Phylogenetic positions of some genera and species of the family Buccinidae (Gastropoda: Mollusca) from China based on ribosomal RNA and COI sequences

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A phylogenetic analysis of members of the family Buccinidae was conducted using 18S rRNA gene, 28S rRNA gene and the mitochondrial cytochrome oxidase I gene. We studied 18 species of Buccinidae that belong to eight different genera and inhabit the China coastal seas. We analyzed the patterns of divergence between an outgroup and basal ingroup taxa, the monophyly of the genus *Neptunea*, and the position of one unnamed species within the Buccinidae. A phylogenetic tree (neighbor-joining (NJ) method) was reconstructed based on the sequences of 18S rRNA, 28S rRNA and COI, with *Rapana venosa* as outgroup. The NJ tree indicated that the 18 species could be divided into five groups. The genus *Buccinum* was monophyletic, whereas *Neptunea* was shown to be paraphyletic since it included *Siphonalia subdilatata* and *Neptunea* sp., a new species. This novel species otherwise clustered consistently with *Neptunea cumingi* in three other phylogenetic trees, showing a low genetic distance and divergence percentage of sequences belonging to the genus *Neptunea*. A smaller genetic distance and a smaller difference of 18S rRNA, 28S rRNA and COI sequences between *Neptunea cumingii* and *Neptunea arthritica cumingii* confirmed them to be the same species.

Buccinidae, molecular phylogenetics, 18S rRNA gene, 28S rRNA gene, COI

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The whelk family Buccinidae contains hundreds of species, which inhabit the oceans of the world. Buccinidae whelks are carnivorous, some being predators of other mollusks and some being scavengers. These large whelks have great commercial value as seafood and the shells are used for ornamental purposes. For the management of these species, knowledge of intraspecific genetic variations are necessary to assess extinction risks, such as inbreeding, and their evolutionary potential in a changing world [1]. At present, the taxonomic status and the phylogenetic relationships of these species are still in dispute. In previous studies, Golikov de-

scribed 86 species and six subspecies from oceans worldwide [2], whereas Tiba and Kosuge described 107 species and nine subspecies from the North Pacific [3], and Higo et al. [4] described 68 species and 13 subspecies from the vicinity of Japan. It has been reported that 31 species of the family Buccinidae are distributed along the coast of China, but some species are threatened by extinction due to environmental deterioration in recent years [5–7]. Cai et al. [8] suggested that the families Buccinidae, Galeodiadae, Nassidae, Fascioliariidae and Columbelloidae belong to the superfamily Buccinoidea, but this term has not been used in recent years. *Buccinum perryi* was allocated to the genus *Buccinum*, but in subsequent studies, *Buccinum perryi* had been

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attributed to the genus *Volutharpa* and was renamed as *Volutharpa ampullaceal perryi* [7,8]. Some taxonomic studies on this family have been reported from the China coast.

The taxonomy of different species and families depends mostly on a high level of homoplasy observed in several morphological characters that are caused by similar lifestyles [9]. Phylogenetic relationships among buccinid species were initially established for several morphoanatomical characters, mainly with respect to characters of the shell [10]. However, more and more molecular markers are used to infer phylogenetic relationships among the Gastropoda (Mollusca) based on DNA and RNA sequences [11,12]. Phylogenetic relationships with a high potential in the absence of sufficient morphological characters can be resolved by molecular phylogenetic techniques according to coded characters [13]. A small-subunit ribosomal gene (18S rRNA) was shown to be an effective tool to estimate phylogenetic relationships in gastropods [14]. Thus, molecular distinctions of two otherwise identical abalone species were made using 18S rDNA sequences, while the phylogeny of the six vetigastropod subgroups and Littorinidae were studied based on 18S rRNA sequences [15–17]. Using the mitochondrial 16S rRNA gene, the phylogenies of eight species of the genus *Buccinum* and of 35 species of the family Buccinidae were analyzed by Iguchi et al. [18] and by Hayashi [19], respectively. 28S rRNA was also used to evaluate the major adaptive radiation of some buccinid life forms [20]. However, there is currently no report on molecular phylogenies of whelk species from the coast of China.

