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Recent vicariant and dispersal events affecting the phylogeny and biogeography of East Asian freshwater crab genus *Nanhaipotamon* (Decapoda: Potamidae)

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ABSTRACT

The molecular phylogeny and biogeography of the East Asian freshwater crabs of the genus *Nanhaipotamon* (Decapoda: Brachyura: Potamidae) were studied, using two mitochondrial (16S rRNA and cytochrome oxidase I) and one nuclear (28S rRNA) markers, and correlated with various vicariant and dispersal events which have occurred in this region. The results showed *Nanhaipotamon* to be a monophyletic taxon with four clades which correspond to the topography of the coastal region of southeastern China and Taiwan Island. Mountains appear to play an important role in the distribution. The genus occurs only from east of the Wuyishan Range (Zhejiang and Fujian) and south of the Nanling Range (Guangdong) in southern China, and is also present west of the Central Range in Taiwan. The molecular and geological data suggest that *Nanhaipotamon* originated in an area between the Wuyishan and Nanling Ranges. In this area, the main and earliest cladogenesis occurred at ~4.8 million years ago (mya), with speciation probably taking place at around 4 mya. The molecular evidence strongly supports the recent invasion of the genus into Taiwan Island from northeastern Fujian, via the paleo-Minjiang River on the landbridge of Taiwan Strait. The presence of the genus in Dongyin Island, however, is through invasion from southeastern Zhejiang, during the Pleistocene glaciation period. *Nanhaipotamon* reached Taiwan and Dongyin Island at ~1.0 and 0.4 mya, respectively. A small population of *Nanhaipotamon formosanum* from Penghu Islands (Pescadores) in the central Taiwan Strait has a slightly different genetic constitution and suggests it is a relict of past Pleistocene glaciations.

1. Introduction

True freshwater crabs spend their entire life cycle within wholly freshwater or terrestrial environments and are effectively landlocked. They have no larvae, with the fertilized eggs developing directly into juvenile crabs that are cared for by the mother (Ng, 1988; Yeo et al., 2008). The relatively low fecundity and poor dispersal abilities of freshwater crabs (Daniels et al., 2003; Yeo et al., 2008) mean that they are easily isolated by barriers like mountains or seas. Geographically isolated populations then become genetically distinct and result in allopatric speciation (Shih et al., 2006; Yeo et al., 2007).

While most freshwater crab species in the main island of Taiwan belong to the potamid genera *Geothelphusa* and *Candidiopotamon* (Shy et al., 1994; Shy and Yu, 1999), there are two other genera: *Somanniathelphusa* (Gecarcinucidae) and *Nanhaipotamon* (Potamidae), which are each represented by a single species. The latter two genera are more speciose in continental Asia (Dai, 1997, 1999; Shy and Yu, 1999; Yeo and Ng, 1999; Shih et al., 2005, 2008). *Nanhaipotamon* Bott, 1968, was originally established for several species from Taiwan, Philippines and Ryukyus (Japan) but was revised by Ng and Shokita (1995), and is now restricted to taxa from continental China and Taiwan. This taxonomic separation has been supported by molecular studies (Shih et al., 2009) and the genus now contains 13 species (Parisi, 1916; Shen, 1940; Dai, 1997, 1999; Shy and Yu, 1999; Cheng et al., 2003, 2009; Shih et al., 2005, 2008; Ng et al., 2008) (cf. Table 1). At present, *Nanhaipotamon* occurs only in western Taiwan, east of the Wuyishan Range (Zhejiang and Fujian) and south of the Nanling Range (Guangdong) in southern China (Fig. 1). *Nanhaipotamon* species dig deep burrows in moist soil in forested areas or near rice paddies and gardens, usually below 500 m above sea level (Ng and Dudgeon, 1992; Dai, 1999; Shih et al., 2005).

