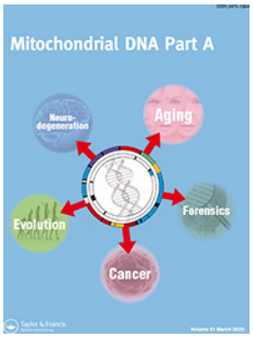

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RESEARCH ARTICLE

Molecular identification of Cerithiidae (Mollusca: Gastropod) in Hainan island, China

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ABSTRACT

A number of same species of Cerithiidae are morphologically unlike, whereas most of species in the same genus are morphologically similar and just exhibit subtle differences. It is difficult to identify them by morphological methods alone. DNA barcoding is a modern molecular technique that can be used to identify species accurately, and is particularly helpful when distinguishing morphologically similar species. In order to identify species of Cerithiidae using DNA barcoding technology based on mitochondrial cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) genes, this study calculated intraspecific and interspecific genetic distance and constructed the phylogenetic trees. A total of 80 COI and 16S rRNA barcode sequences were obtained from 10 species and 3 genera. Some unknown specimens were further identified and a cryptic species may exist in *Cerithium traillii*, showing that DNA barcoding technology has the potential to discover new species and cryptic species. The phylogenetic trees revealed that all of the cerithiids could converge upon a monophyly with high support values and two genera (*Cerithium* and *Clypeomorus*) maybe support the reclassification. It is necessary for traditional morphological methods to combine with the DNA barcoding for classification and identification of Cerithiidae.

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KEYWORDS
Cerithiidae; DNA barcoding; COI gene; 16S rRNA gene; species identification

Introduction

The Cerithiidae is one of the 19 families of Cerithioidea currently recognized and comprises 219 species presently considered valid (Bouchet et al. 2017). The name Cerithium comes from own shell which is often occupied by the pagurian (Houbrick 1978). Shell varies in size, with a lot of spiral wholes and a higher spire. Shell shape is diversified, most of which are conical or spindly. The family is a group of old and evolving slowly prosobranchs. Despite the existence of very old fossils, many living species occur cretaceous fossils (Houbrick 1978; Houbrick 1985; Houbrick 1992). Most of the species in the family live in tropical and subtropical regions and occur in shallow water depth of 100 m, but some cerithiines were discovered in bathyal depths down to 1000–1200 m (Strong and Bouchet 2013; Strong and Bouchet 2018). The majority of species are microherbivorous grazers and generally occur in large populations (Houbrick 1992; Strong and Bouchet 2018).

For a long time, few scholars study the cerithiines, so they are placed ambiguously. A large number of species in the family were discovered, including fossil species and living species. However, because of convergent evolution and phenotypic plasticity of species, many species are misidentified through traditional morphological method, which leads to the synonym or homonym (Wilke and Falniowski 2001). It was not until 1822 that Fleming established the Cerithiidae as an independent family (Bouchet and Rocroi 2005). At present, the family is subdivided into three subfamilies: Argyropezinae, Cerithiinae and Bittiinae (Bouchet et al. 2017), with the Cerithiinae and Bittiinae containing the most of numbers (Strong and Bouchet 2018). As there is uncertain diagnostic feature of shell morphology or internal anatomy to distinguish the Cerithiinae and Bittiinae, species identification of Cerithiidae is difficult due to their morphological variety.

In recent years, with the development of molecular analysis technology, DNA barcoding technology used to identify species has become a focus of attention (Köhler 2007). DNA barcoding is based on the fact that interspecific variation is more than intraspecific variation (Avise 2000). Compared with traditional morphology, DNA barcoding can obtain the molecular data of specimens and identify a mass of samples rapidly (Frezal and Leblois 2008). It can help accurately distinguish some species that have extremely similar external morphological features (Ratnasingham and Hebert 2007; Xing et al. 2018), and is useful for the rapid identification of damaged specimens (Yang et al. 2010) and species of different growth stages (Azmir et al. 2017). DNA barcoding has also facilitated the discovery of cryptic species and new species (Puillandre et al. 2010). So far, DNA barcoding technology

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Supplemental data for this article can be accessed here.

has been widely used in the identification of mollusks (Sun et al. 2012; Barco et al. 2016). Some studies showed that the mitochondrial COI gene had a higher mutation rate and could be used as a standard animal DNA barcode sequence (Stoeckle 2003; Chase and Fay 2009). In mollusks the 16S rRNA gene barcoding has been suggested as a complementary DNA barcoding marker (Feng et al. 2011).

Hainan island is part of the South China Sea and belongs to the shallow sea of the tropical continental shelf in China (Fang and Bailey 1998). The coastal habitats of Hainan island are rock reefs, coral reefs, gravel, sand and mud, which is particularly suitable for the survival and reproduction of gastropod species (Quan et al. 1988; Zuschin and Hohenegger 1998), especially Cerithiidae species. In this study, we sequenced COI and 16S rRNA from 10 species in the Cerithiidae collected from Hainan island to evaluate whether DNA barcoding can be used to distinguish these species, and discuss phylogenetic relationships within Cerithiidae species.