In the present study, the phylogeny of 18 species of the family Buccinidae was investigated using 18S rRNA, 28S rRNA and mitochondrial cytochrome oxidase I (COI) gene sequences. The aim was to assess: (1) the phylogenetic relationships of 18 species of the family Buccinidae; (2) the taxonomic status of 18 Chinese species of the family Buccinidae based on DNA sequences.

1 Materials and methods

1.1 Samples

The 15 species of the family Buccinidae used in this study were collected from the coast of China (Figure 1). The 18S rRNA, 28S rRNA and COI sequence data of 3 other species were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/>). The distribution, collection locations, GenBank accession numbers and abbreviations of the names of the 18 species and *Rapana venosa* as out group are shown in Table 1.

1.2 DNA isolation

Total genomic DNA of five individuals from each species was extracted from muscle tissue. The tissue was minced and lysed in 500 μ L TE buffer (10 mmol L⁻¹ Tris-HCl, 1 mmol L⁻¹ EDTA), after which 25 μ L 10% SDS and 5 μ L

Figure 1 Neighbor-joining (NJ) tree constructed from alignments of 18S rRNA sequences using the Kimura 2-parameter model for 15 Buccinidae species from China. Bootstrap values are shown above branches supported in at least 50% of 1000 replicates. Outgroup: RV, the Asian whelk *Rapana venosa*.

20 mg mL⁻¹ of proteinase K were added, respectively. After incubation for 3 h at 55°C, the suspension was extracted with a standard phenol-chloroform extraction protocol according to Sun et al. [21] and Hou et al. [22]. The genomic DNA was precipitated from the lysate with 100% ethanol, then washed twice with 75% ethanol and solubilized in 100 μ L of bidistilled water. DNA quality and quantity were determined by 0.8% agarose gel electrophoresis.

1.3 PCR amplification

Nearly complete sequences of the 18S rRNA and 28S rRNA genes and a partial sequence of mitochondrial DNA COI were amplified by polymerase chain reaction (PCR). The primer sequences are shown in Table 2. Each PCR had a reaction volume of 20 μ L and contained 2.5 μ L of 10 $\times$ LA PCR buffer (TaKaRa, Japan), 2.5 μ L of 0.2 mmol L⁻¹ of dNTP, 0.4 μ L of each primer, 0.1 μ L of 0.5 U LA *Taq* polymerase (TaKaRa), and 0.8 μ L genomic DNA. PCR was performed using a TC-412 PCR system. The temperature regime used for the amplification of 18S rRNA sequences comprised an initial denaturation step at 94°C for 5 min, followed by 35 circles consisting of a denaturation step at 94°C for 1 min, annealing at 60.5°C for 45 s, and extension at 72°C for 2 min. An additional extension step at 72°C for 10 min was added. The initial denaturation and final extension step of the PCR procedures of 28S rRNA and COI were similar to 18S rRNA. Thirty-five cycles were used for 28S, which included denaturation at 94°C for 35 s, annealing at 50°C for 30 s, and extension at 72°C for 90 s. In addition, 30 cycles were used for COI, which included denaturation at 94°C for 30 s, annealing at 40°C for 30 s, and extension at 72°C for 1 min. The remaining products were purified by a DNA Fragment Purification Kit Version 2.0 (TaKaRa).

1.4 Sequencing

The purified products were cloned using PMD18-T vectors,

Table 1 Distribution, collection locations, and GenBank accession numbers of 18 Buccinidae species used in this study