The affinity of the Taiwanese *Nanhaipotamon formosanum* was studied by Shih et al. (2005), who suggested that *Nanhaipotamon dongyinense* reached Dongyin Island (I.) (an offshore island in the northern Taiwan Strait; Fig. 1) by dispersal through a connecting landbridge during a glaciation event in the recent past. In addition, Taiwan I. was repeatedly connected to China by landbridges during

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Table 1
Haplotypes of 16S rRNA, COI and 28S rRNA genes of the genus *Nanhaipotamon* from Taiwan, Hong Kong, Chinese coastal provinces (Guangdong, Fujian and Zhejiang), and outgroups. The numbers within brackets behind localities correspond to those in Fig. 1.

Species (and distribution)	Localities[a]	Museum catalogue No. [b]	Haplotypes of 16S	DDBJ access. No.	Haplotypes of COI	DDBJ access. No.	Haplotypes of 28S	DDBJ access. No.
N. formosanum (Parisi, 1916) (Taiwan)	Jiji, Nantou Co., Taiwan [1]	NCHUZOOL 13140	Ntw1	AB212864	Ntw-C1	AB433557		
	Dounan, Yunlin Co., Taiwan [2]	NCHUZOOL 13141	Ntw2	AB212865	Ntw-C2	AB433558		
	Dounan, Yunlin Co., Taiwan [2]	NCHUZOOL 13242	Ntw3	AB212866	Ntw-C2	AB433558	Ntw-S1	AB551395
	Dounan, Yunlin Co., Taiwan [2]	NCHUZOOL 13243	Ntw3	AB212866	Ntw-C3	AB470502		
	Botanical Garden, Chiayi City, Taiwan [3]	NCHUZOOL 13142	Ntw3	AB212866	Ntw-C4	AB433559		
	Botanical Garden, Chiayi City, Taiwan [3]	NCHUZOOL 13143	Ntw3	AB212866	Ntw-C5	AB433560		
	Chiayi Univ., Chiayi City, Taiwan [3]	NCHUZOOL 13244	Ntw4	AB470493	Ntw-C2	AB433558		
	Chiayi Univ., Chiayi City, Taiwan [3]	NCHUZOOL 13245	Ntw5	AB212867	Ntw-C7	AB470503		
	Yongkang, Tainan Co., Taiwan [4]	NCHUZOOL 13144	Ntw5	AB212867	Ntw-C6	AB433561		
	Houjia, Tainan City, Taiwan [4]	NCHUZOOL 13246	Ntw5	AB212867	Ntw-C8	AB470504	Ntw-S1	AB551395
	Husi, Penghu Co., Taiwan [5]	NCHUZOOL 13233	Ntw6	AB470494	Ntw-C9	AB470505	Ntw-S2	AB551396
N. dongyinense Shih et al., 2005 (Dongyin I., Taiwan Strait)	Dongyin, Matsu, Taiwan [6]	IZCAS 0401	Ndy1	AB212863	Ndy-C1	AB433562		
	Dongyin, Matsu, Taiwan [6]	NCHUZOOL 13145	Ndy1	AB212863	Ndy-C2	AB433563		
	Dongyin, Matsu, Taiwan [6]	NMNS 4557–009	Ndy1	AB212863	Ndy-C1	AB433562	Ndy-S	AB551397
	Dongyin, Matsu, Taiwan [6]	NMNS 4557–003	Ndy2	AB470495	Ndy-C3	AB470506	Ndy-S	AB551397
N. wenzhouense Dai, 1997 (Zhejiang, China)	Yongjia, Wenzhou, Zhejiang Prov., China [7]	NCHUZOOL 13132	Nwz	AB433543	Nwz-C1	AB433564	Ndy-S	AB551397
	Yongjia, Wenzhou, Zhejiang Prov., China [7]	NCHUZOOL 13247	Nwz	AB433543	Nwz-C2	AB470507		
N. nanriense Dai, 1997 (Fujian, China)	Nanri Is., Putian City, Fujian, China [8]	IZCAS CB05103	Nnr1	AB212868	Nnr-C1	AB433565		
	Shoushan, Fuzhou City, Fujian Prov., China [9]	NCHUZOOL 13133	Nnr2	AB433544	Nnr-C2	AB433566		
	Shoushan, Fuzhou City, Fujian Prov., China [9]	NCHUZOOL 13134	Nnr3	AB433545	Nnr-C2	AB433566	Nnr-S	AB551398
	Shoushan, Fuzhou City, Fujian Prov., China [9]	NCHUZOOL 13248	Nnr3	AB433545	Nnr-C3	AB470508		
N. yongchuense Dai, 1997 (Fujian, China)	Yongchun Co., Fujian Prov., China [10]	IZCAS CB 05104	Nyc	AB433546	Nyc-C	AB433567	Nyc-S	AB551399
N. sp. 1	Nanan, Quanzhou, Fujian Prov., China [11]	NCUDP	Nsp1	AB433547	Nsp1-C	AB433568	Nsp1-S	AB551400
N. hongkongense (Shen, 1940) (Hong Kong)	New Territories, Hong Kong [12]	ZRC	Nhk	AB212869	Nhk-C1	AB433574		
	New Territories, Hong Kong [12]	ZRC 1991.1778–1783	Nhk	AB212869	Nhk-C2	AB470509	Nhk-S	AB551401
N. sp. 2	Dongguan, Guangdong Prov., China [13]	NCHUZOOL 13138	Nsp2	AB433554	Nsp2-C	AB433575	Nhk-S	AB551401
N. wupingense Cheng et al., 2003 (Fujian, China)	Xiaba, Wuping, Fujian Prov., China [14]	NCUDP	Nwp1	AB433548	Nwp-C1	AB433569	Nwp-S	AB551402
	Xiaba, Wuping, Fujian Prov., China [14]	NCUDP	Nwp2	AB470496	Nwp-C2	AB470510		
	Xiaba, Wuping, Fujian Prov., China [14]	NCUDP	Nwp2	AB470496	Nwp-C3	AB470511		
N. sp. 3	Shajian, Huaan Co., Fujian, China [15]	NCHUZOOL 13126	Nsp3	AB433550	Nsp3-C1	AB433570		
	Shajian, Huaan Co., Fujian, China [15]	NCHUZOOL 13127	Nsp3	AB433550	Nsp3-C2	AB433571		
	Shajian, Huaan Co., Fujian, China [15]	NCHUZOOL 13249	Nsp3	AB433550	Nsp3-C3	AB470512	Nsp3-S	AB551403
N. pingyuanense Dai, 1997 (Guangdong, China)	Renju, Pingyuan, Guangdong Prov., China [16]	IZCAS CB 05131	Npy1	AB265237	Npy-C1	AB265249		
	Renju, Pingyuan, Guangdong Prov., China [16]	NCUDP GD020	Npy2	AB470497	Npy-C2	AB470513	Npy-S1	AB551404
	Chagan, Pingyuan, Guangdong Prov., China [16]	NCUDP GD026	Npy3	AB470498	Npy-C3	AB470514	Nwp-S	AB551402
	Dapu, Guangdong Prov., China [17]	NCUDP GD007, 018	Npy4	AB470499	Npy-C4	AB470515	Npy-S2	AB551405

