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Combined morphological and molecular analyses of higher taxa in Ostracoda

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Abstract: 27 morphological characters and 18S rDNA sequences of the 9 main ostracod groups were analyzed by the maximum parsimony method to construct a consensus phylogenetic tree. The results indicate that punciidaen ostracods form a separate clade which may be placed under the subclass 'Punciocopa', with the same status as Podocopa and Myodocopa, but more evidence is still needed to confirm it. The classification status of halocypridian ostracods was undetermined because the topology was different on two phylogenetic trees. Among the three subclasses suggested in the study, Podocopa includes the suborders Cypridocopina, Cytherocopina, Bairdiocopina, Darwinulocopina and the family Cytherellidae; Myodocopa consists of at least two orders, Cladocopida and Myodocopida; and Punciocopa has only one family, Punciidae. This provides new evidence for solving the unstable higher classification of living ostracods.

Key words: Ostracoda; 18S rDNA; morphological characters; classification

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基于形态及分子性状对介形类高级分类体系的分析

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摘要: 运用最大简约法(MP)对介形纲中 9 个主要类群的 27 个形态性状及 18S rDNA 序列进行分析,并构建系统发育一致树,以期为尚存争议的现生介形类高级阶元分类提供新的证据.结果显示 punciidaen 自成一类,其分类地位与尾肢亚纲(Podocopa)和壮肢亚纲(Myodocopa)相当,可命名为"Punciocopa",但仍需更多的证据加以证实;而吸海萤类(halocypridian)由于基于形态与分子证据获得的拓扑结构不一致,其分类地位尚无法得以解决;在介形类 3 个亚纲中,尾肢亚纲应包括金星介(Cypridocopina)、浪花介(Cytherocopina)、巴氏介(Bairdiocopina)、达尔文介(Darwinulocopina)和泡沫介(Cytherellidae)5 个类群, 壮肢亚纲至少包括分肢介(Cladocopida)和壮肢介(Myodocopida)两个类群,而"Punciocopa"仅有 Punciidae 科.

关键词: 介形纲; 18S rDNA 序列; 形态性状; 分类

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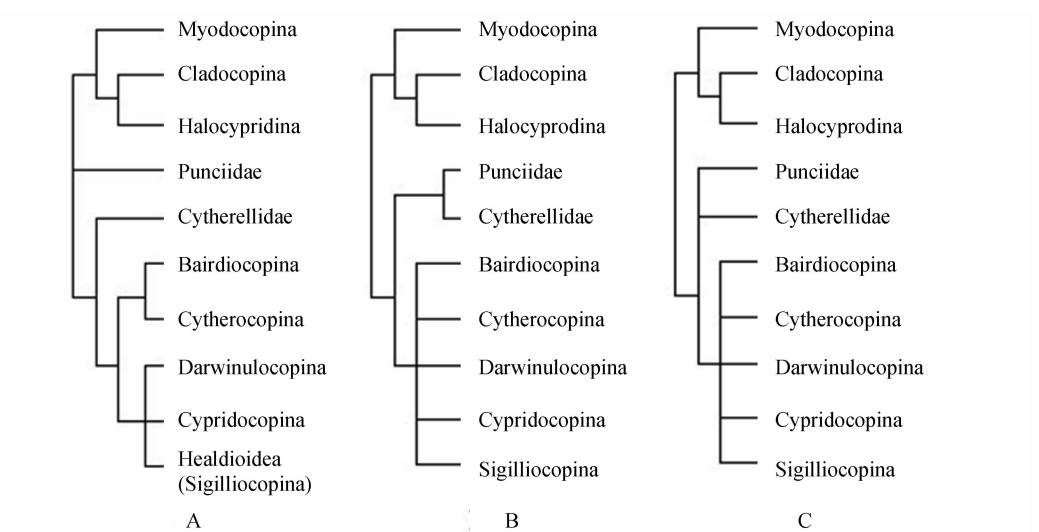
0 Introduction