Genus	Species name	Distribution	Collection location	Abbreviation	18S rRNA	28S rRNA	COI
<i>Cantharus</i>	<i>C. cecillei</i>	South China Sea, Japan, Philippines	Dalian, China	CCI	EU236270.1	EU399879.1	JN053007.1
	<i>N. lyrata decemcostata</i>	North of Yellow Sea in China; North Korea; Japan	Dandong, China	NLD	EU236264.1	EU399877.1	EU883625.1
	<i>N. cumingi</i>	North of Yellow Sea in China; North Korea; Japan	Dalian, China Yantai, China Lianyungang, China	NC	HQ834010.1	EU399876.1	EU883627.1
<i>Neptunea</i>	<i>N. eulimata</i>	North of Yello sea in China; North Korea	Donggang, China	NE	EU236266.1	FJ710103.1	EU883634.1
	<i>N. sp</i>	North of Yellow Sea in China; North Korea	Zhuanghe, China	UN	Not submit	EU399882.1	EU883629.1
	<i>N. kuroshio</i>	East China Sea; South Korea	—	NK	—*	FJ710106.1	AB498772.1
<i>Siphonalia</i>	<i>N. arthritica cumingii</i>	North of Yellow Sea in China; North Korea; Japan	Dalian, China	NAC	GQ290549.1	—	Not submit
	<i>S. spadicea</i>	North of Yellow Sea in China; North Korea; Japan	Dalian, China	SSP	EU236268.1	EU399874.1	EU883626.1
	<i>S. subdilatata</i>	North of Yellow Sea in China; North Korea	Zhuanghe, China	SSU	EU236267.1	EU399878.1	EU883630.1
<i>Buccinum</i>	<i>B. pemphigum</i>	North of Yellow Sea in China; Japan	Dalian, China	BP	EU236269.1	EU399875.1	EU883628.1
	<i>B. yokomaruue</i>	North of Yellow Sea in China; North Korea	Dalian, China	BY	Not submit	—	EU883631.1
	<i>B. tsubai</i>	Japan; South Korea; North of Yellow Sea in China	—	BS	—	FJ712705.1	AB183327.1
<i>Japelon</i>	<i>B. striatissimum</i>	Japan; Korea; North of Bohai Sea in China	—	BST	—	AB439797.1	AB183328.1
	<i>J. latus</i>	North of Yellow Sea in China; Korea; Japan	Lüshun, China	JL	EU236271.1	EU399880.1	EU883633.1
	<i>B. areolata</i>	East China Sea; South China Sea; Japan	Xiamen, China	BA	EU236272.1	EU399873.1	EU883632.1
<i>Babylonia</i>	<i>B. lutosa</i>	East China Sea; South China Sea; Japan	Hainan, China	BL	HQ834022.1	Not submit	JN053010.1
	<i>P. ignea</i>	South China Sea in China; Philippines	Hainan, China	PI	Not submit	Not submit	Not submit
	<i>Volutharpa</i>	North of Yellow Sea in China; Japan	Dalian, China	VP	HQ834009.1	FJ710108.1	JN053003.1
<i>Rapana</i>	<i>R. venosa</i>	North of Yellow Sea in China; East China Sea	Zhuanghe, China Donggang, China	RV	HQ834011.1	HE584197.1	HE584367.1

* No sequence available in GenBank.

Table 2 Primers used in this study

Gene	Sequences	References
18S rRNA	5'-CCTggTTgATCCTgCCA-g-3'	[16]
	5'-TAATgATCCTTCCgCaggTTA-3'	
28S rRNA	5'-gACCCgTCTTgAAACACgga-3'	[20]
	5'-AgCCAATCCTTATCCCgAAg-3'	
COI	5'-ggTCAACAAATCATAAAgATATTgg-3'	
	5'-TAAACTTCagggtgACCAAAAAATCA-3'	

thus transforming DH5 α -*E. coli* cells. The plasmid was isolated from recombinant cells using the alkaline lysis method [23]. It was sequenced directly using the dye-terminator cycle sequencing reaction (PerkinElmer CO. USA) with an ABI PRISM™ 377XL DNA sequencer and ABI PRISM™ 3730XL DNA analyzer. Sequencing reactions were performed with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using the primers of two universal M13-47 and RV-M primers supplied with the kit (TaKaRa). The fragments were sequenced from both strands to obtain

accurate sequences. All final sequences were obtained from both strands for verification. All sequences were submitted to GenBank (<http://www.ncbi.nlm.nih.gov>).

1.5 Phylogenetic analyses

Sequences were aligned using ClustalX [24] and the extent of sequence difference between individuals was calculated by averaging pairwise comparisons of sequence differences across all individuals. For congruence evaluation the parti-

tion homogeneity test of the software program PAUP 4.0b [25] was used. Phylogenetic trees were constructed using the Neighbor-Joining tree provided by the software program MEGA 4.1 [26], based on the Kimura 2-parameter method [27]. The data was set to 1000 bootstraps in the test of inferred phylogenies to estimate the internal stability of the tree nodes.