	Yinjiang, Dapu, Guangdong Prov., China [17]	NCUDP GD014	Npy5	AB470500	Npy-C5	AB470516	Npy-S2	AB551405
	Bingcuen, Meixian, Guangdong Prov., China [19]	NCUDP GD010	Npy6	AB470501	Npy-C6	AB470517	Nwp-S	AB551402
N. huaanense Dai, 1997 (Fujian, China)	Gaoan, Huaan Co., Fujian Prov., China [18]	IZCAS CB 05105	Nha	AB212870	Nha-C	AB433572		
N. hepingense Dai, 1997 (Guangdong, China)	Heping Co., Guangdong Prov., China [20]	IZCAS CB 05106	Nhp	AB433552			Nhp-S	AB551406
N. pinghense Dai, 1997 (Guangdong, China)	Heping Co., Guangdong Prov., China [20]	IZCAS CB 05132	Nhp	AB433553				
Outgroups								
Huananpotamon angulatum (Dai and Lin, 1979)	Dongqiao, Minqing Co., Fujian Prov., China	NCHUZOOL 13139	HUag	AB433555	HUag-C	AB433576	HUag-S	AB576807
H. nanchengense Dai, Zhou and Peng, 1995	Xiaowan, Nancheng, Jiangxi, China	NCUDP	HUnc	AB551390	HUnc-C	AB551392		
Geothelphusa dehaani (White, 1847)	Koyama, Tottori, Japan	NCHUZOOL 13192	Gd	AB551391	Gd-C	AB551393		
G. olea Shy et al., 1994	Botanical Garden, Chiayi City, Taiwan	NCHUZOOL 13009	Go	AB266150	Go-C	AB266263	Go-S	AB576808
Candidiopotamon rathbunae (De Man, 1914)	Wulai, Taipei Co., Taiwan	NCHUZOOL 13146	Cr-1	AB208591	Cr-C1	AB433579	Cr-S	AB576809
	Daren, Taitung Co., Taiwan	TMCD	Cr-2	AB208617	Cr-C2	AB551394		