The genus name *Cypris* was first proposed for freshwater European ostracods in 1776[1]. The history of ostracod studies has thus spanned more than 200 years. As early as 1865, Sars had divided the order Ostracoda into four suborders: Myodocopa, Cladocopa, Platycopa, and Podocopa[2]. Today, the taxon Ostracoda has been promoted to the rank of Class with more new species discovered. Because there are many similarities among the main groups of Ostracoda, many previous workers have considered ostracods to be a monophyletic assemblage. This perspective has been adopted here and is held by a majority of current workers in the field. A variety of classification systems have been produced, however, based on different morphological characters[3-6], which has led to an unstable and unreliable higher classification for ostracods. At present, three representative views based on morphological evidence have been widely adopted (see Fig. 1). All of these views agree that: ① the subclass Myodocopa includes the suborders Myodocopina, Cladocopina and Halocypridina; and ② the order Podocopida consists of the suborders Bairdiocopina, Cytherocopina, Darwinulocopina, Cypridocopina and Sigilliocopina. Furthermore, there are still some controversies on the status of the family Punciidae and the relationship between the family Cytherellidae and the order Podocopida (see Fig. 1).



Note: A. Bowman & Abele, 1982; B. Martin & Davis, 2001; C. Liebau, 2005

Fig. 1 The classification of higher taxa in Ostracoda

Early classifications of ostracods have always been based on morphological evidence, including the bivalve carapace and soft-body characters. Since Spears & Abele[7] suggested the phylogentic relationship of Crustacea which included a paraphyletic Ostracoda by analyzing the nucleotide sequences, some scholars have made important contribution to the

classification and phylogeny of ostracods based on the molecular evidences[8-10]. But faunal morphological characters are the result of the combined effect of genotype and environmental factors during the whole ontogenetic process, while the rate of evolution can be affected by these environmental factors[11]. Morphology could be misleading when structural similarity resulted from convergent evolution, while molecular tree estimation could also be misleading when the underlying sequence evolution assumptions violated actual sequence evolution parameters[12]. Thus, classifications have differed according to whether morphological or molecular characters were given higher weight[13]. In this paper, we present a tentative analysis to the classification of Ostracoda's higher taxa by putting together the morphological characters with the molecular characters in order to offer new evidence for the classification and phylogeny of Ostracoda.

1　Materials and methods

1.1　Materials

All 18S rDNA sequences of ostracods in the GenBank database were analyzed to find sequence divergences by the Kimura two-parameter method with Mega 4.0 software[14]. A total of 111 sequences had been submitted to the GenBank database as of August 2007, but we eliminated some sequences when assessing genetic distances because they were considered uncertain. We thus used 72 sequences, including 16 myodocopidan, 3 bairdiocopinan, 29 cytherocopinan, 2 darwinulocopinan, 18 cypridocopinan, 1 halocypridinan, 1 cladocopinan, 1 punciidan and 1 cytherellid Ostracoda. The distances among several groups were larger than all within-group distances, indicating that each main group was monophyletic. We thus selected a representative species from each group to reconstruct phylogenetic trees.

In this study, selected material included almost all taxa reported by Martin & Davis[5], but did not include the suborder Sigilliocopina because of incomplete classificatory and molecular data. *Philomedes* sp., *Conchoecia* sp., *Polycope* sp., *Neonesidea haikangensis*, *Tanella opima*, *Darwinula stevensoni*, *Cypridopsis adusta* and *Cytherelloidea munechikai* were selected to represent the suborders Myodocopina, Halocypridina, Cladocopina, Bairdiocopina, Cytherocopina, Darwinulocopina, Cypridocopina and the families Punciida and Cytherellidae, respectively (see Tab. 1).

1.2　Selection and analysis of morphological characters

Twenty-seven characters related to carapace and appendage morphology were used. These are listed in Table 2. The data matrix was analyzed using PAUP 4.0 software[17]. All characters were designated 'Dollo. Up', meaning that we assumed character states could change from plesiomorphic to apomorphic conditions, and they were irreversible[18]. All characters were given equal weight (1). Trees were built using the maximum parsimony (MP) and maximum likelihood (ML) methods with the branch-and-bound routine (furthest taxon input). Bootstrapping used the fast step-wise addition method with 1 000 rep-

licates.

