Biological Journal of the Linnean Society, 2004, **83**, 39–45. With 2 figures

A phylogenetic study of chthamaloids (Cirripedia; Thorcica; Chthamaloidae) based on 16S rDNA and COI sequence analysis

T. FISHER, D. J. KATCOFF and Y. ACHITUV*

Faculty of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel

Received 22 October 2003; accepted for publication 19 January 2004

The sequences of gene fragments encoding cytochrome *c* oxidase subunit I (COI) and 16S rDNA were obtained and used to construct phylograms of eight taxa of chthamaloid barnacles using the scalpelloid *Calantica* as an out-group. The phylograms support the basal position of *Catomerus* within the chthamaloids. Analysis of 16S rDNA shows that *Octomeris* and the four-plated barnacle *Chamaesipho* are located on the same clade, while *Chthamalus*, *Euraphia* and *Tetrachthamalus* are located on a second clade, indicating that reduction in the number of shell plates occurred twice in the evolution of the chthamaloids. The topology of phylograms based on COI sequences is poorly resolved: 93% of third position nucleotides in this fragment are polymorphic while the amino acid sequences are strictly conserved. We assume that in the chthamaloids, at least at the generic level, polymorphism in the COI gene is saturated beyond phylogenetic information and cannot resolve the phylogenetic relationships within this superfamily. © 2004 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2004, **83**, 39–45.

ADDITIONAL KEYWORDS: barnacles – evolution – molecular systematics.

INTRODUCTION

Traditionally, it is accepted that genera belonging to the superfamily Chthamaloidae exhibit the most plesiomorphic characters within the suborder Balanomorpha. In this superfamily, there are two genera, *Catophragmus* and *Catomerus*, whose shells consist of a wall of eight solid plates and several whorls of small imbricating plates and *Octomeris*, with eight solid plates, as well as several genera with either six or four plates. Darwin (1854) established the homology of the plates and the imbricating plates to those found in the scalpelloids, stating (p. 39): 'this subfamily (Chthamalinae) is so closely related to the ancient genera *Pollicipes* and *Scalpellum,* whence all the Thoracic Cirripedia may be said to radiate'. Darwin (1854) proposed that *Catophragmus*, having eight plates and whorls of imbricating plates, displays the basic form among the Balanomorpha. *Catophragmus s.l.* fossils are the oldest balanomorphs, known from the late Cretaceous period. The structure of their plates represents the most plesiomorphic feature found in the Balanomorpha. The chthamaloids show other plesiomorphic characters, such as a membranous basis and a scalpelloid-like oral cone and mouthparts, the labrum is bullate, palps are simple and the mandibles are tri- or quadridentoid with well developed incisor tooth and strong setose or pectinated molar process (Anderson, 1983). In the chthamaloids, only the first and second cirri are modified to serve as maxillipeds and assist the transfer of food captured by the four posterior pairs of cirri. In contrast, in the other balanomorphs the three anterior pairs serve as maxillipeds. Based on comparative functional morphology, Anderson (1983) re-stated the hypothesis of evolution of catophragmid balanomorphs from calanticine scalpellids.

The line of diversification within the Chthamaloidea included the loss of the whorl of imbricating plates of *Catophragmus* and *Catomerus*, and the reduction of plate number from eight in *Octomeris* to six in *Chthamalus* and *Euraphia*. A further reduction in the number of wall plates, owing to plate fusion, is evident in

*Corresponding author. E-mail: achity@mail.bi.ac.il

the four-plated genera *Tetrachthamalus, Chamaesipho* and *Jehlius* (Zullo, 1963; Newman, Zullo & Withers, 1969; Ross, 1971). Newman (1996) divided the superfamily Chthamaloidea into two families: the more plesiomorphic Catophragmatidae, including two genera *Catomerus* and *Catophragmus*; and the Chthamalidae, which he subdivided into three subfamilies: Euraphiinae that includes *Octomeris* and *Euraphia,* Notochthamalinae that includes *Chamaesipho,* and the Chthamalinae with *Chthamalus* and *Tetrachthamalus*.