[a] Co., County; Prov., Province.
[b] IZCAS: Institute of Zoology, Chinese Academy of Sciences, Beijing, China; NCHUZOOL: Zoological Collections of the Department of Life Science, National Chung Hsing Univ., Taichung, Taiwan; NCUDP: the Department of Parasitology, Nanchang Univ., Jiangxi, China; TMCD: National Taiwan Museum, Taipei, Taiwan; ZRC: Zoological Reference Collection, Raffles Museum, National Univ. of Singapore.

past glacial periods (Boggs et al., 1979; Voris, 2000). This route has also been suggested as the way in which the ancestors of the Taiwanese *Somanniathelphusa taiwanensis* dispersed from Fujian in China (Shih et al., 2007). Therefore, we hypothesize that the ancestors of the endemic Taiwanese *N. formosanum* dispersed from China by a similar route (i.e., the paleo-Minjiang River [R.] on the landbridge of Taiwan Strait). Their present distribution supports this; both genera are restricted to lowland areas in western Taiwan (cf. Fig. 1).

Nanhaipotamon species have very restricted distributions, and this is almost due to isolation caused by the emergence of mountain ranges and/or past sea level rises. The terrain the Chinese *Nanhaipotamon* now inhabit are geologically relatively stable with little orogenic activity during the Cenozoic (Yi, 1996; Zhou and Li, 2000), except for small scale mountain deformations in the region around southwestern Fujian and northeastern Guangdong about 5 million years ago (mya) (Xu and Xie, 2005). We therefore hypothesize that cladogenesis of *Nanhaipotamon* occurred in this area during this period.

The present study focuses on the phylogeny and biogeography of *Nanhaipotamon* using the mitochondrial markers 16S rRNA and cytochrome oxidase I (COI), and the nuclear gene 28S rRNA. Based on the phylogenetic analyses, divergence time estimation and geological events, we aim to: (1) investigate phylogenetic relationships among *Nanhaipotamon* species; (2) test the hypothesis that Taiwanese *N. formosanum* dispersed from China through the landbridge of Taiwan Strait during glaciation; and (3) determine the possible center of origin of this genus.

2. Materials and methods

2.1. Specimen collection

Specimens from Taiwan, Zhejiang, Fujian and Guangdong were recently collected and preserved in 75–95% ethanol, and deposited in museums or universities in Taiwan and China (Table 1). The remaining specimens used were from the museum collections (IZCAS and ZRC in Table 1). Muscle tissues of all known species of *Nanhaipotamon* (except *Nanhaipotamon aculatum* Dai, 1997 [Hong Kong] and *Nanhaipotamon xiapuense* Cheng et al., 2009 [Xiapu, Fujian, China]) were utilized for DNA extraction. No DNA could be extracted from the holotype of *Nanhaipotamon guangdongense* Dai, 1997 (IZCAS CB05141), its hard tissues suggesting it was originally preserved in formalin. The specimen of *N. guangdongense* was provided by Zhongshan Medical University, Guangdong (X.-M. Zhou, personal observation), but without locality or collection date. The COI sequence also could not be obtained for *Nanhaipotamon hepingense* and *Nanhaipotamon pinghense* even after several attempts (including different combinations of primers, internal primers and lower annealing temperatures), and is probably due to the poor condition and age of the specimens (both collected on 7 May 1965). *Huananpotamon*, *Geothelphusa* and *Candidiopotamon* were selected as outgroup taxa based on the overall phylogenetic study of the Potamidae (Shih et al., 2009) (Table 1).