Tab. 1 Samples used and DNA sequenced in the Study

Taxon	Species	Source of morphological data	GenBank accession Nos. of molecular data
Ostracoda			
Myodocopa			
Myodocopida			
Myodocopina	*Philomedes* sp.	East Sea, China	DQ531747
Halocyprida			
Halocypridina	*Conchoecia* sp.	East Sea, China	AF363296
Cladocopina	*Polycope* sp.	East Sea, China	AF363310
Podocopa			
Podocopida			
Bairdiocopina	*Neonesidea haikangensis*	Taiwan Sea, China	AY863437
Cytherocopina	*Tanella opima*	East Sea, China	AY863434
Darwinulocopina	*Darwinula stevensoni*	Taihu Lake, China	AY622197
Cypridocopina	*Cypridopsis adusta*	Shanghai, China	AY622193
Platycopida			
Punciidae	*Manawa staceyi*	Van Morkhoven, 1963[15]	AF363295
Cytherellidae	*Cytherelloidea munechikai*	Van Morkhoven, 1963[16]	AB076612

Tab. 2 Morphological characters of ostracods used in this study

Characters	Character states
Ⅰ. Carapace	
1. Incisure	Present (0) Absent (1)
2. Types of hinge	Adont (0) Merodont (1) Both (2)
3. Numbers of traces of adductor muscle	Not more than 6 (0) More than 6 (1)
4. Numbers of traces of antennule muscle	Usually one (0) Usually two (1)
5. Size of bivalves	Left > right (0) Right > Left (1) Equal (2)
Ⅱ. Frontal organ and Visual organs	
6. Frontal organ	Present (0) Absent (1)
7. Compound eyes	Present (0) Absent (1)
8. Larval eyes	Present (0) Absent (1)
Ⅲ. Antennule, first antenna	
9. Numbers of segment	Not more than 6-segmented (0) Not less than 6-segmented (1) Both (2)
10. Natatory setae	Well developed (0) Reduced (1)
Ⅳ. Antenna, second antenna	
11. Types	Biramous (0) Endopod or exopod missing, uniramous (1)
12. Numbers of endopodite segment	Not more than 3-segmented (0) 3 to 4-segmented (1)
Ⅴ. Mandible	
13. Number of segments	3 to 4-segments (0) Usually 5-segments (1)
14. Functions of first segment palpus	With branchial process (0) Without branchial process (1)
15. With branchial process	Present (0) Absent (1)
Ⅵ. Thoracic appendages	
16. Numbers	3 pairs (0) 2 pairs (1) 1 pair (2)
17. Function of first pair	Maxillipeds (0) Crawling legs (1)
18. Function of second pair	Maxillipeds (0) Crawling legs (1) Absent (2)
19. Function of third pair	Cleaning legs (0) Crawling legs (1) Absent (2)

续表 2

Characters	Character states
Ⅶ. Caudal furca (Based on the assumption that the furca in all ostracod taxa is homologus)	
20. Shaped	Laminar (0)　Stick-shaped (1)　Absent (2)
21. On phase A-7 of ontogeny	Producing one claw (0)　Producing 2 claws (1)　Not change (2)
22. With slender bristle-like protuberance which has only half long of claw on phase	Present (0)　Absent (0)
Ⅷ. Heart	
23. Heart	Absent (0)　Present (1)
Ⅸ. Sex organs and Procreation	
24. Position of gonads	Located within body (0)　Located between valves of shell (1)
25. Numbers of tubules of seminal receptacle	Two (0)　One (1)
26. Types of reproduction	Amphigenesis (0)　Parthenogenesis (1)　Mixed types of reproduction (2)
27. A-8 phase of ontogeny	With 3 pairs of appendages (0)　With 4 pairs of appendages (1)

1.3　18S rDNA sequencing and analyses

We conducted our own sequencing of the 18S rDNA of all experimental species except *Polycope* sp., *Conchoecia* sp., *M. staceyi* and *C. munechikai*. For each species, a single fresh specimen was used for DNA preparation. Genomic DNA was prepared by grinding the bodies in a microcentrifuge tube with digesting solution, and heated at 50 ℃ for 4 h and 94 ℃ for 4 min. DNA samples were then stored at 4 ℃ .