2 Results

2.1 Characterization of 18S, 28S rRNA and COI gene sequences of 18 whelk species

The length of 18 species of 18S rRNA sequence ranged from 1828 bp in *Neptunea arthritica cumingii* to 2031 bp in *Buccinum pemphigum*, while the 28S rRNA sequence ranged from 686 bp in *Buccinum subai* to 1640 bp in *Japelon latus*,

and the COI sequences ranged from 490 bp in *Buccinum tsu-bai* and *Buccinum striatissimum* to 698 bp in *Neptunea* sp.

The genetic distance and the ratio of transitions/transversions and genetic distance of these 18 whelk species based on the 18S rRNA, 28S rRNA and COI gene sequences are listed in Tables 3–5, respectively. The experimental results showed that the longest distance between SSP and JL was 0.022 based on 18S rRNA, and the longest distance value between BST and CCI based on 28S rRNA was 0.372. In addition, the shortest distance based on 18S rRNA was 0.002 between BL and BA, NC and NAC, respectively. The shortest distance based on 28S rRNA was 0.003 between NLD and NK. Based on the COI sequences, the longest genetic distance was 0.465 between BA and BST and the shortest genetic distance was 0.000 between UN and NC, NC and NAC, UN and NAC and BA and BY, respectively (Table 5).

Table 3 Ratio of transitions/transversions (above diagonal) and genetic distances (below diagonal) under Kimura’s 2-parameter model of 15 species of the family Buccinidae inferred from 18S rRNA sequences

	NC	NAC	NLD	JL	BA	BL	SSU	UN	SSP	NE	BP	BY	CCI	VP	PI
NC		3/0	10/1	8/9	11/5	11/5	25/10	24/9	20/10	18/8	22/9	18/9	18/9	9/3	18/6
NAC	0.002		7/1	5/9	8/5	8/5	22/10	21/9	17/10	15/8	19/9	15/9	15/9	6/3	15/6
NLD	0.007	0.005		10/10	13/6	13/6	27/11	26/10	22/11	20/9	24/10	20/10	20/10	11/4	18/7
JL	0.012	0.010	0.015		9/8	9/8	25/13	24/12	20/13	18/13	20/12	16/12	16/12	7/6	15/11
BA	0.010	0.008	0.014	0.012		0/0	26/9	25/8	21/9	19/9	21/8	17/8	17/8	8/4	15/7
BL	0.010	0.008	0.014	0.012	0.000		26/9	25/8	21/9	19/9	21/8	17/8	17/8	8/4	15/7
SSU	0.014	0.012	0.017	0.019	0.014	0.014		11/1	7/2	9/4	15/3	11/3	11/3	17/9	31/11
UN	0.014	0.012	0.017	0.019	0.014	0.014	0.000		6/1	8/3	14/2	10/2	10/2	16/8	30/10
SSP	0.017	0.015	0.020	0.022	0.017	0.017	0.003	0.003		4/4	10/3	6/3	6/3	12/9	26/11
NE	0.015	0.014	0.019	0.020	0.015	0.015	0.005	0.005	0.008		10/3	6/3	6/3	10/7	24/9
BP	0.019	0.017	0.022	0.020	0.015	0.015	0.008	0.008	0.012	0.010		8/2	8/2	12/8	26/10
BY	0.014	0.012	0.017	0.015	0.010	0.010	0.003	0.003	0.007	0.005	0.005		0/0	8/8	26/10
CCI	0.014	0.012	0.017	0.015	0.010	0.010	0.003	0.003	0.007	0.005	0.005	0.000		8/8	22/10
VP	0.008	0.007	0.012	0.010	0.005	0.005	0.008	0.008	0.012	0.010	0.010	0.005	0.005		14/5
PI	0.014	0.012	0.017	0.014	0.007	0.007	0.017	0.017	0.020	0.019	0.019	0.014	0.014	0.008	

Table 4 Ratio of transitions/transversions (above the diagonal) and genetic distances (below the diagonal) of 28S rRNA sequences from 15 Buccinidae species

