. The growth characteristics of Pyrgomatidae were confirmed by Chemedanov (personal communication) in *Wanella* and *Trevathana* using the alizarin staining method. The shell plates must withstand the lateral pressure of the growing coral, and this can be achieved by the concrescent shell. A fused shell, without sutures, seems to be better adapted to competition with the skeletal growth of the host coral. Fusion of scutum and tergum and modified tergum are also homoplasious characters in the pyrgomatids. This modification usually results from the increase of basal margins of scuta or elongation of the spur of terga, probably due to the cone shaped basis.

There are three obligatory symbiotic barnacles that live on the surface of living colonies of the hydrocoral *Millepora*, two species of *Megabalanus, M. stultus* and *M. ajax*, and *Wanella milleporae* (Ross, 1999). *Wanella* is regarded as a pyrgomatid (Darwin, 1854; Ross and Newman, 1973) and exhibits many characteristics that are common with other pyrgomatids, i.e., fused shell and elongated scuta. On the basis of our analysis, we conclude that these traits are homoplasious with those found in the "*Savignium–Pyrgopsella*" clade and are the result of convergence due to adaptation to a symbiotic life with coral.

Our phylogenetic results allowed us to reject previous classifications of Pyrgomatidae that were based on morphological data. They also raise new phylogenetic hypotheses including the paraphyly of Pyrgomatidae and the placement of *Armatobalanus* within Pyrgomatidae. However, in spite the large data set analyzed, including more than 2500 bp of ribosomal sequences, many nodes were only weakly supported. Sequencing of additional genes carrying more highly variable regions is thus needed to confirm our observations. However, there are limited primers available for genes suitable for amplification and sequencing of cirripedes. A potential gene for such an analysis is cytochrome oxidase 1 (COI), which is widely used for phylogenetic analysis of a variety of taxa, including cirripedes (Van Syoc, 2001; Wares, 2001; Puspasari et al., 2001). However, in the chthamalids it was found that at the intergeneric level, this gene is saturated and contains limited phylogenetic information (Fisher et al., 2004). Recently, Moulton and Wiegmann (2004) used the nuclear coding gene, CAD, to infer the phylogeny of flies. This gene, not yet widely used, possesses a moderate level of non-synonymous divergence among taxa of intermediate evolutionary age and may be a suitable phylogenetic marker for future work on the Pyrgomatidae.

Acknowledgments

This study was supported by the Israel Science Foundation (ISF) (Grant No. 430/03-3). We acknowledge Mr. Assi Arbiv of ASEA Holon, Israel, for help in obtaining coral barnacles used in this study. Dr. Itzhak Brickner and Dr. Sarit Karako-Lampert helped us with sample collection. The material from Thailand was obtained with the help of Dr. Niphon Phangsuwan from the Phuket Marine Biological Centre. We thank the Interuniversity Institute for Marine Studies in Eilat for assistance and use of its facilities. Dr. G. Kolbasov helped with the identification of *Armatobalanus*. We thank Prof. W.A. Newman of The Scripps Institution of Oceanography for critical reading and comments on the manuscript. We are indebted to the anonymous reviewers for valuable suggestions and remarks.

References

Anderson, D.T., 1992. Structure function and phylogeny of coral-inhabiting barnacles (Cirripedia, Balanoidea). Zool. J. Linn. Soc. London 106, 277–339.

Anderson, D.T., 1993. Addendum/corrigendum. Zool. J. Linn. Soc. London 107, 377.

Darwin, C., 1854. A Monograph on the Sub-class Cirripedia with Figures of All the Species. The Balanidae the Verrucidae etc. Ray Society, London.

Do, C.B., Mahabhashyam, M.S.P., Brudno, M., Batzoglou, S., 2005. ProbCons: probabilistic consistency-based multiple sequence alignment. Genome Res. 15, 330–340.

Fisher, T., Katcoff, D.J., Achituv, Y., 2004. Phylogenetic study of Chthamaloids (Cirripedia; Thorcica; Chthamaloidae) based on 16S rDNA and COI sequence analysis. Biol. J. Linn. Soc. London. 83, 39–45.

Galkin, S.V., 1986. The system of coral-inhabiting barnacles (Cirripedia, Balanomorpha). Zool. Zh. 65, 1285–1295.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis. Available from: <http://www.mbio.ncsu.edu/BioEdit/bioedit.html/>.

