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Phylogeny and divergence time estimation of cheilostome bryozoans based on mitochodrial 16S rRNA sequences

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Abstract **The mitochondrial 16S rDNA sequences of 40 species of cheilostome bryozoans including those of 24 species newly determined were used to reconstruct the phylogenetic tree using neighboring-joining and maximum-parsimony methods. By applying molecular clock technique on the basis of the appropriate phylogeny and the fossil record, the divergence times of the two main cheilostome groups, Anasca and Ascophora *sensu stricto*, were estimated. The results show that the molecular phylogeny of the higher taxonomic groups (superfamilies and higher taxa) of cheilostome bryozoans is mostly in conflict with the morphology-based phylogenetic trees; the divergence of the extant groups of Anasca and those of Ascophora *sensu stricto* is estimated to have happened about 263 Ma (Permian Guadalupian Epoch) and 183 Ma (Early Jurassic), respectively.**

Keywords: bryozoans, cheilostomes, 16S rRNA, molecular phylogeny, phylochronology.

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Cheilsotomes comprise the dominant group of bryozoans, which contains more than four thousand species, accounting for about 95% of the extant marine bryozoans. Their colonies are highly variable in shape and mostly calcified to a more or less extent. Cheilostome zooecia are typically box-shaped with frontal orifice closed by the operculum. The classification of cheilostomes has been, to the best of our knowledge, established almost exclusively on the basis of the morphological features and remains controversial among bryozoologists[1—4]. At present, the higher taxonomy scheme commonly recognized by the International Bryozoologists Association (IBA) is the one proposed by Gorden[2,3]. However, some taxonomic problems at the levels of suborder and family, especially within suborder Neocheilostomina, remain unresolved[4], and for this reason some other criteria such as molecular sequence data are needed.

16S rRNA gene is the large subunit ribosomal RNA encoding gene of mitochondrial DNA, and has been widely used in the molecular phylogenetic analyses of many metazoan groups in recent years owing to its abundance in animal tissues and moderate evolutionary rate[5—7]. As for bryozoans, only one study using 16S rDNA sequence data has been reported[8], in which preliminary phylogenetic analyses of gymnolaemates including Cyclostomida, Ctenostomida and Cheilostomida were made. In the present study, new sequence data of 24 species and sequences of other 16 species from GenBank were combined to reconstruct their molecular phylogenetic trees by using neighboring-joining and maximum-parsimony methods, aiming to reveal their phylogenetic relationships. In recent more than ten years, some progresses have been made in organism groups' origin and divergence estimation[9—11], however, little studies have been done about the lower metazoan groups. In this study, we tried to construct the 16S rDNA molecular clock based on evolutionary distance and select fossil records to estimate the divergence time of their main lineages.

1 Materials and methods

1.1 Cheilostome bryozoans used in this study and their sources (Table 1)

1.2 Isolation and purification of total genomic DNA

About 0.5 g clean bryozoan material was ground into powder and put into one 10 mL centrifuge tube containing 2.0 mL DNA isolation buffer (0.5% SDS, 15 mmol/L EDTA, 5 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.6) and 20 μL Proteinase K (20 μg/mL), bathed at 55℃ for 2—3 h, centrifuged at 4000 rpm for a few seconds; then about 400 μL of the supernant was removed into one 1.5 mL Eppendorf tube containing 500 μL 8 mol/L GuSCN and 100 μL 50% clean glass milk liquor, bathed at 37℃ for 2—3 h; centrifuged at 4000 rpm for 2 min, the supernatant was removed , and the sediments were cleaned with 70% alcohol twice, acetone once, dried in a vacuum concentrator at 45℃ for about 15 min; then 100 μL of TE (10 mmol/L Tris-Cl, 1 mmol/L EDTA, pH 8.0) was bathed at 55℃ for 30 min, centrifuged at 8000 rpm for 2 min. Supernatant was removed and preserved at –20℃ till use.

1.3 The designing and synthesizing of primers

One pair of universal oligonucleotide primers for metazoans[12]: 16Sa (5′-CGCCTGTTTATCAAAAACAT-3′) and 16Sb (5′-CCGGTCTGAACTCAGAT-3′) were synthesized by Shanghai Sangon Biotechnology Company Ltd.