Spears, Abele & Applegate (1994) were the first to apply molecular tools to study the phylogenetic relationship within the cirripedes, using the 18S rDNA sequence. Their study was followed by the research of Mizrahi *et al*. (1998) and Perl-Treves *et al*. (2000), with the addition of three suborders to the analysis; their data were re-analysed by Pérez-Losada *et al*. (2002). Spears *et al*. (1994) and Harris *et al*. (2000) noted that the 18S rDNA is not suitable for resolving closely related taxa, such as species within a genus, because of its high conservation. Other genes were used for the separation of barnacle species, or populations within the same species. The sequence of the gene encoding cytochrome *c* oxidase subunit I (COI) has been widely used to distinguish the interspecific and intraspecific patterns of evolution in a variety of organisms (Folmer *et al*., 1994). This mitochondrial gene has the advantages of large regions of variable sequences, spanning about 300–500 bases, between highly conserved regions. van Syoc, 1995) used the sequence of this gene to determine the genetic relationships among populations of *Pollicipes elegans* Lesson from the western coast of America, and produced a phylogeny of three species of the genus *Pollicipes.* The COI gene was also used to construct cladograms of the three species of *Tetraclita* (Hasegawa *et al*., 1996) and six species of the *Balanus amphitrite* Darwin complex found in Japan (Puspasari, Yamaguchi & Kojima, 2001). Wares (2001) used the sequence of CO1 together with 16S rRNA to infer the age and pattern of speciation in North American species of *Chthamalus*. Mokady *et al*. (1999), using 12S rDNA analysis and micromorphology, showed that the three varieties of *Trevathana dentata* (Darwin) recognized by Darwin (1854) cluster according to their host species. In another study, Mokady *et al*. (2000) used 12S rDNA and CO1 for intra and interspecific comparison of populations of *Chthamalus anisopoma* Pilsbry from Mexico.

In this study, we used molecular tools to examine the phylogenetic relationship within the chthamaloids. Partial sequences of the mitochondrial genes encoding COI and 16S rDNA were analysed in eight taxa of chthamaloid barnacles using the scalpelloid *Calantica* as an out-group.

MATERIAL AND METHODS

ANIMALS

Eight species of chthamaloids were used for this analysis. The list of species used for analysis and collection sites is given in Table 1. We selected *Calantica spinosa* as an out-group. The animals were dissected immedi-

Table 1. Material used for DNA extraction and sequencing

Species	GenBank accession number	Locality
Calantica spinosa (Quoy & Gaimard)	COI: AY428047	Timaru, New Zealand
	16S: AY428051	
Catomerus polymerus (Darwin)	COI: AY428048	Sydney, Australia
	16S: AY428045	
Octomeris angulosa Sowerby	COI: AY428049	Cape Town, South Africa
	16S: AY428042	
Octomeris brunnea Darwin	COI: AY430812	Phuket Island, Thailand
	16S: AY428041	
Chthamalus stellatus (Poli)	COI: AY392451	Michmoret, Israel
	16S: AY394087	Sdot-Yam, Israel
	16S: AY428039	Plymouth, UK
Euraphia depressa (Poli)	COI: AY428050	Michmoret, Israel
	16S: AY428040	Pantelleria, Italy
Euraphia withersi (Pilsbry)	COI: AY430814	Townsville, Queensland, Australia
	16S: AY428043	
Tetrachthamalus oblitteratus Newman	COI: AY430813	Eilat, Red Sea, Israel
	16S: AY428044	
Chamaesipho brunnea Moore	COI: AY430811	Auckland, New Zealand
	16S: AY428046	

ately after collection and preserved in 95% ethanol. For the analysis of 16S rDNA from *Chthamalus stellatus* we used material from two localities: Sdot-Yam, Israel, and Plymouth, UK.