Materials and methods

Specimen collection and identification

The samples of Cerithiidae used in this study were collected from the coast of Hainan island, China during March–December 2018 (Figure 1). A total of 13 sites of the Hainan Island were surveyed for Cerithiidae species. All specimens were preserved in 95% alcohol and transported to laboratory for identification. A total of 40 Cerithiidae samples was chosen for the research, and one to nine individual specimens were collected for each Cerithiidae species. The detailed information of specimens was showed in Table 1. Specimens were identified based on morphological features. In order to identify the unknown specimens accurately, we constructed the phylogenetic topologies between the known species and the unknown specimens by using the method of molecular systematics. Morphological characteristics of the 10 species are shown in Figure 2.

DNA isolation, amplification and sequencing

A small piece of tissue (about 100 mg) was removed from the foot of each sample or from the entire animal. DNA was extracted using standard cetyltrimethyl ammonium bromide (CTAB) and chloroform-isoamyl alcohol protocols (Winnepenninckx et al. 1993). The extracted DNA were preserved in TE solution and frozen at –20 °C until used.

We selected two mitochondrial genes (COI and 16S rRNA) that had worked well in previous studies on gastropods. A 640 base pairs COI fragment was amplified using universal primers (LCO1490 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′ and HCO2198 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) designed by Folmer et al. (1994). A 480 base pairs 16S rRNA fragment was amplified using universal primers (16Sar 5′-CGC CTG TTT ATC AAA AAC AT-3′ and 16Sbr 5′-CCG GTC TGA ACT CAG ATC ACG T-3′) designed by Palumbi et al. (1991). The primers used in this study were synthesized by Sangon Biotech. The total polymerase chain reaction (PCR) volume was 25 μL, including 2.5 μL 10× PCR Buffer (Mg^{2+} Plus), 0.5 μL 2.5 mM dNTP, 1 μL of each 10 μmol/L prime, 0.2 μL 5 U/μL *Taq* DNA polymerase, 1 μL 100 ng/μL template DNA and 18.8 μL sterile distilled water. The PCR reaction procedure was performed under the following conditions: an initial denaturation for 3 min at 94 °C, 35 cycles of 94 °C for 30 s, 48 °C for 1 min (COI) or 30 s (16S) and 72 °C for 1 min, with final extension of 5 min at 72 °C, and then preserved 4 °C refrigerator. PCR products were detected by 1.5% agarose gel, and the qualified products were sent to Sangon Biotech for purification and bidirectional sequencing.

Data analysis

All COI and 16S rRNA gene sequences were entered into the SeqMan program of DNASTAR software (Pettengill and Neel 2010) to edit sequences and manually delete primer sequences. The corrected COI and 16S rRNA gene sequences were aligned using the Clustal W in BioEdit v.7.2.6.1 (Hall 1999), and then uploaded to GenBank (Table 1). Some parameters were analyzed using the software MEGA 6.0 (Tamura et al. 2013),

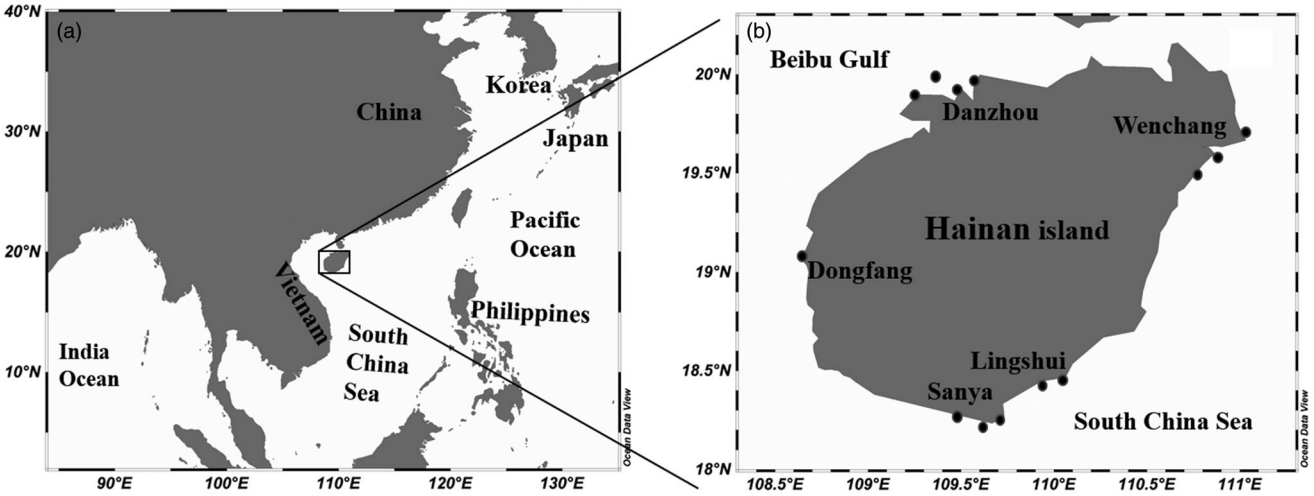

Figure 1. Distribution of the sampling localities for the specimens collected in this study. Locations of Hainan island (a) and sampling sites (b).

Table 1. The details of Cerithiidae specimens used in this study.