1.4 PCR amplification, DNA sequencing and Phylogenetic analyses

PCR was performed in 50 μL volumes containing about 5—10 ng DNA, 10×Buffer, 2.5 mmol/L $MgCl_2$, 0.1 μmol/L each dNTP, 0.1 μmol/L each primer, 5% BSA and

Table 1 List of cheilostome bryozoan species used in this study

Suborder	Infraorder	Superfamily	Family	Spcies	Sources and Acession No. of GenBank
Ascophorina	Lepraliomorpha	Schizoporelloidea	Schizoporellidae	***Schizoporella erratoidea****	Dongshan Island, Fujian Prov, China (AY789102)
				Schizoporella sp.	Gulf Specimen Co., Florida, USA (AF156296)
			Smittinidae	***Smittoidea spinigera****	Dongshan Island, Fujian Prov, China (AY789104)
				Parasmittina sp.*	Dongshan Island, Fujian Prov, China (AY789098)
			Bitectiporidae	***Schizomavella acuta***	Pacific Bio-Marine,California, USA (AF156289)
				Schizomavella australis*	Xinchun Port, Hainan Province, China (AY789100)
				Schizomavella sp.*	Xinchun Port, Hainan Province, China (AY789101)
			Lanceoporidae	*Calypotheca* sp.*	Dongshan Island, Fujian Prov, China (AY789090)
			Watersiporidae	***Watersipora subtorquata****	South Australia Museum, Australia (AY789108)
				Watersipora arcuata*	South Australia Museum, Australia (AY789107)
			Microporellidae	***Microporella ciliata***	Pacific Bio-Marine, California, USA (AF156286)
		Celleporoidea	Phidoloporidae	*Phidolopora pacifica*	Pacific Bio-Marine, California,, USA (AF156287)
			Cleidochasmatidae	*Cleidochasma* sp.*	Xinchun Port, Hainan Province, China (AY789094)
			Celleporidae	*Celleporina souleae*	Pacific Bio-Marine, California, USA (AF156279)
	Umbonulomorpha	Umbonuloidea	Lepraliellidae	*Celleporaria aperta**	Xinchun Port, Hainan Prov., China (AY789093)
				Celleporaria magnifica	Gulf Spcimen Co.,Florida, USA (AF156277)
				Celleporaria sp.*	Linchang Island, Hainan Prov., China (AY789092)
	Hippothoomorpha	Hippothoidea	Hippothoidae	*Celleporella hyaline*	California , USA (AF155939)
				Celleporella sp.	Pacific Bio-Marine, California, USA (AF156278)
	Cribriomorpha	Cantenicelloidea	Savigniellidae	*Savigniella lafontii**	Xinchun Port, Hainan Province, China (AY789099)
		Cribrilinoidea	Cribrilinidae	*Cribrilina annulata*	Pemaquid Neck, Maine, USA (AF156280)
				Reginella hippocrepis	Pemaquid Neck, Maine,USA (AF156288)
Neo-cheilostomina	Cellulariomorpha	Candioidea	Candidae	*Scrupocellaria maderensis**	Xinchun Port, Hainan Province, China (AY789103)
				Scrupocellaria varians	Pacific Bio-Marine, California, USA (AF156291)
				*Tricellaria monotrypa**	South Australia Museum, Australia (AY789106)
		Bugulioidea	Bugulidae	*Bugula stolonifera**	Dongshan Island, Fujian Prov, China (AY789087)
				*Bugula neritina**	Dachen Island, Zhejiang Prov,China (AY789086)
			Beaniidae	*Beania magellanica**	The south Australia Museum, Australia (AY789085)
	Cryptocystomorpha	Microporoidea	Thalamorphorelli-dae	*Thalamoporella californica*	Pacific Bio-Marine, California, USA (AF156293)
			Steginoporellidae	*Steginoporella truncata**	South Australia Museum, Australia (AY789105)
			Onychocellidae	*Onychocella* sp.*	Xinchun Port, Hainan Province, China (AY789097)
		Cellarioidea	Cellariidae	*Cellaria immersa**	South Australia Museum, Australia (AY789091)
				Cellaria mandibulata	Pacific Bio-Marine, California, USA (AF156276)
	Psudomalacostegom orpha	Calloporoidea	Calloporidae	*Callopora* sp.*	Xinchun Port, Hainan Province, China (AY789089)
			Cupuladriidae	*Discoporella* sp.*	Isthmus of Panama, USA (AY123621)
				Cupuladria surinamensis	Isthmus of Panama, USA (AY123498)
			Flustridae	*Antropora tincta*	Pacific Bio-Marine, California, USA (AF156274)
Malacostegina			Membraniporidae	*Membranipora grandicella**	Dongshan Island, Fujian Prov, China (AY789096)
				*Membranipora tuberculata**	Sanya Port, Hainan Province, China (AY789095)
			Electridae	*Electra bellula**	Dongshan Island, Fujian Prov, China (AY789088)