DNA PREPARATION AND AMPLIFICATION

DNA was prepared from alcohol-preserved specimens according to a protocol modified from Dellaporta, Wood & Hicks (1983) and previously used by us (Mizrahi *et al*., 1998). Fifty nanograms of DNA were used for amplification by the polymerase chain reaction (PCR) (Saiki *et al*., 1988). Forward and reverse primers used to amplify the 16S rDNA gene fragments were 16SAR/SBR, 5′-CGCCTGTTTAACAAAAACAT-3′ and 5′-CCGGTTTGAACTCAGATCATGT-3′ (Palumbi, 1996). For the COI gene fragment, the 'universal' primers of Folmer *et al*. (1994) were used; HCOI2198, 5′-TAAAC TTCAGGGTGACCAAAAAATCA-3′, and LCOI1490, 5′-GGTCAACAAATCATAAAGATATTGG-3′. Amplification was carried out in a Crocodyle II thermocycler (Appligene Cie, Illkirch, Germany) by performing 29 cycles of 2 min at 92°C, 2 min at 54°C and 3 min at 72°C, followed by a final extension of 10 min at 72°C. PCR products were purified by centrifugation through a QIA quick spin column (Qiagen GmbH, Hilden, Germany).

DNA SEQUENCING

Forward and reverse sequencing of PCR products was performed at MBC Laboratories, Nes Ziona (Israel), using an ABI automated sequencer model and the *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, CA, USA). Sequences were subsequently manually inspected and edited using the BioEdit program.

SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS

The 16S DNA and COI sequences from eight chthamaloids and *Calantica spinosa* were multiply aligned using the ClustalX program (Thompson *et al*., 1997). Minor adjustments were made manually. PAUP 4Beta was used for phylogenetic analyses. To determine the most appropriate model of evolution of these genes, we used the approach proposed by Posada & Crandall, 1998). Using the PAUP program, we obtained a preliminary phylogenetic tree using neighbour-joining and assuming the Jukes & Cantor (1969) model. Likelihood scores for various models of evolution were then calculated using this tree, and the raw data were compared statistically using Modeltest version 3.06 (Posada & Crandall, 1998). The most likely model of evolution selected by the program was used to derive phylograms using maximum parsimony, neighbour-joining and maximum likelihood estimates of the phylogenetic relationship, using 100 replicates heuristic search with random sequence addition. Data were bootstrapped for evaluation of confidence of results, using the BOOTSTRAP option of PAUP, with 100 replicates for maximum likelihood and 1000 replicates for maximum parsimony and neighbour-joining.

RESULTS

The 16S rDNA and COI gene fragments of about 650 bp were sequenced and deposited in the GenBank (for Accession numbers, see Table 1). The aligned sequences of 16S rDNA of eight chthamaloids and *Calantica spinolosa* were 479 bp long, of which 112 positions were informative. The alignment of the COI gene for the same taxa was 526 bp long, and 162 were parsimony-informative. The sequence alignments used for our analysis are available on the web site http://www.biu.ac.il/ls/staff/Achituv.

The best fit model of nucleotide substitution was selected using Modeltest (Posada & Crandall, 1998). The evolutionary model selected for the 16 rDNA sequence was TVM + G (Table 1). The model assumes unequal base frequencies (A = 0.3505; C = 0.1094; G = 0.1652; T +0.3749). The assumed substitution rate differences were found to be R(a) [A–C] = 0.7771; R(b) [A–G] = 7.4457; R(c) [A–T] = 2.3269; R(d) [C–G] = 0.0000; R(e) [C–T] = 7.4457; and R(f) [G–T] = 1.0000. Gamma distribution shape parameter = 0.2339.