The primer pairs were 5′-CCT GGT TGA TCC TGC CAG -3′and 5′-TAA TGA TCC TTC CGC AGG TT -3′ for the initial amplification of all sequences. Amplification of 18S ribosomal RNA was carried out in a 25 μL reaction solution with 10 μL rTaq buffer, 15 μL $MgCl_2$, 2 μL dNTP, 2 μL each primer, 0.2 μL rTaq DNA polymerase, 4～6 μL template genomic DNA, and dddH_2O to make up any shortfall. Polymerase chain reaction (PCR) was carried out in a Hybaid PCR Sprint thermal cycler (Hybaid, Middlesex, UK). The cycling protocol included an initial denaturation step at 94 ℃ for 4 min, and PCR was performed over 35 cycles. Each cycle consisted of denaturation at 94 ℃ for 1 min, annealing at 50 ℃ for 1 min, and extension at 72 ℃ for 1 min. The reaction was completed with 10 min incubation at 72 ℃.

The PCR products were purified for sequencing reactions, using the Gel Purification MiniKit (Watson Biotechnologies, Inc., Shanghai, China), and then sequenced by using two primers in the ABI PRISM™ 377 DNA sequencer (Shanghai GeneCore Biotechnologies, Inc., Shanghai, China). The nucleotide sequences determined in this study have been submitted to GenBank, with the following accession numbers: DQ531747, AF363310, AB076658, AY863437, AY863434, AY622197, AY622193, AF363295, and AB076612 (see Tab.1).

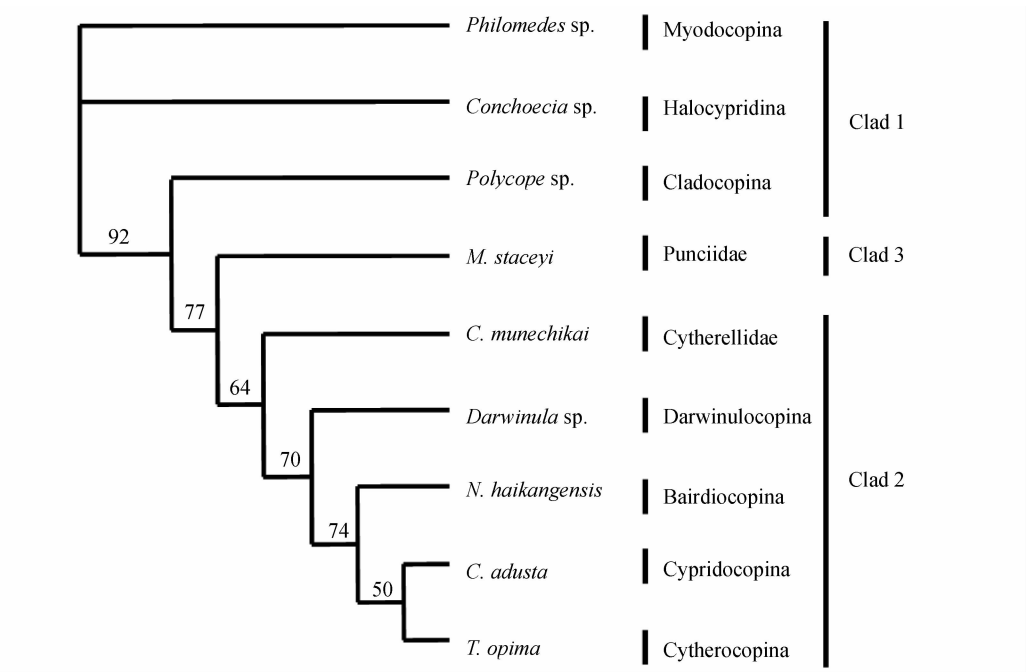
The DNA sequences were assembled and edited using the software program EditSeq in DNAStar (DNAStar, Inc., USA, 1996), and preliminary alignment was achieved using

MegAlign in DNAStar with default gap penalties. The alignment sequences were manually proofed. Regions including inserts, missing sites and alignment-ambiguous regions were excluded from subsequent phylogenetic analyses.