1.0 units Taq DNA polymerase using the standard technique. The cycling parameters were as follows: pre-denaturation at 94℃ for 10 min; then for a total of 40 cycles: denaturation at 94℃ for 1 min; annealing at 55℃ for 45 s and extension at 72℃ for 2 min; final extension at 72℃ for 10 min. PCR products were stored at –20℃ until use. The PCR products were purified with the PCR purification mini kit (Shanghai Watson Biotechnology Company Ltd.). The purified DNA amplification fragments were directly cloned into the bacteriophage M13 vector, then 1—2 μL of the ligation reaction were transformed into the commercially prepared HB101 *E. coli* bacterial cells. Positive colonies identified by antibiotic resistance and blue/white selection were cultured in LB medium. DNA preparations from 24 positive clones were extracted and sequenced with the universal M13 primers (reverse 5′-TTCACACAGGAAAA-3′) on an ABI-377 automated DNA sequencer.

The sequences we determined were verified through Blast analysis of GenBank databases and sequence alignment was performed using ClustalX[13]. For the reason that ctenostomes and cheilostomes belong to the same class

and may be sister to each other on the criteria of some morphological and a few molecular characters, so ctenostome *Farrella repens* (GenBank Accession No. AF156283) was selected as the outgroup[14], and the molecular trees were reconstructed in MEGA (version 2.0)[15] and PHYLIP (version 3.5c)[16] software packages using neighbour-joining (NJ)[17] and maximum-parsimony (MP) methods[18]. NJ analysis is based on the distance model of Kimura's- two parameters[19]. MP analysis was done using a branch-and-bound search. Bootstrap analyses (500 replicates) with internal branch test were performed to estimate the support levels for the nodes in the resultant topology from both the NJ and MP analyses. Gaps were treated as missing data in all analyses in this study.

1.5 Divergence time estimation

The NJ tree constructed by MEGA software was used to conduct the relative rate tests with Tajima's non-parameter method[20]. The taxa (branches) that failed in the tests (cutoff value $[p] = 0.001$) were excluded and those that passed were used in divergence time estimation. The genetic distances between lineage pairs were calculated from the Kimura-2α, Jukes-Cantor[21], and Tamura-Nei[22] models. The evolutionary rate of 16S rRNA gene was then estimated by the following formula:

$$r_r = h_r / t_r.$$

where h_r is the node height of the selected reference taxon, expressed as

$$h_r = \frac{\sum_{i=1}^{|A|}\sum_{j=1}^{|B|} d_{ij}}{2\,|A|\,|B|}$$

A and B were the number of sequences in clade A and clade B respectively; t_r is the first fossil emergence time of the reference taxon we selected.

Under the condition of the relative rate test, we assumed that there exists the molecular clock of this small natural groups. Then h_u was calculated on the same grounds as before and t_u was calculated using the formula: $t_u = h_u / r_r$.

2 Results

2.1 Sequences and sequence alignment

The 16S sequence lengths of the 24 bryozoan species we sequenced were between 547 and 583 bp. All the sequences of 40 species including the outgroup were aligned for phylogenetic analysis. The total length of the aligned sequences is 620 bp, of which 120 are conserved, 480 variable, 359 parsimony informative. Furthermore, the sequence alignment with five hypervariable regions (61—67 bp, 251—257 bp, 295—319 bp, 362—378 bp, 403—421 bp) excluded was also done, the resultant alignment sequence being 543 bp long with 120 conserved, 412 variable and 298 parsimony informative sites.

2.2 Phylogenetic analysis

The 40 species of cheilsotomes in this study belong to 3 suborders, 7 infraorders, 12 superfamilies and 25 families. The tree topologies obtained with different methods are similar except for a few minor differences. Here, only NJ tree is presented (Fig. 1), which is apparently in general agreement with the morphological classification schemes, although some discrepancies also exist, as will be discussed below.