The three methods of analysis, maximum parsimony (MP), neighbour-joining (NJ) and maximum likelihood (ML), resulted in similar phylograms. The heuristic search using MP found two most parsimonious trees with 399 steps (consistency index = 0.652; retention index = 0.335). The difference between the two trees involves the internal nodes between the two eight-plated chthamaloids and *Chamaesipho* and the internal nodes between *Chthamalus, Euraphia* and *Tetrachthamalus*. One of the two MP trees is presented in Figure 1. The topology of the NJ and ML trees is similar to that of the MP trees (Fig. 1). Bootstrap consensus trees for each of the three methods are shown in Figure 2. Bootstrap analysis did not support grouping of *Chamaesipho* and *Octomeris angulosa* in the same clade. The short branch that grouped *Chamaesipho* and *Octomeris angulosa* in Figure 1 'collapsed' in the bootstrap analyses and the two eight-plated barnacles and *Chamaesipho* were placed on separate branches, separated from *Catomerus*, the six-plated barnacles and *Tetrachthamalus*.

The evolutionary model selected by Modeltest for the COI gene was TVM + I + G (Table 2). The base frequencies were (A = 0.2947; C = 0.1625; G = 0.1356;

Table 2. Likelihood-ratio tests of models of molecular evolution of the 16S rDNA sequence of eight taxa of chthamaloids and *Calantica*, using Modeltest version 3.06 (Posada & Crandall, 1998)

Null hypothesis	Models compared	– ln L0	– ln L1	2 (lnL1–lnL0)	d.f.	*P*-value
Equal base frequencies	H_0: JC H_1: F81	2427	2366	121	3	< 0.000001
Ti = Tv	H_0: F81 H_1: HKY	2366	2328	76	1	< 0.000001
Equal Ti rates	H_0: HKY H_1: TrN	2328	2327	1.8	1	0.170458
Equal Tv rates	H_0: HKY H_1: TrN	2328	2316	22	1	< 0.000002
Only two Tv rates	H_0: K81uf H_1: TVM	2316	2294	45	2	< 0.000001
Equal rates among sites	H_0: TVM H_1: TVM+G	2294	2160	267	1	< 0.000001
No invariable sites	H_0: TVM+G H_1: TVM+I+G	2160	2157	4	1	0.015656

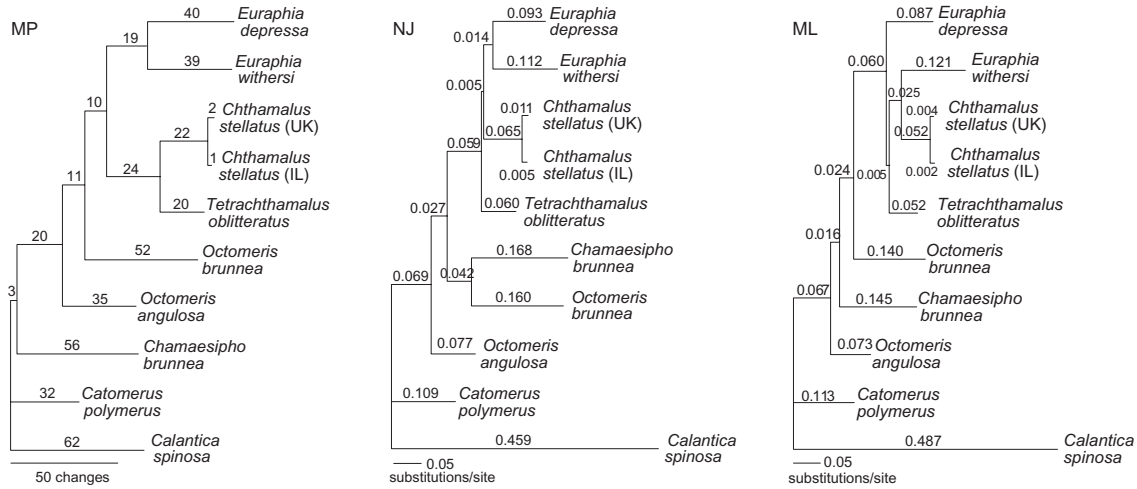

Figure 1. Maximum parsimony (MP), neighbour-joining (NJ) and maximum likelihood (ML) estimates of phylogenetic relationships among eight chthamaloids, based on 16S rDNA sequences. There are two samples of *Chthamalus stellatus*, one from Sdot Yam, Israel, and one from Plymouth, UK. Numbers above the branches indicates the amount of changes along the branch, and branch length is proportional to these changes.