Sample number	Species name	Collecting locality	Accession number	
			COI	16S rRNA
13	*Cerithium zonatum*	Banshanbandao, Sanya	MN249981	MN249994
14	*Cerithium zonatum*	Yangpu, Baimajing	MN249982	MN249995
15	*Cerithium zonatum*	Luhuitou, Sanya	MN249983	MN249996
17	*Cerithium zonatum**	Shitou park, Wenchang	MN249984	MN249997
51	*Cerithium zonatum**	Linchangjiao, Danzhou	MN249985	MN249998
56	*Cerithium zonatum**	Linchangjiao, Danzhou	MN249986	MN249999
55	*Cerithium traillii**	Shitou park, Wenchang	MN249972	MN250002
57	*Cerithium traillii*	Shenchong, Danzhou	MN249958	MN250003
58	*Cerithium traillii*	Yinghao, Danzhou	MN249988	MN250004
63	*Cerithium traillii*	Shenchong, Danzhou	MN249973	MN250005
64	*Cerithium traillii*	Shenchong, Danzhou	MN249974	MN250006
65	*Cerithium traillii*	Shitou park, Wenchang	MN249975	MN250007
66	*Cerithium traillii*	Shitou park, Wenchang	MN249976	MN250008
67	*Cerithium traillii*	Shitou park, Wenchang	MN249977	MN250009
68	*Cerithium traillii*	Shitou park, Wenchang	MN249978	MN250010
5-1	*Cerithium coralium*	Linchangjiao, Danzhou	MN249953	MN249989
6-2	*Cerithium coralium*	Gangdong, Wenchang	MN249954	MN249990
142	*Cerithium coralium*	Lingzi, Lingshui	MN249955	MN249991
143	*Cerithium coralium*	Lingzi,Lingshui	MN249956	MN249992
146	*Cerithium coralium*	Lingzi, Lingshui	MN249957	MN249993
61	*Cerithium punctatum*	Lingzi, Lingshui	MN249950	MN250011
62	*Cerithium punctatum*	Gangdong, Wenchang	MN249979	MN250012
140	*Cerithium mangrovum*	Lingzi, Lingshui	MN249987	MN250000
147	*Cerithium mangrovum*	Linchangjiao, Danzhou	MN249949	MN250001
36-1	*Clypeomorus petrosa*	Shitou park, Wenchang	MN249965	MN250022
37-1	*Clypeomorus petrosa*	Yulinzhou, Dongfang	MN249966	MN250023
39-1	*Clypeomorus petrosa*	Yuerongzhuang, Sanya	MN249967	MN250024
137	*Clypeomorus petrosa*	Yuerongzhuang, Sanya	MN249968	MN250025
39	*Clypeomorus subbrevicula*	Shajing, Danzhou	MN249962	MN250018
41	*Clypeomorus subbrevicula*	Banshanbandao, Sanya	MN249963	MN250019
47	*Clypeomorus subbrevicula*	Shajing, Danzhou	MN249952	MN250020
48	*Clypeomorus subbrevicula*	Shenchong, Danzhou	MN249964	MN250021
38	*Clypeomorus pellucida*	Shenchong, Danzhou	MN249959	MN250013
43	*Clypeomorus pellucida*	Shajing, Danzhou	MN249960	MN250014
49	*Clypeomorus pellucida*	Baoshi, Wenchang	MN249971	MN250015
50	*Clypeomorus pellucida*	Baoshi, Wenchang	MN249961	MN250016
54	*Clypeomorus pellucida*	Baoshi, Wenchang	MN249969	MN250017
31-1	*Clypeomorus batillariaeformis*	Baoshi, Wenchang	MN249970	MN250026
59-2	*Clypeomorus batillariaeformis*	Chiling, Lingshui	MN249951	MN250027
45-1	*Rhinoclavis sinensis*	Chiling, Lingshui	MN249980	MN250028

Some specimens that cannot be identified by morphology were indicated by ''.

including the base composition, the genetic distance and the neighbor-joining (NJ) tree of COI and 16S rRNA gene sequence. The Kimura 2-parameter (K2P) model (Kimura 1980) was used to calculate genetic distance of intraspecific and intergeneric and create NJ tree. Tree topology and branch lengths were optimized artificially. Node support was assessed by performing bootstrapping analysis with 1000 replicates (Felsenstein 1985). *Batillaria zonalis* was selected as outgroup.

Results

A total of 40 individuals from 10 species of Cerithiidae were used for sequence analysis of COI and 16S rRNA genes, which yielded 80 sequences. All sequences were deposited in GenBank (Accession Number: MN249949- MN249988 for COI, MN249989- MN250028 for 16S rRNA).

COI gene analyses

After editing, the consensus length of all barcoding sequences of COI gene was 640 base pairs. No stop codons, insertions or deletions were observed in any of the sequences. The mean nucleotide frequencies are 35.7% (T), 22.2% (A), 21.2% (C) and 20.9% (G). The base composition analysis for the COI sequence showed that the mean T content was highest and the mean G content was the lowest; the AT content (average 58.0%) was higher than the GC content (average 42.0%) (Table 2). Among these species, *C. punctatum* showed the highest GC content (43.6%), while *C. batillariaeformis* showed the lowest GC content (41.0%). As showed in Table 3 and Table S1, Supplementary material, the K2P distances of the COI sequence within species ranged from 0 to 2.6%, with an average distance of 0.5%; the highest distance of 2.6% was found in *C. traillii*. The interspecific genetic distance ranged from 1.0% to 24.8%, with an average of 18.5%, which was 37 times the mean intraspecific genetic distance. The largest interspecies distance of 24.8% was found between *C. punctatum* and *C. subbrevicula* and the lowest was found between *C. pellucida* and *C. subbrevicula*.