Aligned data sets were assessed for the phylogenetic signal based on the g1 statistic[19] for 10 000 random trees using the random trees option in PAUP 4.0[20]. Phylogenetic analyses were carried out using PAUP 4.0[17], and the same topology was supported by MP. Bootstrap analyses[21] for the MP tree used the fast step-wise addition method with 1 000 replicates. It should be noted that ML trees which were constructed with 100 bootstrap replicates in PAUP 4.0, were not shown because of some similar topologies with MP tree in this paper.

2 Results

Phylogenetic trees were constructed for morphological and 18S rDNA data, respectively. The MP tree from the morphological data is shown in Figure 2, the consensus tree from the 18S rDNA sequence data is shown in Figure 3, and the topology tree from the joint molecular / morphological data is shown in Figure 4.



Note: The strict consensus of 2 equally parsimonious trees found in the heuristic search, with the following results, tree length=53 steps, CI=0.735 8, RI=0.641 0, RC=0.471 7; Numbers at nodes are % bootstrap values of 1 000 replicas (only values >50 are given)

Fig. 2 Strict consensus tree of two maximum parsimony trees based on 27 morphological characters

2.1 Morphological analysis

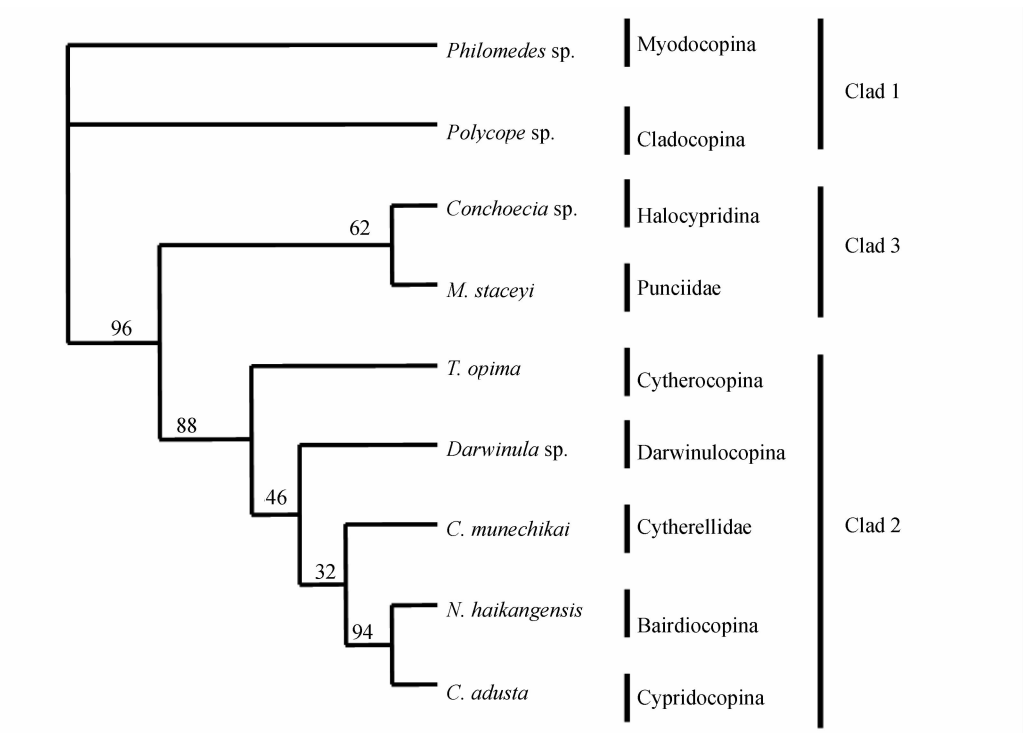
The phylogenetic reconstruction from the morphological data set resulted in two e-

qually parsimonious trees; the strict consensus tree of these was shown in Figure 2. Ostracods were divided into three branches:

(1) *Philomedes* sp. and *Conchoecia* sp. cluster most closely with high bootstrap support (92%), and these two species further cluster with *Polycope* sp. by 77% bootstrap support. These results supported Martin & Davis's[5] arrangements of Myodocopa.