and T = 0.4072). The assumed substitution rate differences were R(a) [A–C] = 0.0000; R(b) [A–G] = 17.2948; R(c)[A–T] = 6.2544; R(d) [C–G] = 0.3055; R(e) [C–T] = 17.2948; and R(f)[G–T] = 1.0000. Proportion of invariable sites was 0.6078, with a gamma distribution parameter of 6.9347. All three methods of analysis, MP, NJ and ML, and their bootstrap consensus trees, grouped *Catomerous polymerus* and *Octomeris angulosa* in the same clade, separated from all other chthamaloids. Apart from this, the three different methods of analysis yielded different trees that also differed from those obtained by 16S rDNA analyses (not shown). Parsimony analysis resulted in one most parsimonious tree with 523 steps (consistency index = 0.5430; retention index = 0.2240). In this tree, *Octomeris brunnea* and *Chamaesipho brunnea* clustered with the six-plated chthamaloids and *Tetrachthamalus,* but bootstrap analysis did not support this tree and dissolved the grouping of the other chthamaloids, placing *Octomeris brunnea* and *Chamaesipho brunnea* on two separate branches stemming from the basal branch, while the six-plated chthamaloids and *Tetrachthamalus* were found on another clade. Bootstrap analysis collapsed the basal

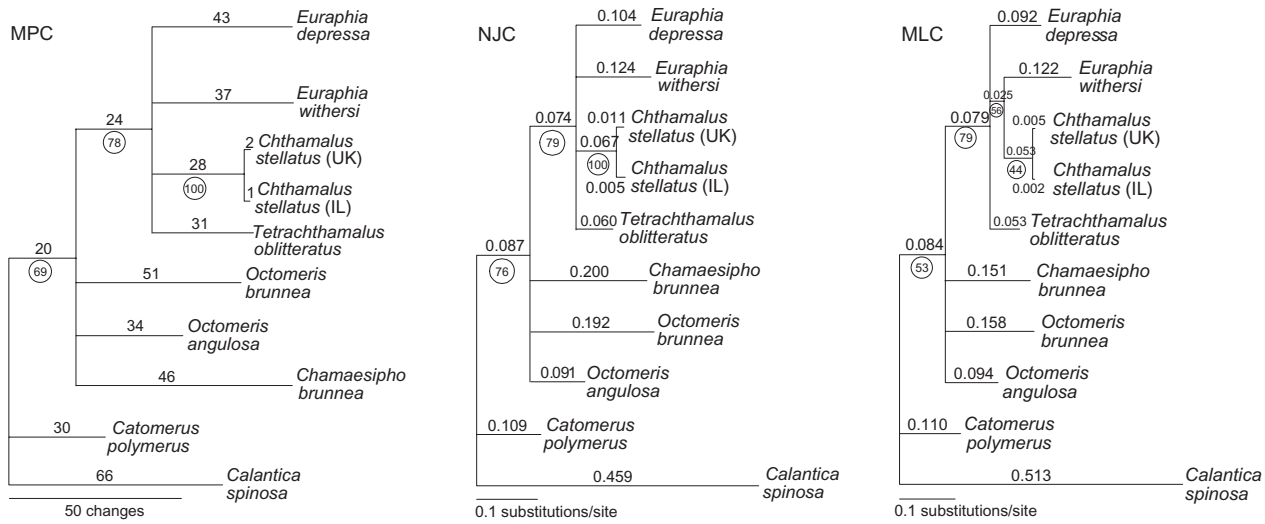

Figure 2. Maximum parsimony consensus (MPC), neighbour-joining consensus (NJC) and maximum likelihood consensus (MLC) estimates of phylogenetic relationships among eight chthamaloids based on 16S rDNA sequences. There are two samples of *Chthamalus stellatus*, one from Sdot Yam, Israel, and the other from Plymouth, UK. Numbers above the branches indicates the amount of changes along the branch, and branch length is proportional to these changes. Bootstrap values based on 1000 replicates for MPC and NJC and 100 replicates for MLC are shown as a percentage in circles below the branches.