2.2. DNA extraction, amplification and sequencing

Genomic DNA was isolated from the muscle tissue of legs by using the GeneMark tissue and cell genomic DNA purification kit (Taichung, Taiwan). A region of ~550 basepairs (=bp) of the 5′-end of the 16S gene was selected for amplification with polymerase chain reaction (PCR) using the primers 1471 and 1472 (Crandall and Fitzpatrick, 1996). A portion of the COI gene was amplified with PCR using the primers LCO1490 and HCO2198 (Folmer et al., 1994). The PCR conditions for the above primers were denaturation for 50 s

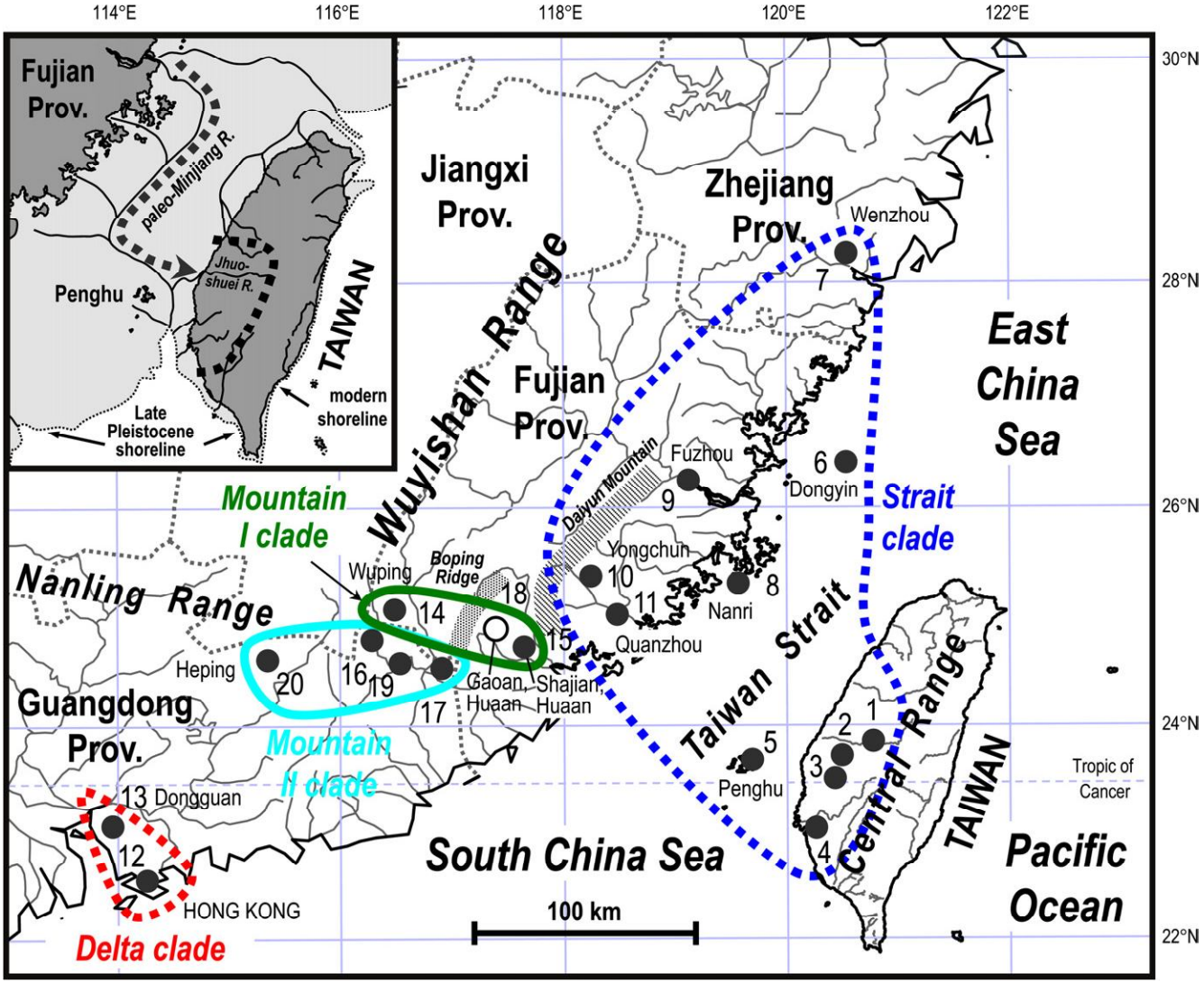

Fig. 1. Collection sites (gray circles) for the genus *Nanhaipotamon* from Taiwan, Hong Kong and the Chinese coastal provinces (Guangdong, Fujian and Zhejiang) used in this study. The numbers beside circles represent the collection sites in Table 1. The open circle (No. 18) means the locality of *N. huaanense* is uncertain (see Section 4.4). The different lines indicate the possible biogeographic boundaries for clades based on the molecular results (Figs. 2 and 3) in our study. Inserted figure indicates a postulated paleo-drainage system on the Taiwan continental shelf during glaciation in Late Pleistocene. Sea level is assumed to be 140 m below the present level (modified from Boggs et al., 1979). The broken line in west-central Taiwan indicates the present distribution of *Nanhaipotamon formosanum*. The arrow from China to Taiwan indicates the possible dispersal pathway of the *N. formosanum* subclade during glaciation. Prov., Province.

at 94 °C, annealing for 70 s at 45–47 °C, and extension for 60 s at 72 °C (40 cycles), followed by extension for 10 min at 72 °C. The primers for 28S were 28L4 and 28H4 (Ragionieri et al., 2009) with the annealing temperature being 47–50 °C in PCR condition. Because of the low variability of 28S compared with 16S and COI (Ragionieri et al., 2009), this gene was sequenced from 19 specimens representing