16S rRNA gene analyses

A total of 480 base pairs of aligned sequences of 16S rRNA gene were studied in the 10 species of Cerithiidae. The average nucleotide frequencies were calculated to be $A = 30.1\%$, $T = 29.3\%$, $G = 22.3\%$ and $C = 18.2\%$ (Table 4). The sequences of 16S rRNA were AT-rich, with an average of

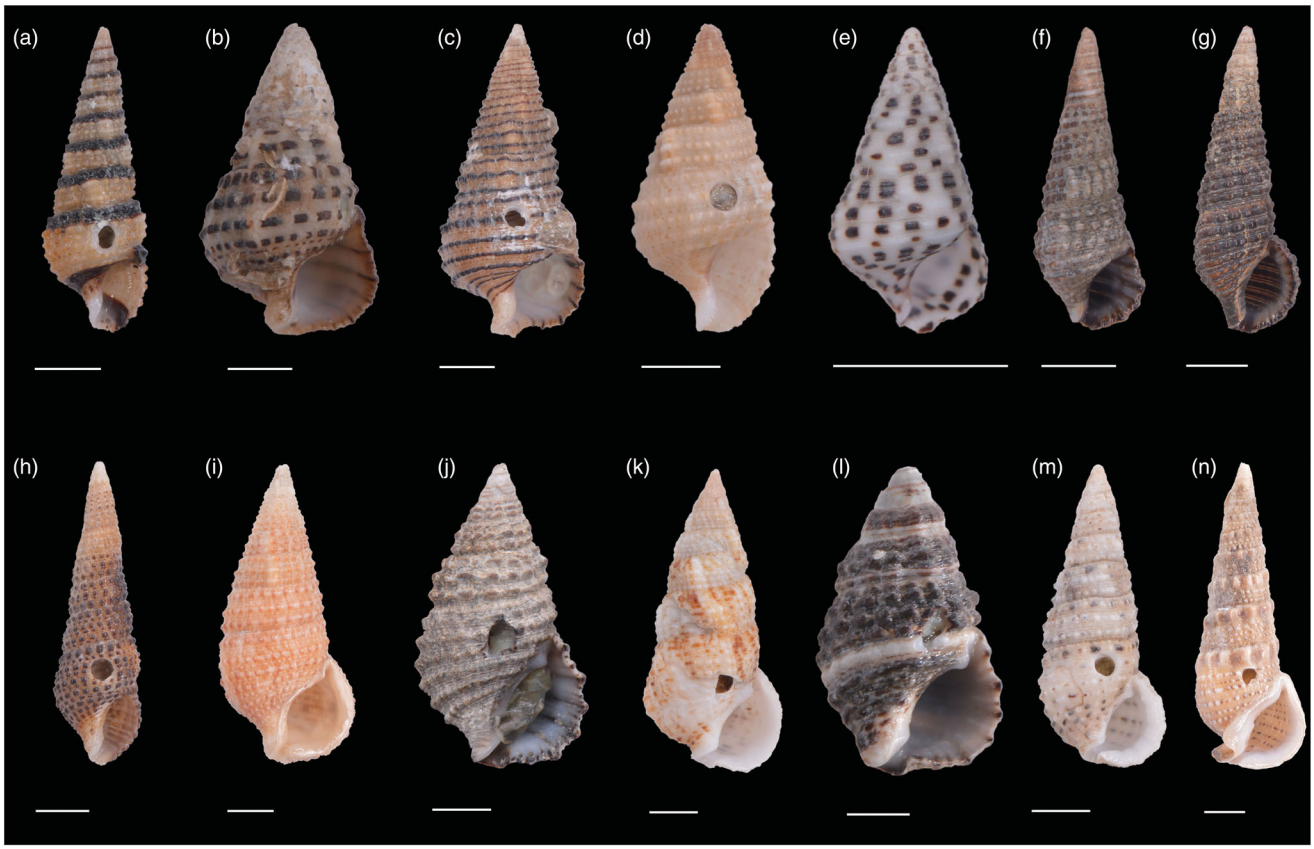

Figure 2. The samples of Cerithiidae were collected in Hainan island, China. (a–d) *Cerithium zonatum*, (b–d) uncertain specimens by morphology, (b) 17 *Cerithium zonatum*, (c) 51 *Cerithium zonatum*, (d) 56 *Cerithium zonatum*; (e) *Cerithium punctatum*; (f) *Cerithium mangrovum*; (g) *Cerithium coralium*; (h,i) *Cerithium traillii*, (h) uncertain specimen by morphology, 55 *Cerithium traillii*; (j) *Clypeomorus pellucida*; (k) *Clypeomorus petrosa*; (l) *Clypeomorus subbrevicula*; (m) *Clypeomorus batillariaeformis*; (n) *Rhinoclavis sinensis*. Scale bars: 5 mm.

Table 2. Mean percent base composition and GC content of the first, second and third codon position from COI sequences of Cerithiidae.