The anascans, which was once considered as a natural group, is obviously polyphyletic according to our molecular analysis. There are obvious three main lineages in this polyphyletic clade: (i) *Callopora* sp. (superfamily Calloporoidea, infraorder Pseudomalacostegomorpha), *Thalamorporella californica*, *Steginoporella truncata* and *Onychocella* sp. (superfamily Microporidea, infraorder Cryptocystomorpha) of suborder Neocheilostomina; the three species *Membranipora grandicella*, *Membranipora tuberculata* and *Electra bellula* (superfamily Membraniporoidea) of suborder Malacostegomorphina constitute one lineage, within which four species of suborder Neocheilostomina is the sister group of three species of suborder Malacostegomorphina; (ii) *Cellaria mandibulata* and *Cellaria immersa* (superfamily Cellaroidea, infraorder Cryptocystomorpha), *Scrupocellaria manderensis*, *Scrupocellaria varians* and *Tricellaria monotrypa* (superfamily Candioidea, infraorder Cryptocystomorpha), *Antropora tincta* (superfamily Calloporoidea, infraorder Pseudomalacostegomorpha); *Bugula neritina*, *Bugula stolonifera* and *Beania magellanica* (superfamily Buguloidea, infraorder Cellulariomorpha) of suborder Neocheilostomina constitute the second lineage, within which three species of infraorder Cellulariomorpha is sister to the others in this lineage; (iii) *Cupuladria surinamensis* and *Discoporella* sp. (family Cupuladridae, superfamily Calloporoidea, infraorder Pseudomalacostegomorpha) of suborder Neocheilostomina make up the third lineage (Fig. 1).

This study indicates that Ascophora is not a natural group, within which *Celleporella* sp. and *Celleporella hyaline* of family Hippothoidae make up a small lineage nested in the anascans; the other species makes up another large monophyletic lineage, namely Ascophora *sensu stricto* here. Within the latter, *Microporella ciliata* (family Microporellidae, infraorder Lepraliomorpha) is the basal branch. As for the relationships among these ascophorans except for *Microporella ciliata*, some patterns are incongruent with those of morphological results and therefore further analyses are needed to clarify these discrepancies (Fig. 1).

Interestingly, in the NJ and MP trees obtained, the third lineage of anascans (family Cupuladridae), which includes unique free-living bryozoans, forms a monophyletic group with Ascophora *sensu stricto*, probably indicating that this monophyletic group shares a common trochophore-like