	VP	BS	NK	UN	NLD	NE	SSU	JL	BA	SSP	BP	BL	NC	BST	CCI
VP		6/3	13/8	17/22	17/22	17/22	16/21	17/22	17/22	17/22	17/21	17/22	16/22	15/21	16/23
BS	0.115		13/8	16/22	17/21	16/22	16/21	17/22	17/22	17/21	17/21	17/21	16/21	16/20	16/23
NK	0.259	0.253		17/22	17/21	17/22	17/21	17/22	17/22	17/21	18/21	18/21	17/21	16/21	16/22
UN	0.215	0.235	0.010		3/1	3/2	5/2	7/14	12/8	6/12	11/8	12/10	12/7	16/26	16/26
NLD	0.012	0.327	0.003	0.015		4/1	6/1	8/15	13/7	7/11	9/9	10/11	13/6	16/26	16/25
NE	0.021	0.210	0.015	0.010	0.015		4/0	5/15	14/7	7/11	9/10	10/12	14/8	16/26	16/25
SSU	0.015	0.220	0.018	0.119	0.015	0.009		7/11	14/4	7/9	12/7	13/9	15/7	16/26	16/25
JL	0.217	0.256	0.215	0.211	0.126	0.210	0.210		11/16	9/15	13/12	13/14	17/16	17/27	16/26
BA	0.219	0.239	0.281	0.215	0.221	0.215	0.215	0.226		6/9	10/7	11/9	19/9	17/26	16/26
SSP	0.231	0.271	0.310	0.210	0.215	0.210	0.210	0.221	0.265		8/6	9/8	15/13	17/26	16/26
BP	0.187	0.023	0.221	0.136	0.231	0.186	0.136	0.247	0.231	0.326		2/0	20/13	17/26	16/26
BL	0.192	0.265	0.275	0.231	0.226	0.231	0.231	0.242	0.026	0.221	0.205		21/15	17/26	16/26
NC	0.219	0.216	0.126	0.015	0.021	0.226	0.126	0.236	0.231	0.226	0.253	0.347		16/26	16/25
BST	0.292	0.009	0.167	0.255	0.269	0.217	0.297	0.279	0.233	0.255	0.291	0.291	0.287		16/23
CCI	0.321	0.275	0.316	0.311	0.305	0.326	0.326	0.013	0.287	0.305	0.366	0.366	0.323	0.372	

Table 5 The ratio of transitions/transversions (above the diagonal) and genetic distances (below the diagonal) of COI sequences from 18 Buccinidae species

	NC	UN	NAC	NE	SSU	NLD	NK	BP	BY	BS	BST	VP	PI	CCI	JL	SSP	BA	BL
NC		0/0	0/0	25/2	31/2	36/2	36/4	39/22	39/22	42/21	32/31	40/22	32/31	45/30	39/22	42/24	44/40	46/48
UN	0.000		0/0	25/2	31/2	36/2	36/4	39/22	39/22	42/21	32/31	40/22	32/31	45/30	39/22	42/24	44/40	46/48
NAC	0.000	0.000		25/2	31/2	36/2	36/4	39/22	39/22	42/21	32/31	40/22	32/31	45/30	39/22	42/24	42/42	46/48
NE	0.114	0.114	0.114		21/2	30/2	29/4	38/22	38/22	39/21	36/31	37/22	36/31	46/30	35/22	40/24	48/40	44/50
SSU	0.131	0.131	0.131	0.091		36/2	33/2	47/20	47/20	44/19	41/31	44/20	41/31	52/30	39/22	48/24	47/38	50/48
NLD	0.139	0.139	0.139	0.114	0.140		40/4	46/22	46/22	44/21	41/31	38/22	41/31	48/30	45/22	41/24	52/40	53/48
NK	0.155	0.155	0.155	0.138	0.156	0.155		49/20	49/20	50/19	41/29	43/20	41/29	49/30	39/20	49/22	47/38	48/46
BP	0.210	0.210	0.210	0.210	0.269	0.219	0.239		0/0	35/5	35/5	38/10	45/35	53/36	43/20	46/30	47/38	50/44
BY	0.210	0.210	0.210	0.210	0.269	0.219	0.239	0.000		35/5	35/5	38/10	45/35	53/36	43/20	46/30	47/38	50/44
BS	0.257	0.257	0.257	0.257	0.279	0.228	0.312	0.165	0.165		30/4	35/9	39/34	45/33	43/19	45/25	56/39	58/45
BST	0.239	0.239	0.239	0.312	0.337	0.323	0.303	0.222	0.222	0.166		42/7	45/34	53/35	50/17	51/25	52/39	57/45
VP	0.238	0.238	0.238	0.247	0.290	0.210	0.239	0.180	0.180	0.208	0.271		43/33	44/34	38/20	44/24	46/37	47/45
PI	0.224	0.224	0.224	0.251	0.270	0.289	0.253	0.262	0.262	0.243	0.283	0.266		36/15	31/21	40/25	47/39	51/47
CCI	0.334	0.334	0.334	0.334	0.408	0.323	0.370	0.343	0.343	0.324	0.393	0.316	0.213		37/26	47/26	50/47	54/55
JL	0.235	0.235	0.235	0.226	0.255	0.284	0.210	0.257	0.257	0.267	0.312	0.230	0.160	0.247		35/26	48/40	51/48
SSP	0.253	0.253	0.253	0.253	0.304	0.262	0.307	0.244	0.244	0.245	0.305	0.275	0.227	0.340	0.216		42/36	43/44
BA	0.361	0.361	0.361	0.405	0.394	0.420	0.351	0.410	0.410	0.491	0.465	0.377	0.389	0.481	0.363	0.341		4/8
BL	0.391	0.391	0.391	0.437	0.425	0.452	0.380	0.419	0.419	0.515	0.488	0.394	0.431	0.529	0.393	0.359	0.026	