Species	T	C	A	G	GC	1st	2nd	3rd
Cerithium coralium	35.9	20.9	21.8	21.2	42.1	41.3	29.9	55.3
Cerithium mangrovum	35.7	21.4	21.7	21.2	42.6	41.3	31.3	55.5
Cerithium zonatum	36.3	21.0	22.0	20.7	41.7	41.3	26.2	57.6
Cerithium traillii	35.7	20.8	22.5	20.9	41.7	41.8	27.4	56.2
Cerithium punctatum	34.4	22.6	22.0	21.0	43.6	42.2	31.4	57.2
Clypeomorus petrosa	35.9	21.2	22.8	20.1	41.3	41.3	27.4	55.3
Clypeomorus subbrevicula	35.0	21.7	22.2	21.1	42.8	41.5	31.8	55.1
Clypeomorus pellucida	34.9	21.6	22.2	21.3	42.9	41.5	32.1	55.1
Clypeomorus batillariaeformis	37.0	20.5	22.0	20.5	41.0	42.1	24.1	57.0
Rhinoclavis sinensis	35.4	21.7	22.0	20.8	42.5	40.8	31.2	55.7
Avg.	35.7	21.2	22.2	20.9	42.1	41.5	28.9	56.0

Table 3. Mean inter-species and mean intra-species genetic distance (K2P percent) based on COI gene of Cerithiidae.

	1	2	3	4	5	6	7	8	9	10
Cerithium coralium	0.7									
Cerithium mangrovum	21.9	0.8								
Cerithium zonatum	16.2	18.2	0.8							
Cerithium traillii	19.8	23.1	17.2	1.3						
Cerithium punctatum	22.2	22.6	19.1	18.6	0.0					
Clypeomorus petrosa	18.7	21.6	18.2	20.5	21.3	0.2				
Clypeomorus subbrevicula	17.2	18.7	17.5	22.6	24.7	13.1	0.2			
Clypeomorus pellucida	17.4	18.8	18.1	22.6	24.3	12.9	1.2	0.1		
Clypeomorus batillariaeformis	16.0	18.6	14.6	17.2	17.9	16.0	16.6	16.6	0.0	
Rhinoclavis sinensis	21.6	21.4	19.7	21.4	24.3	21.9	21.4	20.3	20.6	–

Table 4. Mean percent base composition and GC content of the first, second and third codon position from 16S rRNA sequences of Cerithiidae.

Species	T	C	A	G	GC	1st	2nd	3rd
Cerithium coralium	29.5	18.6	29.3	22.6	41.2	47.5	37.5	38.7
Cerithium mangrovum	27.6	18.0	30.0	24.4	42.4	46.5	40.1	40.5
Cerithium zonatum	28.3	19.0	29.5	23.2	42.2	48.9	40.3	37.3
Cerithium traillii	30.4	17.8	30.2	21.6	39.4	42.9	39.0	36.2
Cerithium punctatum	29.3	17.4	30.2	23.1	40.5	43.5	39.3	38.8
Clypeomorus petrosa	29.7	18.0	30.5	21.8	39.8	45.7	36.6	37.0
Clypeomorus subbrevicula	29.0	18.5	30.6	21.9	40.4	44.7	37.7	38.8
Clypeomorus pellucida	29.0	18.5	30.6	21.8	40.3	45.2	37.8	38.1
Clypeomorus batillariaeformis	29.6	17.7	30.7	21.9	39.6	45.3	38.6	35.1
Rhinoclavis sinensis	28.4	17.8	30.6	23.3	41.1	45.7	39.3	38.0
Avg.	29.3	18.2	30.1	22.3	40.6	45.5	38.5	37.6

Table 5. Mean inter-species and mean intra-species genetic distance (K2P percent) based on 16S rRNA gene of Cerithiidae.

	1	2	3	4	5	6	7	8	9	10
Cerithium coralium	0.2									
Cerithium mangrovum	9.8	0.4								
Cerithium zonatum	6.1	9.6	0.1							
Cerithium traillii	9.9	8.6	11.9	0.7						
Cerithium punctatum	8.9	8.3	11.1	8.3	0.2					
Clypeomorus petrosa	6.1	8.8	7.3	8.1	7.3	0.4				
Clypeomorus subbrevicula	7.1	9.4	7.7	9.8	7.7	4.8	0.0			
Clypeomorus pellucida	7.3	9.1	8.0	9.5	7.8	4.8	0.5	0.1		
Clypeomorus batillariaeformis	8.8	10.4	10.2	9.1	10.5	6.9	7.2	6.9	0.9	
Rhinoclavis sinensis	10.7	11.2	10.4	10.5	11.6	9.1	9.7	9.6	12.7	–

59.4% AT content. Among these species, the highest GC content was *C. mangrovum* (42.4%), while the lowest was *C. traillii* (39.4%). None of the sequences contained insertions, deletions or stop codons. The intra- and interspecific genetic distance of 10 species of Cerithiidae were showed in the Table 5 and Table S2, Supplementary material. The results indicated that intraspecific K2P divergence of 16S rRNA gene ranged from 0 to 1.1%, with an average of 0.3%, and that of interspecific genetic distance ranged from 0.4% to 12.5% (average 8.4%). The highest intraspecific genetic distance was found in *C. traillii*. The highest interspecies distance was discovered between *C. zonatum* and *C. traillii* and the lowest was found between *C. pellucida* and *C. subbrevicula*. The interspecific genetic distance was significantly higher than the intraspecific genetic distance (about 28 times).

Neighbor-joining tree

The phylogenetic NJ trees were generated based on all individuals' COI and 16S rRNA sequences (Figure 3). All of individuals from each species formed distinct clusters in the NJ tree. However, the NJ tree analyses of the COI sequences indicated that several species of same genus (*Cerithium* and *Clypeomorus*) were not gathered together. In addition, the species of *Cerithium* in the NJ tree based on 16S rRNA sequences were not monophyletic.