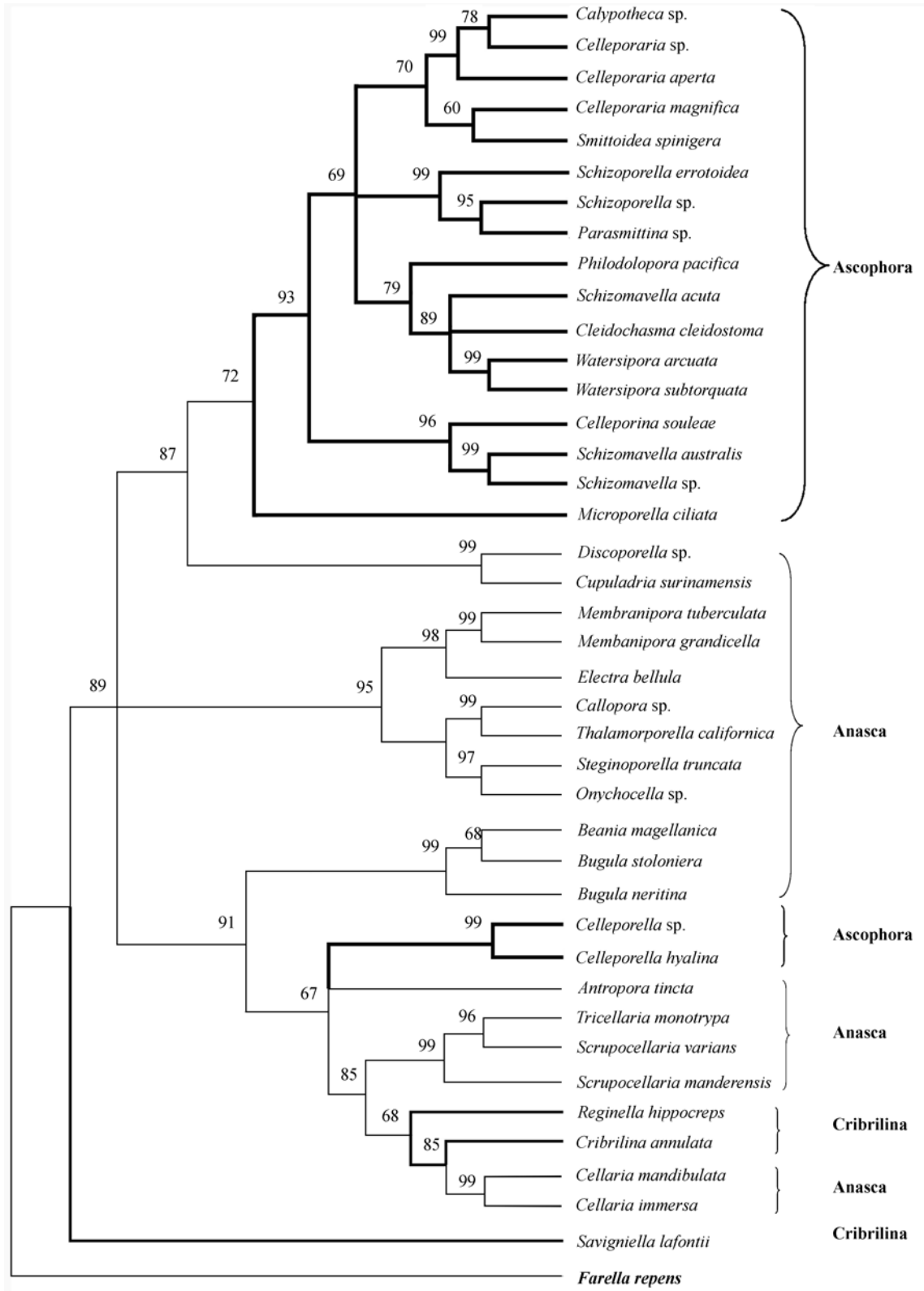

Fig. 1. The strict consensus tree of cheilostome bryozoans based on 16S rDNA data (NJ analysis, MEGA 2.0). Number on each node indicates the bootstrap values (500 replicates).

ancestor (Fig. 1).

This study shows *Savignella lafontii* (superfamily Cantenicelloidea) is basal to all cheilostomes according to their 16S rDNA sequence data (Fig. 1).

2.3 The divergence time estimation

After careful selection, the family Cupuladriidae with the earliest fossil records occurring at Danian Stage (Paleocene, about 65 Ma)[23] was used as the reference taxon. On the basis of the reference fossil date and the distances among the taxa used, the divergence times of Anasca (not including suborder Inovicellina and Scrupariina) and Ascophora (not including *Celleporella* of family Hippothoidae) were estimated. The results indicate that the Anasca originated during Guadalupian Period (Permian to Early Triassic, about 263 Ma) and the Ascophara *s. s.* diverged from Anasca during Toarcian Age (Early Jurassic, about 183 Ma) (Table 2 and Fig. 2).

Table 2 Divergence time estimated for Anasca and Ascophora *sensu stricto* of Cheilostomida based on 16S rDNA molecular data

Divergent node	Genetic distance	Evolutionary rate/Substitutions·site^{-1}·Ma^{-1}	Node height	Divergence time/Ma
Anasca	Kimura-2α	$5.85\pm1.23\times10^{-4}$	0.14351±0.01733	245.3±29.6
	Jukes-Cantor	$6.15\pm1.31\times10^{-4}$	0.18553±0.02683	282.5±39.5
	Tamura-Nei	$6.23\pm1.38\times10^{-4}$	0.16243±0.02771	260.7±44.5
Ascophora *s.s.*	Kimura-2α	$5.85\pm1.23\times10^{-4}$	0.10143±0.01337	173.4±22.9
	Jukes-Cantor	$6.15\pm1.31\times10^{-4}$	0.11410±0.01650	185.5±26.8
	Tamura-Nei	$6.23\pm1.38\times10^{-4}$	0.11870±0.01900	190.1±30.4