2.2 Analysis of phylogeny based on the 18S rRNA, 28S rRNA and COI genes sequences

A neighbor-joining (NJ) tree was constructed using genetic distances by means of the Kimura 2-parameter [27]. Phylogenetic trees of the 15 species were constructed by using 18S rRNA (Figure 1) and 28S rRNA sequences (Figure 2), whereas a phylogenetic tree of 18 species was inferred from COI gene sequences (Figure 3), respectively.

In the 18S rRNA, 28S rRNA and COI sequences, transitions and transversions were the main form of transformations of bases. The average ratios of transitions to transversions were 2.52, 1.01 and 2.56, respectively. The nucleotide compositions were T: 25.2%, C: 22.9%, A: 24.3%, G:

Figure 2 Neighbor-joining (NJ) tree constructed from alignments of 28S rRNA sequences using the Kimura 2-parameter model for 15 Buccinidae species from China. Bootstrap values are shown above branches supported in at least 50% of 1000 replicates. Outgroup: RV, the Asian whelk *Rapana venosa*. **Figure 3** Neighbor-joining (NJ) tree constructed from alignments of COI sequences using the Kimura 2-parameter model for 18 Buccinidae species from China. Bootstrap values are shown above branches supported in at least 50% of 1000 replicates. Outgroup: RV, the Asian whelk *Rapana venosa*.

27.6% in the 18S rRNA sequence, T: 23.9%, C: 31.4%, A: 20.2%, G: 24.5% in the 28S rRNA sequence, and T: 37.3%, C: 17.6%, A: 25.9%, G: 19.2% in the COI sequence.

The three resulting phylogenetic trees were very similar. In the NJ tree resulting from all three gene sequences the individuals were divided into five groups: *Neptunea eulimata* (NE), *Siphonalia subdilata* (SSU), *Neptunea lyrata decemcostata* (NLD), *Neptunea cumingi* (NC). *Neptunea arthritica cumingii* (NAC) and an unnamed species (UN) clustered as group 1; *Volutharpa perryi* (VP) *Buccinum pemphigum* (BP), *Buccinum yokomaru* (BY), *Buccinum tsubai* (BS) and *Buccinum striatissimum* (BST) were grouped in group 2; group 3 accommodated *Japelon latus* (JL) *Pisania ignea* (PI) and *Cantharus cecillei* (CCI); group

4 included only *Siphonalia spadicea* (SSP); group 5 contained *Babylonia areolata* (BA) and *Babylonia lutosa* (BL). The unnamed species formed a sister clade with NC in all three phylogenetic trees. BY and BP of the genus *Buccinum* were consistently in the same group. However, SSU did not cluster with SSP, although they belonged to the same genus.

To detect the differences within species, the phylogenetic tree of the 50 individuals of 10 most common buccinid species was inferred from 28S rRNA gene sequences. A neighbor-joining (NJ) tree was constructed using genetic distances by means of the Kimura 2-parameter [27] (Figure 4).