(2) *M. staceyi* forms a single branch itself. This is the representative species of Punciidae, which was designated one family of the Platycopida in Martin & Davis's[5] classification.

(3) The remaining five species form one branch with low bootstrap support (64%) and show the phylogenetic relationship of (*C. munechikai* plus (*D. stevensoni* plus (*N. haikangensis* plus (*C. adusta* plus *T. opima*)))). These results indicate that Cytherellidae of Platycopida and Podocopida might comprise Podocopa.



Note: Analysis of 18S rDNA sequences in the heuristic search, with the following results, tree length=1 331 step, CI=0.703 2, RI=0.350 3, RC=0.246 4; The bootstrap confidence levels based on 1 000 replications are shown at each branching point of the MP tree

Fig. 3　Maximum parsimony tree based on 18S rDNA characters

2.2　Molecular analysis

After the sequences were aligned and the inserts and/or missing sites and ambiguously aligned sequences were discarded, 1 733 sites were available for phylogenetic analyses. The results of 9 species found 671 (39.47%) variable sites and 328 (19.29%) phylogenetic informative sites, shown in the 18S rDNA sequence matrix. The distances among all se-

quences range from 0.099 to 0.303, average 0.182. This is greater than the distances among the myodocopidan (0.001～0.076), bairdiocopinan (0.008～0.023), cytherocopinan (0.004～0.092), darwinulocopinan (0.003), and cypridocopinan (0.000～0.099) ingroups. The values of Ts/Tv (R) range from 1.200 to 2.357, which indicate that transitions were more frequent than transversions in the 18S rDNA sequence taxa set.

The skewnew test statistic (g1) value[19] was −0.594 601 after evaluating 10 000 trees equiprobably sampled from the set of all possible trees, which indicated much useful information among the taxa used in this study. Phylogenetic relationships among ostracods with the MP method are shown in Figure 3. All 9 sequences form three clades:

(1) *Philomedes* sp. and *Polycope* sp. cluster on one clade with high support values (96%), which indicated that the suborder Halocypridina in Martin & Davis's[5] classification was divided from the order Myodocopa.

(2) There was one (halocypridinan, punciidaen) clade with support values of 62%.

(3) The remaining five species clustered into one clade, which was similar to the results from the morphological data, with slight differences in the relationship of ingroup species. The phylogenetic relationships were (*T. opima* plus (*D. stevensoni* plus (*C. munechikai* plus (*C. adusta* plus *N. haikangensis*)))) with 88% support values.