Discussion

Morphological differences between individuals of the same species

Many of the same species of Cerithiidae are morphologically unlike and exhibit diverse characteristics (Houbrick 1992). In this study, we found that there are morphological differences among individuals of the same species, such as *C. zonatum* and *C. traillii* species. The shell of *C. traillii* is usually stout, straight-sided with indistinct suture and acute apex. Three spiral ribs are sculptured on each whorl, crossed by narrow axial ribs to form granules on upper whorls. The colour of shell is white, with tan spiral bands or rows of tan fine blotches. The shell of *C. zonatum* is similar to *C. trailli*, but smaller, with distinct granules on spiral ribs. The colour and sculpture of shell is extremely variable. Many synonyms are present (Okutani 2017). Some specimens used this study have diverse morphological characteristics. Shell shapes vary from conical to tower-shaped, the shell is also very diverse in colour, with the presence of white, brown and dark brown. Some individuals have irregular varices on each whorl and distinct granules on spiral ribs. It is quite difficult to identify them by morphological features alone.

Cryptic species

DNA barcoding that use the COI gene as a marker for identifying species, especially shellfish species, have recently attracted attention (Feng et al. 2011; Sun et al. 2012; Barco et al. 2016). One of the main reasons for the selection of COI as the standard barcode gene is that many species have the typical pattern of variation, which shows that there is little overlap between intraspecific genetic distance and interspecific genetic distance (Hebert, Cywinska, et al. 2003; Hebert, Ratnasingham, et al. 2003). To further improve the efficiency of identification, 16S rRNA gene was used as another marker. In general, the intraspecific genetic distance of animals based on K2P model is less than 1%, and rarely more than 2% (Hebert, Cywinska, et al. 2003; Hebert, Ratnasingham, et al. 2003), including shellfish (Barco et al. 2016). In the present study, the intraspecific genetic distance of most species was less than 2%, except for *C. traillii*. The boundary between intraspecific genetic distance and interspecific genetic distance has always been the focus of DNA barcoding research. The mean interspecific difference was 10 times of intraspecific difference, which was considered as the threshold of animal species identification (Hebert et al. 2004). The result of this study was consistent with this value. Nevertheless, a genetic differentiation was found among *C. traillii* specimens collected from different localities. Phylogenetic and genetic differentiation analysis showed the genetic diversity of these clades, indicating the possible existence of cryptic species. The intraspecific genetic distances among 16S rRNA sequences, as well as COI sequences, were exceeded the intraspecific threshold values detected other mollusks (Feng et al. 2011; Zou et al. 2012). According to the genetic evidence, *C. traillii* from Yinghaocun may represent a new lineage of the *C. traillii* species complex.

GC content variation

The base composition analysis for the COI and 16S rRNA sequences from Cerithiidae showed that the A + T content