branches and the phylograms that resulted were phylogenetically meaningless.

We concluded that in the chthamaolids, at intergeneric level, COI was saturated with substitutions beyond phylogenetic information. This assumption is supported by the high proportion of polymorphic sites, the rather short basal branches and long-terminal branches, and the collapse of the basal branches in the bootstrap analyses. Examining the alignment used for our COI analysis, we found that most of the changes were at the third position within the codon. Among the third codon positions there were only 12 invariable sites among all nine taxa, while 163 were polymorphic. Of these, 93 positions had two different characters at the same site (of which 67 were transitions and 26 transversions), 62 cases had three different bases at the same position, and in eight cases all four nucleotides were present among the nine taxa. There were also 27 cases of polymorphism in the first codon position and not a single polymorphism in the second codon position. The vast majority of these substitutions were, however, silent and did not affect the amino acid sequence of the encoded protein. The alignment that we used encodes 175 amino acid residues (http://www.biu.ac.il/ls/staff/Achituv). Within the 175 amino acid residues, the out-group *Calantica* differed from the chthamaloids studied by only two amino acids. Within the chthamaloids, *Euraphia withersi* and *Chamaesipho brunnea* each differed by a single amino acid from the other chthamaloids, showing that this gene fragment is highly conserved at the amino acid level but very diverged at the nucleotide level.

DISCUSSION

The phylograms based on 16S rDNA are generally consistent with the traditional phylogeny of the Chthamaloidea, but there are contradictions with some of the details of the traditional phylogeny that depend on strong morphological evidence. The topology of the phylograms does not support the subdivision of the Chthamalidae into three subfamilies as suggested by Newman (1996). Anderson (1983), using comparative functional morphology, tested and restated the hypothesis of scalpelloid ancestry of *Catomerus*. Ross & Newman (2001) affirmed that the catophragmatid barnacles retain plesiomorphic attributes, such as the whorls of basal imbricating plates and eight wall plates. The three methods of analysis of 16S rDNA and COI support the basal position of *Catomerus* within the chthamaloids. In the 16S rDNA analyses, the two eight-plated chthamaloids, *Octomeris angulosa* and *O. brunnea,* and four-plated *Chamaesipho*, were found on the same clade. The six-plated taxa *Chthamalus* and *Euraphia,* and the four-plated *Tetrachthamalus,* were found on a separate clade, indicating a sister group relationship between the two clades. The separation of the chthamaloids into two lineages can be supported by a morphological character, the lack of caudal appendages in *Catomerus* and *Octomeris*. In the NJ tree, the two eight-plated

chthamaloids *Octomeris brunnea* and *O. angulosa* were found on two separate, but close, clades. *Chamaesipho* was found on the same clade as *O. brunnea*. Bootstrap analyses do not support the common node of *Chamaesipho* and *O. brunnea*, and place the two *Octomeris* and *Chamaesipho* at a rather basal position of the tree, separated from the other four-plated chthamaloid *Tetrachthamalus*. This may indicate that reduction in the number of plates may have occurred at least twice within this family. This finding, however, does not agree with the traditional notion (Ross, 1971; Newman, 1996) that the three genera with four-plated wall evolved from the six-plated following a reduction of plate number. The separation between *Chamaesipho* and *Tetrachthamalus* is probably reflected in the different mechanisms of reduction in the number of wall plates in these two genera. In *Tetrachthamalus,* the reduction in wall plate number was attained by fusion of the rostrolateral plates with the rostrum, whereas in *Chamaesipho*, the rostrolateral plates fused with the lateral ones (Ross, 1971).