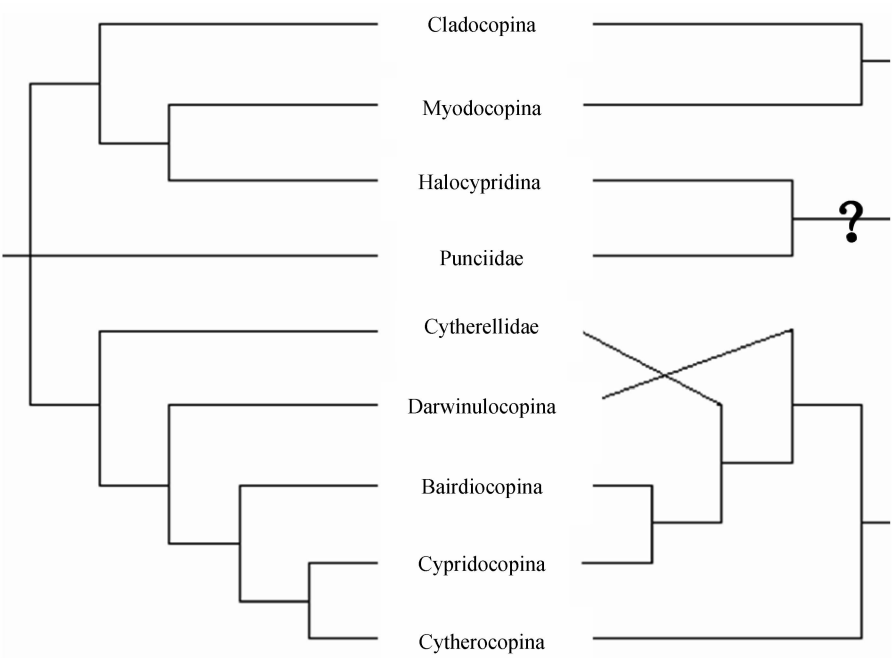


Fig. 4 Comparison of morphological (left) and molecular (right) phylogenies

2.3 Joint phylogenies

Comparison of the morphological and molecular 18S rDNA trees is shown in Figure 4, which indicates that both joint topologies shared a high degree of congruence. The last topology was changed from an unrooted to a rooted tree by analyzing previous classifica-

tions[22], such as Martin & Davis[5]. The clad 2 group mentioned above was always represented and supported by high bootstrap values, where the suborders Cytherocopina, Darwinulocopina, Bairdiocopina, Cypridocopina, and the family Cytherellidae cluster together. The differences between morphological and molecular trees are evident in the positions of Cytherocopina, Darwinulocopina and Cytherellidae in Podocopa, and the relationship between halocypridinan and myodocopan ostracods. The morphological evidence suggests that podocopan ostracods form a (Cytherellidae (Darwinulocopina (Bairdiocopina (Cypridocopina, Cytherocopina)))) clade, halocypridinans are closely related with myodocopins, thus the overall relationship is (Cladocopina (Myodocopina, Halocypridina)). In contrast, the molecular evidence shows that the phylogenetic relationship of podocopan ostracods is (Cytherocopina (Darwinulocopina (Cytherellidae (Bairdiocopina, Cypridocopina)))), and halocypridinan ostracods is separated from Myodocopa.

3　Discussion

Punciidae has been designated an amphibolous family in the classification of Ostracoda, and its status has long puzzled taxonomists. Some have classified it into Podocopida[23,24], while others have considered it to be one group of Platycopida[5]. The results of the present study indicate that the punciidae might be independent of Podocopida and Platycopida, which is consistent with Bowman & Abele's conclusion[4]. However, there are still differences in some details. For example, our results showed that recent Ostracoda might be divided into three groups, among them, punciidaen forming a single taxon. Bowman & Abele's classification[4] based on bivalve morphological characters, in contrast, concluded that Punciidae was only one living group in the subclass Palaeocopa, which formed the class Ostracoda with Myodocopa and Podocopa. Similar results were also found by Liebau[6], who argued that the punciidaen were one independent group, and upgraded the family Punciidae to the order Punciocopida with the same status as Platycopida, Podocopida, Cypridinida and Halocypridida. Therefore, based on these previous studies and our results, it seems reasonable to postulate the punciidaen ostracods as an independent group whose taxonomic status was equal with the subclasses Myodocopa and Podocopa. We have tried to designate this group the subclass "Punciocopa". But the exact status of the punciidaen ostracods is still uncertain because of insufficient evidence in this study and more evidence is necessary.

Cytherellidae has traditionally been the only family in the order Platycopida, while Bairdiocopina, Cytherocopina, Darwinulocopina and Cypridocopina comprise the order Podocopida. The orders Platycopida and Podocopida, in turn, formed the subclass Podocopa[4,5]. Platycopida and Podocopida were thus considered sister groups. The results of this study, however, showed that Podocopa consisted of five main groups, including cytherellidaen ostracods. In other words, the order Podocopa included the suborders Bairdiocopina, Cytherocopina, Darwinulocopina, Cypridocopina and the family Cytherellidae. There were

some differences between the morphological and molecular results of our study. The morphological evidence showed that Cytherellidae was a sister group with the traditional Podocopida and that the five groups of Podocopa formed a ((((cypridocopinan, cytherocopinan) bairdiocopinan) darwinulocopinan) cytherellidaen) clade, while the molecular evidence suggested that cytherellidaen was located in the traditional podocopidan ostracods and the relationship ((((cypridocopinan, bairdiocopinan) cytherellidaen) darwinulocopinan) cytherocopinan) was formed.