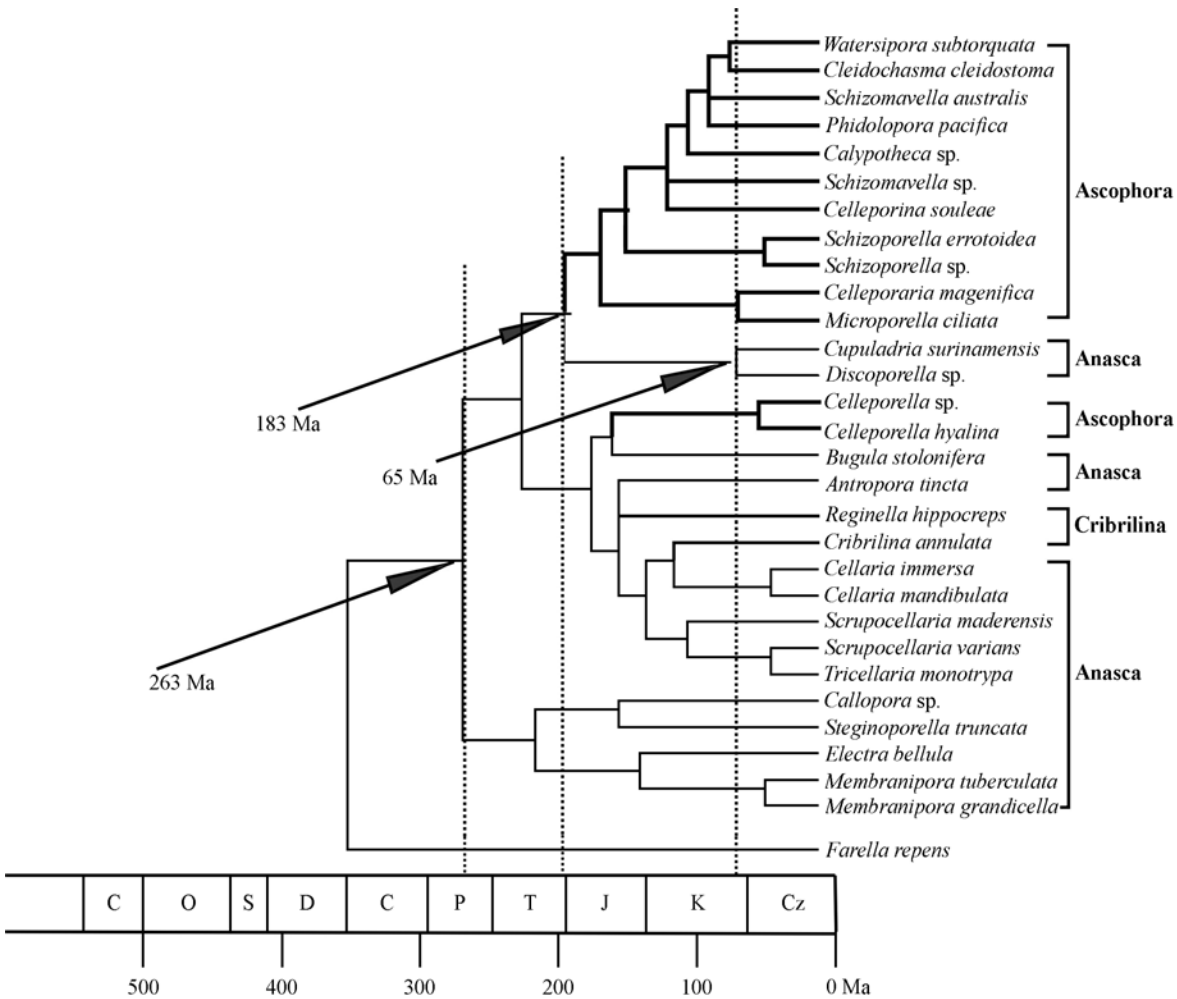

Fig. 2. The divergence time of Anasca and Ascophora *sensu stricto* estimated on the basis of 16S rDNA and calibrated with fossil record of Cupuladriidae.