Healy, J.M., Anderson, D.T., 1990. The sperm ultrastructure in the Cirripedia and its phylogenetic significance. Records Aust. Mus. 42, 1–26.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X., Wilson, A.C., 1989. Dynamic of mitochondrial evolution in animals: amplification and sequencing with conserve primers. Proc. Natl. Acad. Sci. USA 86, 6196–6200.

Mizrahi, L., Achituv, Y., Katcoff, D.C., Perl-Treves, R., 1998. The phylogenetic position of Ibla (Cirripedia, Thoracica) based on 18s rDNA sequence analysis. J. Crust. Biol. 18, 363–368.

Mokady, O., Loya, Y., Achituv, Y., Gefen, E., Grauer, D., Rozenblatt, S., Brickner, I., 1999. Speciation versus phenotypic

plasticity—Darwin's observations in an ecological context. J. Mol. Evol. 49, 367–375.
Moulton, J.K., Wiegmann, B.M., 2004. Evolution and phylogenetic utility of CAD (rudimentary) among Mesozoic-aged Eremoneuran Diptera (Insecta). Mol. Phylogen. Evol. 31, 363–378.
Newman, W.A., Ladd, H.S., 1974. Origin of coral-inhabiting Balanids Cirripedia, Thoracica. Verhandl. Naturf. Ges. Basel. 84, 381–396.
Newman, W.A., Jumars, P.A., Ross, A., 1976. Diversity trend in coral-inhabiting barnacles (Cirripedia, Pyrgomatidae). Micronesia 12, 69–82.
Ogawa, K., Matsuzaki, K., 1992. An assay on host specificity, systematics taxonomy and evolution of the coral-barnacles. Bull. Biogeogr. Soc. Jpn. 47, 87–101.
Palumbi, S.R., 1996. Nucleic acids II: the polymerase chain reaction. In: Hillis, M., Moritz, C., Aable, B.K. (Eds.), Molecular Systematics. Sinauer, Sunderland, Massachusetts, pp. 205–247.
Pérez-Losada, M., Høeg, J.T., Crandall, K.A., 2004. Unraveling the evolutionary radiation of the thoracican barnacles using molecular and morphological evidence: a comparison of several divergence time estimation approaches. Syst. Biol. 53, 244–264.
Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitutions. Bioinformatics 14, 817–818.
Puspasari, I.A., Yamaguchi, T., Kojima, S., 2001. Phylogeny of the Balanus amphitrite complex occurring in Japan (Cirripedia: Balanidae) inferred from mitochondrial COI gene nucleotide sequences and morphology. Sessile Organisms 18, 7–17.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Ross, A., 1999. On the occurrence of obligatory symbiotic barnacles Megabalanus stultus (Darwin), 1854 (Cirripedia: Balanomorpha) in Taiwan: a reappraisal. Zool. Stud. 38, 275–278.
Ross, A., Newman, W.A., 1973. Revision of the coral-inhabiting barnacles (Cirripedia: Balanidae). Trans. San Diego Soc. Nat. Hist. 17, 137–173.
Ross, A., Newman, W.A., 1995. A coral eating barnacle revisited (Cirripedia, Pyrgomatidae). Contrib. Zool. 65, 129–175.
Ross, A., Newman, W.A., 2002. Coral barnacles: cemozoic decline and extinction in the Atlantic/East Pacific versus diversification in the Indo West Pacific. In: Proc. 9th Int. Coral Reef Symp., Bali Indonesia, 1, pp. 179–184.
Saiki, R., Gelfand, D.H., Stofell, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Ehlich, H.A., 1988. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science 239, 487–491.
Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.
Spears, T., Abele, L.G., Kim, W., 1992. The monophyly of brachyuran crabs – a phylogenetic study based on 18S ribosomal RNA. Sys. Biol. 41, 446–461.
Spears, T., Abele, L.G., Applegate, M.A., 1994. Phylogenetic study of cirripedes and selected relatives (Thecostraca) based on 18S rDNA sequence analysis. J. Crust. Biol. 14, 641–656.
Swofford, D.L., 2000. PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods), Version 4b10. Sinauer Associates, Sunderland, MA.
Van Syoc, R.J., 2001. Barnacle mitochondrial DNA: determining genetic relationship among species of Pollicipes. In: Schram, F.R., Høeg, J.T. (Eds.), New Frontier in Barnacle Evolution, Crustacean issues, vol. 10. Balkema, Rotterdam, pp. 269–296.
Wares, J.P., 2001. Patterns of speciation inferred from mitochondrial DNA in North American Chthamalus (Cirripedia: Balanomorpha: Chthamaloidea). Mol. Phylogenet. Evol. 18, 104–116.