In the traditional Ostracoda classification, the phylogenetic relationships of podocopan ostracods remained unstable. The controversy centered on the phylogenetic relationships of the traditional Podocopida, which included only the suborders Cypridocopina, Darwinulocopina, Bairdiocopina and Cytherocopina. For example, Maddocks[25,26] used soft-body morphological characters to argue that the relationship of Podocopida was ((Cypridocopina, Darwinulocopina) (Bairdiocopina, Cytherocopina)), while Scott & Sylvester-Bradley[3] used carapace characters to conclude that the relationship was (((Cypridocopina, Darwinulocopina) Bairdicocopina) Cytherocopina). Both of these conclusions posit a close relationship between Cypridocopina and Darwinulocopina, but other scholars have disagreed. Smith & Kamiya[27] found that some morphological characters of phase A-7 of ostracod ontogeny were plesiomorphic, and thus suggested that Cypridocopina had a close relationship with Cytherocopina, followed by Bairdiocopina. Darwinulocopina was unfortunately not included in their study. Yamaguchi & Endo[9] and Yu et al[28] agreed with the close relationship between Cypridocopina and Cytherocopina by analyzing 18S rDNA sequences of podocopidan ostracods. Among them, Yu et al[28] argued that the podocopidan ostracods formed a (Darwinulocopina (Bairdiocopina (Cytherocopina, Cypridocopina))) clade. These studies leave the exact status of cytherellidaen in Podocopa unclear, but we could suggest that the subclass Podocopa consists of five main groups, including the family Cytherellidae.

The morphological results obtained in this study agree with some traditional classifications of Myodocopa[2,29-31]. Among these classification systems, the subclass Myodocopa has usually been considered a single monophyletic assemblage containing two sister groups, the orders Cladocopida and Myodocopida. The suborders Myodocopina and Halocypridina have been included in the order Myodocopida, while the order Cladaocopida has traditionally included only one suborder, Cladocopina. Sars[5,29] believed that the order Myodocopida included only the two groups Cypridinidae and Conchoeciidae, while Martin & Davis[5] have argued that the two groups were separate families in the suborders Myodocopina and Halocypridina. After Kornicker & Sohn[32] first suggested separation of the halocypridinan ostracod from the order Myodocopida and formed the order Halocyprida with the suborder Cladocopina[5], this view was accepted (albeit with slight revisions) by many scholars[4,5]. Liebau[6], however, put forward a different view, replacing the status of the order Myodocopida with the order Cypridinida, which included the three families

Cypridinidae, Saraiellidae and Cylindroleberididae. Liebau also suggested that the order Halocypridida should include the suborders Cladocopina, Thaumatocypridina and Halocypridina; and finally, that the orders Cypridinida and Halocypridida formed the "Myodocopomorpha". The morphological and molecular results of the present study differed, with the morphological evidence suggesting that the subclass Myodocopa consisted of the orders Cladocopina, Myodocopina and Halocypridina, while the molecular evidence indicated that the order Halocypridina was free from the subclass Myodocopa. Therefore, we can ascertain that the subclass Myodocopa includes the orders Cladocopina and Myodocopina, while the status of the suborder Halocypridina requires further investigation.

At present, many works suggested it was advantageous to solve the problems of animal classification by finding new evidences, increasing the number of new samples, and analyzing various evidences together in the present study. In this study, we tried to combine morphologic and molecular evidences to analyze the classification of Ostracoda's higher taxa, despite some results were uncertain, such as classification status of punciidaen ostracods. It was a significative work to the classification of Ostracoda.

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