3 Discussions

3.1 The phylogenetic relationships among the major groups

In general, the Cheilostomida was classified in two suborders (Anasca and Ascophora) or three suborders (Anasca, Cribriomorpha and Ascophora) according to the calcification degrees and types of their zooidal frontal membranes. These taxonomic schemes reflected the evolutionary trends of their zooecial hydrostatic regulating mechanism, but not their zooidal frontal wall developing types[4]. In a recent taxonomic scheme by Gorden[2,3], the Cribriomorpha was assigned to suborder Ascophora, and Ascophora was further divided into four infraorders: Cribriomorpha, Hippothoomorpha, Umbonulomorpha and Lepraliomorpha, whereas Anasca was classified into four suborders: Inovicellina, Scrupariina, Malastogiina and Neocheilostomina, the last one being further divided into three infraorders: Pseudomalacostegomorpha, Cellulariomorpha, Cryptocystomorpha. The results of our analyses mostly corroborate with their morphological classifications at the taxonomic levels of family and superfamily. However, on the levels of suborder and infraorder, there are some severe conflicts between them; thus, the higher taxonomic classification of cheilostomes mainly according to whether or no the frontal membrane was calcified is questionable.

As for Ascophora, morphologically, they have three different frontal wall developmental types (gymnocystal, umbonuloid and lepralioid)[24] which may reflect their genealogical differences[25]. The frontal wall of *Celleporella* is gymnocystal, but those of other ascophorans are either umbonuloid or lepralioid. Some researchers proposed that the latter two may have a common ancestor or lepralioid derived from umbonuloid[26,27]. Dick et al. showed that *Celleporella* sp. did not constitute a monophyletic group with other ascophorans; it appeared between *Reginella hippocreps* and *Thalamoporella californica* in the MP tree, and sister to *Reginella hippocreps* in the ML tree. Our analysis by adding another species of *Celleporella* shows similar result.

For Cribriomorpha, whose frontal membranes are covered by spine-like structures, the phylogenetic position has long been a topic of debate[1,4,28−31]. In morphological view, there are two kinds of frontal wall developmental types[5,6]: spinocystal for Cribrilinoidea and gymnocystal for Catenicelloidea. Our results show that Cribrilinoidea are imbedded within anascans; therefore, it is reasonable to assign them to anascans again. However, Catenicelloidea came out as a basal branch and close to the outgroup in the molecular trees, whereas it was imbedded within the ascophorans in MP trees (not shown here); therefore, their phylogenetic positions need to be further investigated.

3.2 The divergence time estimation

In the studies of molecular clock, it is critical to obtain a constant molecular evolutionary rate. Although molecular clock does not hold in an exact sense, it is reasonable to obtain a reliable one by some special methods, such as relative rate tests in a relatively small groups[32] exampled in this study. In our work the evolutionary rates of 16S rDNA of cheilostome bryozoans were shown to be consistent with those of another metazoan groups obtained by other researchers[7,13,15,33]. The divergence time of Anasca from the outgroup is estimated to be 245—282 Ma (Table 2, Permian to Early Triassic). However, their earliest fossil record (*Pyroporopsis portlandensis* of Electridae) is dated at Late Jurassic Tithonian time (ca. 145—150 Ma) and most of this group emerged during Albian (100—112 Ma, Early Cretaceous)[23,34]. Thus, the anascans apparently had evolved over 120 million years before they radiated in the middle Cretaceous. As for Ascophora *s. s.*, they diverged from anascans at about 173—190 Ma (Table 2, Early to Middle Jurassic), but their earliest fossil (*Boreasina nowickii* of Hippothoidae) is dated to be Turonian to Coniacian time (85—93 Ma, Late Cretaceous)[22], so they also underwent an evolutionary period of nearly 100 million years before they radiated during Late Cretaceous time.

3.3 The origin of Cheilostomida

Morphological studies indicate that Cheilostomida originated from Order Ctenostomida of Class Gymnolaemata, or they share a most recent common ancestor[35−37]. Some research workers postulated that Cheilostomida arose from genus *Salpingoidea* with avicularia-like structures of Cyclostomida, Class Stenostomata[38]. However, Xia and a few European and American researchers proposed that they may have descended from a new genus of Family Fenestraporidae, Order Fenestrida of Class Stenolaemata through analyzing *Aviculofenestella* from the Qoima-Co Formation (Bajocian, Middle Jurassic), North Tibet in China together with the *Fenestropora* from Devonian found in North America and Transcaucasia[39]. Although palaeontological views differ greatly, the divergence time of Cheilostomida in late Paleozoic on the ground of molecular phylogenetic dating in this study is in support of Xia's hypothesis.

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