3 Discussion

18S rRNA is very conservative gene and has therefore been suggested by some researchers as a phylogenetic marker to trace the relationships among distantly related taxa [15,16, 28–30]. The 28S rRNA gene has a low transitional ratio and is more conservative in evolutionary transitions. This makes them more suitable for the study of phylogenetic relationships of taxa higher than the genus level [31]. However, the 18S rRNA sequence has also been shown to be useful in the phylogenetic analyses of congeneric species [32–37]. By

using single sequences for the evaluation of phylogenies, resulting information seems limited, and the results are arbitrary. In order to avoid conflicting phylogenies among 18S rRNA, 28S rRNA and COI gene sequences, combined sequences are generally used for phylogenetic reconstructions [38]. In an earlier study, phylogenetic relationships among 21 species of stromateoid fishes, representing five families and 13 genera, were reconstructed using 3263 bp of mitochondrial DNA sequences, including the posterior half of the 16S rRNA and the entire COI and Cytb genes [39]. The experimental results of the present study showed no conflicts among the 18S rRNA, 28S rRNA, and COI gene sequences. This provides evidence for the reliability and usefulness of combining the 18S rRNA, 28S rRNA, and COI gene sequences as molecular markers in the present study. However, since 18S rRNA or 28S rRNA sequences were not available for some species, an analysis of the phylogeny of all 18 species of the family Buccinidae could not be carried out using the combined sequence information.

Knight and Mindell suggested that a mutant gene sequence reaches saturation if the ratio of transitions/ transversions (Ts/Tv) was less than 2.0 [40]. In the present study, the Ts/Tv value of the 18S rRNA gene sequence was 1.015 as determined by MEGA 4.0, which means that it was

Figure 4 Concatenated neighbor-joining (NJ) tree constructed from alignments of 18S rRNA sequences using the Kimura 2-parameter model for 50 individuals of 10 common Buccinidae species from China.

evolutionarily saturated. This result was probably caused by a single or multiple transitions, leading to the accumulation of transitions that finally reached an equilibrium [41].

For the phylogeny of 18 Buccinidae whelk species from China, the species in group 1 were distributed along the east coast of the Liaoning Peninsula and north of the Yellow sea. The unnamed species was allocated to the genus *Neptunea* based on this phylogenetic analysis, as the three phylogenetic trees indicated that this species belonged to the genus *Neptunea*. The genetic distances between the unnamed species and other species of the genus *Neptunea* were both at the congeneric level. Consequently, this species should be included in this genus and has been named *Neptunea* sp. until described as a new species.

It is worth mentioning that SSU clustered with NE (bootstrap value=85), and was allocated to the genus *Neptunea*, which conflicts with its contemporary taxonomic status. Although SSU showed a larger distance to SSP than to other species of *Neptunea*, they both belong to the same genus. This is in accordance with major biological features, such as the shell shape of *Neptunea* species. Conventional characters also indicate that the genus *Neptunea* has a close evolutionary relationship to the genus *Siphonalia*. The noticeable factor that NE and SSP had a very similar shell shape also supported this conclusion.

BP, BY, BST and BS belong to the genus *Buccinum*. Because of the minimal genetic distances among the species, they are clustered in one clade in all phylogenetic trees. They therefore belong to the monophylum *Buccinum*.

JL was only distributed in the area near Lüshun where it was an original species, and where it evolved independently in the north of China. The phylogenetic tree, which was derived from 18S rRNA, 28S rRNA, and COI gene sequences showed that the genus *Japelion* had a closer relationship with the genus *Neptunea*. Both genera are found in the north of China.

BA and BL were distributed along the southern coast of China with a distant relationship to other species in the north of China (Figures 1–3). These results indicate that this geographic distance could explain the resulting phylogeny due to its effect on geographic isolation, genetic drift and phylogeny [42].

Qi et al. [7] and Okutan [43] suggested that *Neptunea arthritica cumingii* is a subspecies of *Neptunea arthritica*, whereas Min considered *Neptunea cumingi* as a unique species [44]. In the present study, a shorter genetic distance and a smaller difference of 18S rRNA, 28S rRNA and COI sequences between *Neptunea cumingi* and *Neptunea arthritica cumingii* demonstrated that they belong to the same species.

We conclude that the sequences of 18S rRNA, 28S rRNA and COI are appropriate as molecular markers to study relatively recent cladogenetic events among the family Buccinidae. This became evident by studying some aspects of the phylogeny of the Buccinidae. Some controversial issues, such as the paraphyletic phylogeny of the Buccinidae and

particularities of their evolutionary history, should be further analyzed in future studies.

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