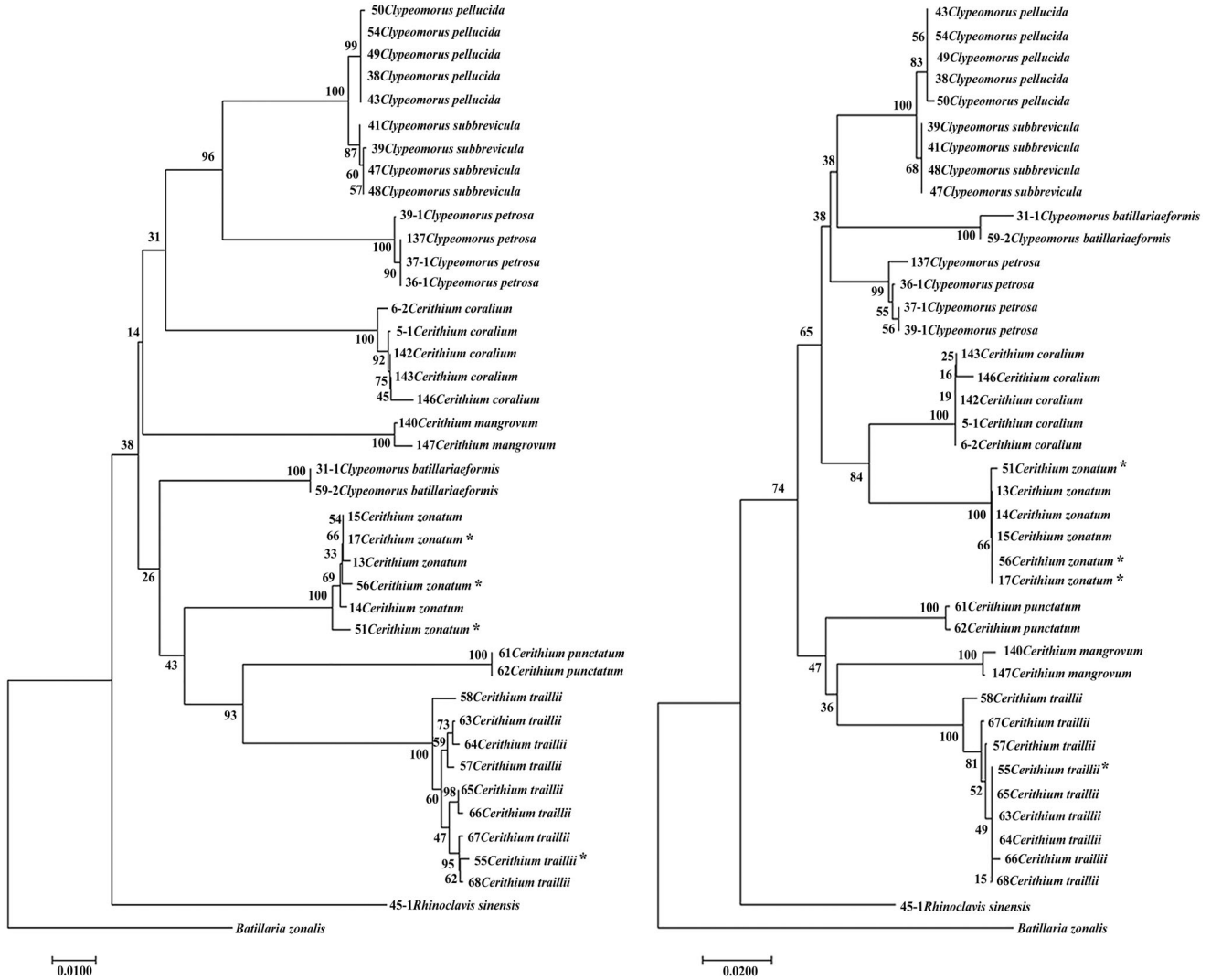

Figure 3. NJ tree of Cerithiidae species based on the partial COI (left) and 16S rRNA (right) gene. Several specimens that cannot be identified by morphology were indicated by '*'.

was higher than the G + C content, which is consistent with some research on mollusks (Sun et al. 2012). The variation of GC content affects different codon positions. The mean CG content of COI sequences for Cerithiidae in the first, second and third codon positions were 41.5%, 28.9% and 56.0%, while the mean content of 16S rRNA were 45.5%, 38.5% and 37.6%, respectively. Differences between these codon positions may reflect the degree of selective constraint, so GC content could provide an important insight into the nature of selective pressures that influence nucleotide usage (Clare et al. 2008). In the process of species evolution, selection pressure of base-mutation may affect the codon positions of mitochondrial genes to some extent, and base usage bias may be caused by base-mutation pressure in codon positions. In addition, GC content has been shown to be associated with some biological functions, such as gene expression (Quax et al. 2015). The variation of GC content in base composition can be explained by mutational biases, natural selection (Mooers and Holmes 2000) and other factors, such as ambient temperature (Bernardi and Bernardi 1986).

Utility of DNA barcoding in species identification

In this study, we found several species are difficult to identify through morphological identification (Figure 2). Using molecular identification methods, they were quickly identified to the species level, showing that DNA barcoding is a powerful method for identification of Cerithiidae species and has the potential to discover new species. The morphological differences of the same species may be caused by different living environment or different growth stages. The same species were gathered on the same nodes and supported by high bootstrap values in the NJ trees. Up to now, although the study of phylogeny was not documented, the gathering of some genera in NJ tree probably support the reclassification in the Cerithiidae. The genus *Rhinoclavis* showed monophyletic and differed from others obviously, while *Cerithium* was polyphyletic in the NJ trees. Meanwhile, *Clypeomorus* was not supported as a monophyletic group in the NJ tree based on the COI gene. This indicated that the current Cerithiidae classification cannot validly reflect a natural subdivision. The phylogenetic analysis supported the reclassification between *Cerithium* and *Clypeomorus*, although more

comprehensive taxa sampling is required to assess the affinities of the two genera. In a word, all of the species formed clusters obviously, confirming the utility of the DNA barcoding method in Cerithiidae identification. In addition, the sequencing results revealed some genus that may have been placed ambiguously and need further study.

In summary, DNA barcoding technology based on COI and 16S rRNA genes has achieved significant effect in the identification of Cerithiidae. Meanwhile, some uncertain specimens were further identified by molecular methods, indicating that DNA barcoding could be used as a rapid and efficient identification tool for the species of Cerithiidae.

Disclosure statement

The authors declare that they have no conflict of interest.

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References

Avise JC. 2000. Phylogeography: the history and formation of species. Cambridge: Harvard University Press.

Azmir IA, Esa Y, Amin SMN, Md Yasin I.S, Md Yusof FZ. 2017. Identification of larval fish in mangrove areas of Peninsular Malaysia using morphology and DNA barcoding methods. J Appl Ichthyol. 33(5):998–1006.

Barco A, Raupach MJ, Laakmann S, Neumann H, Knebelsberger T. 2016. Identification of North Sea molluscs with DNA barcoding. Mol Ecol Resour. 16(1):288–297.

Bernardi G, Bernardi G. 1986. Compositional constraints and genome evolution. J Mol Evol. 24(1–2):1–11.

Bouchet P, Rocroi JP. 2005. Classification and nomenclator of gastropod families. Malacologia. 47:1–368.

Bouchet P, Rocroi JP, Hausdorf B, Kaim A, Kano Y, Nützel A, Parkhaev P, Schrödl M, Strong EE. 2017. Revised classification, nomenclator and typification of gastropod and monoplacophoran families. Malacologia. 61(1–2):1–526.

Chase MW, Fay MF. 2009. Barcoding of plants and fungi. Science. 325(5941):682–683.