Within the clade of the six-plated chthamaloids and *Tetrachthamalus,* all three methods of analysis of 16S rDNA sequence supported the basal position of *Euraphia*; the distance between *Euraphia* and *Tetrachthamalus* was smaller than between this taxon and *Chthamalus*. It appears therefore that *Tetrachthamalus* is an offshoot of *Euraphia* as can be concluded from the bootstrap consensus trees. This however, contradicts the morphological data. Based on general morphology, Ross (1971) concluded that *Tetrachthamalus* is 'an offshoot of *Chthamalus*'. The external appearance of *Tetrachthamalus* is closer to *Chthamalus*; *Tetrachthamalus* like *Chthamalus* has a quadridentate mandible, whereas *Euraphia* has a tridentate mandible.

The cladograms based on 16S rDNA show slight differences between *Chthamalus stellatus* from Plymouth, UK and from Israel. These data are consistent with the findings of Pannacciulli, Bishop & Hawkins (1997) that showed genetic variations in terms of heterozygosity and allelic diversity when comparing the Mediterranean and eastern Atlantic populations of *Chthamalus stellatus*.

The analyses of COI resulted in phylogenetically meaningless trees, with most branches stemming from the common basal node. The COI gene encodes a subunit of cytochrome oxidase, which is a part of the electron transport chain. Its amino acid sequence is highly conserved across phyla. Thus, only silent changes that do not affect the protein structure are allowed. The third position of codons evolves faster than the first and second positions, as many third position substitutions are silent and do not alter the amino acid sequence. It seems that COI is not informative at the higher taxonomic level due to the fast rate of nucleotide substitutions that do not affect the encoded protein. Palumbi (1996) suggests that the amino acid sequences of COI are useful only in phylogenetic construction of deep evolutionary branches. Wares (2001) found that in the North American species of *Chthamalus* 42% of third position characters were polymorphic, and that there is little bootstrap support for the deeper nodes of phylogeny. He concluded that the third position appeared to be strongly saturated with mutations, recommending their exclusion from further phylogenetic analysis. Wares (2001) found that in North American *Chthamalus* the rate of genetic divergence of the 16S rDNA gene is 0.67% divergence/million years, while that of COI is about five times faster, at 3.1% divergence/million years. However, some authors have used the COI gene in Cirripedia to elucidate the phylogenetic relationship between closely related species or to examine populations of the same species (van Syoc, 1995; Hasegawa *et al*., 1996; Puspasari *et al*., 2001). In *Pollicipes* van Syoc (1995) used a 540 bp long sequence encoding 280 amino acids, and reported that 38% of the third position characters were polymorphic. Hasegawa *et al*. (1996) studied three species of *Tetraclita* and reported 42% polymorphism at the third position nucleotide and no difference in the amino acid sequences; *Tetraclita* species differed from the out-group, *Capitulum mitella,* by only four amino acid substitutions. Puspasari *et al*. (2001) analysed six species of the *Balanus amphitrite* complex found in Japan and reported 62.5% polymorphism in a 552 bp segment, but in the *Balanus amphitrite* complex these substitutions were reflected in the amino acid composition.

The high rate of substitutions in COI, mainly in the third position of the codons, indicates that this position is strongly saturated beyond phylogenetic information and casts some doubt on the validity of using this gene to study the phylogeny of cirripedes, particularly at higher taxonomic levels.

ACKNOWLEDGEMENTS

The sample of *Octomeris angulosa* used in this study was collected and sent to us by Prof. G. Branch, Department of Zoology, Cape Town University, and we thank him for his help. We thank Dr R. Perl-Treves, Bar Ilan University, for helpful comments on this manuscript. This study was supported by a grant from the Committee for advancement of Research, Bar Ilan University.

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