Clare EL, Kerr KCR, Von Königslöw TE, Wilson JJ, Hebert PD. 2008. Diagnosing mitochondrial DNA diversity: applications of a sentinel gene approach. J Mol Evol. 66(4):362–367.

Fang Z, Bailey RL. 1998. Height-diameter models for tropical forests on Hainan Island in southern China. Forest Ecol Manag. 110(1–3):315–327.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39(4):783–791.

Feng Y, Li Q, Kong L, Zheng X. 2011. DNA barcoding and phylogenetic analysis of Pectinidae (Mollusca: Bivalvia) based on mitochondrial COI and 16S rRNA genes. Mol Biol Rep. 38(1):291–299.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 3(5):294–299.

Frezal L, Leblois R. 2008. Four years of DNA barcoding: current advances and prospects. Infect Genet Evol. 8:727–736.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 41:95–98.

Hebert PDN, Cywinska A, Ball SL, de Waard JR. 2003. Biological identifications through DNA barcodes. Proc R Soc Lond B. 270(1512):313–321.

Hebert PDN, Ratnasingham S, de Waard JR. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc R Soc B. 270:S96–S99.

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM. 2004. Identification of birds through DNA barcodes. PLoS Biol. 2(10):e312–1663.

Houbrick RS. 1978. The family Cerithiidae in the Indo-Pacific. Part 1: the genera *Rhinoclavis, Pseudovertagus* and *Clavocerithium*. Monogr Mar Mollusca. 1:1–130.

Houbrick RS. 1985. Genus *Clypeomorus* Jousseaume (Cerithiidae: Prosobranchia). Smithsonian Contrib Zool. 403:1–131.

Houbrick RS. 1992. Monograph of the Genus *Cerithium* Bruguiere in the Indo-Pacific (Cerithiidae: Prosobranchia). Smithsonian Contrib Zool. 510:1–211.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 16(2):111–120.

Köhler F. 2007. From DNA taxonomy to barcoding: how a vague idea evolved into a biosystematic tool. Zool Reihe. 83(S1):44–51.

Mooers AO, Holmes EC. 2000. The evolution of base composition and phylogenetic inference. Trends Ecol Evol. 15(9):365–369.

Okutani T. 2017. Marine mollusks in Japan. Tokyo: Tokai University Press.

Palumbi SR, Martin A, Romano S, McMillan WO, Stice L, Grabowski G. 1991. The simple fool's guide to PCR, Version 2.0. Honolulu: Department of Zoology and Kewalo Marine Laboratory, University of Hawaii.

Pettengill JB, Neel MC. 2010. An evaluation of candidate plant DNA barcodes and assignment methods in diagnosing 29 species in the genus Agalinis (Orobanchaceae). Am J Bot. 97(8):1391–1406.

Puillandre N, Cruaud C, Kantor YI. 2010. Cryptic species in Gemmuloborsonia (Gastropoda: Conoidea). J Mollus Stud. 76(1):11–23.

Quan SQ, Wang GZ, Lu B. 1988. The recent carbonate sedimentary facies and sedimentation of the muddy water in the paipu fringing coral reef, Hainan Island. Oceanol Limnol Sin. 19:179–187.

Quax TEF, Claassens NJ, Söll D, Van der Oost J. 2015. Codon bias as a means to finetune gene expression. Mol Cell. 59(2):149–161.

Ratnasingham S, Hebert P. 2007. BOLD: the barcode of life data system. Mol Ecol Notes. 7(3):355–364. http://www.barcodinglife.org.

Stoeckle M. 2003. Taxonomy, DNA and the barcode of life. Bioscience. 53(9):796–797.2.0.CO;2]

Strong EE, Bouchet P. 2013. Cryptic yet colorful: anatomy and relationships of a new genus of Cerithiidae (Caenogastropoda, Cerithioidea) from coral reef drop-offs. Inverrebr Biol. 132(4):326–351.

Strong EE, Bouchet P. 2018. A rare and unusual new bittiine genus with two new species from the south pacific (Cerithiidae, gastropoda). ZooKeys. 758:1–18.

Sun Y, Li Q, Kong L, Zheng X. 2012. DNA barcoding of Caenogastropoda along coast of China based on the COI gene. Mol Ecol Resour. 12(2): 209–218.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 30(12):2725–2729.

Wilke T, Falniowski A. 2001. The genus Adriohydrobia (Hydrobiidae: Gastropoda): polytypic species or polymorphic populations? J Zool Syst. 39(4):227–234.

Winnepenninckx B, Backeljau T, De Wachter R. 1993. Extraction of high molecular weight DNA from molluscs. Trends Genet. 9(12):407.

Xing B, Lin H, Zhang Z, Wang C, Wang Y, Wang J. 2018. DNA barcoding for identification of fish species in the Taiwan Strait. PLoS One. 13: e0198109.

Yang R, Wu XB, Yan P, Li XQ. 2010. Using DNA barcodes to identify a bird involved in a bird strike at a Chinese airport. Mol Biol Rep. 37(7): 3517–3523.

Zou S, Li Q, Kong L. 2012. Multigene barcoding and phylogeny of geographically widespread Muricids (Gastropoda: Neogastropoda) along the coast of China. Mar Biotechnol. 14(1):21–34.

Zuschin M, Hohenegger J. 1998. Subtropical coral-reef associated sedimentary faciès characterized by molluscs (Northern Bay of Safaga, Red Sea, Egypt. Facies. 38(1):229–254.