the available species (Table 1). Sequences were obtained by automated sequencing (Applied Biosystems 3730) and were aligned with the aid of ClustalW (v. 1.4, Thompson et al., 1994), after verification with the complimentary strand. Sequences of the different haplotypes have been deposited in the DNA Data Bank of Japan (DDBJ) (accession numbers shown in Table 1).

2.3. Phylogenetic analyses

For a combined analysis of mitochondrial (16S and COI) and nuclear (28S) markers, phylogenetic congruence among the two dataset partitions was tested under the maximum parsimony (MP) criterion using the incongruent length difference (ILD) test (Farris et al., 1994) implemented in the PAUP* program (v. 4.0b10, Swofford, 2003) as the partition homogeneity test. The parameters included 1000 reiterations of a heuristic search with 100 randomly added sequence replications, TBR branch-swapping, using Steepest Descent and the MULTREES option enabled. The topologies of the two data sets were congruent ($P = 0.892$) and as such, the sequences were combined.

For the combined 16S, COI and 28S dataset, the best-fitting models for sequence evolution of individual datasets were determined by MrModeltest (v. 2.2, Nylander, 2005), selected by the Akaike information criterion (AIC). The obtained best models were GTR + G, GTR + I + G and GTR + I, respectively, and were subsequently used for the partitioned Bayesian inference (BI) analysis. For the combined 16S and COI dataset, both the best models of individual datasets were GTR + I + G and were partitioned in BI analysis. The BI analysis was performed with MrBayes (v. 3.1.1, Ronquist and Huelsenbeck, 2003). The search was run with 4 chains for 10 million generations and 4 independent runs, with trees sampled every 1000 generations. The convergence of chains was determined by the effective sample size (ESS) (>200 as recommended) in Tracer (v. 1.5, Rambaut and Drummond, 2009) and the first 1000 trees were

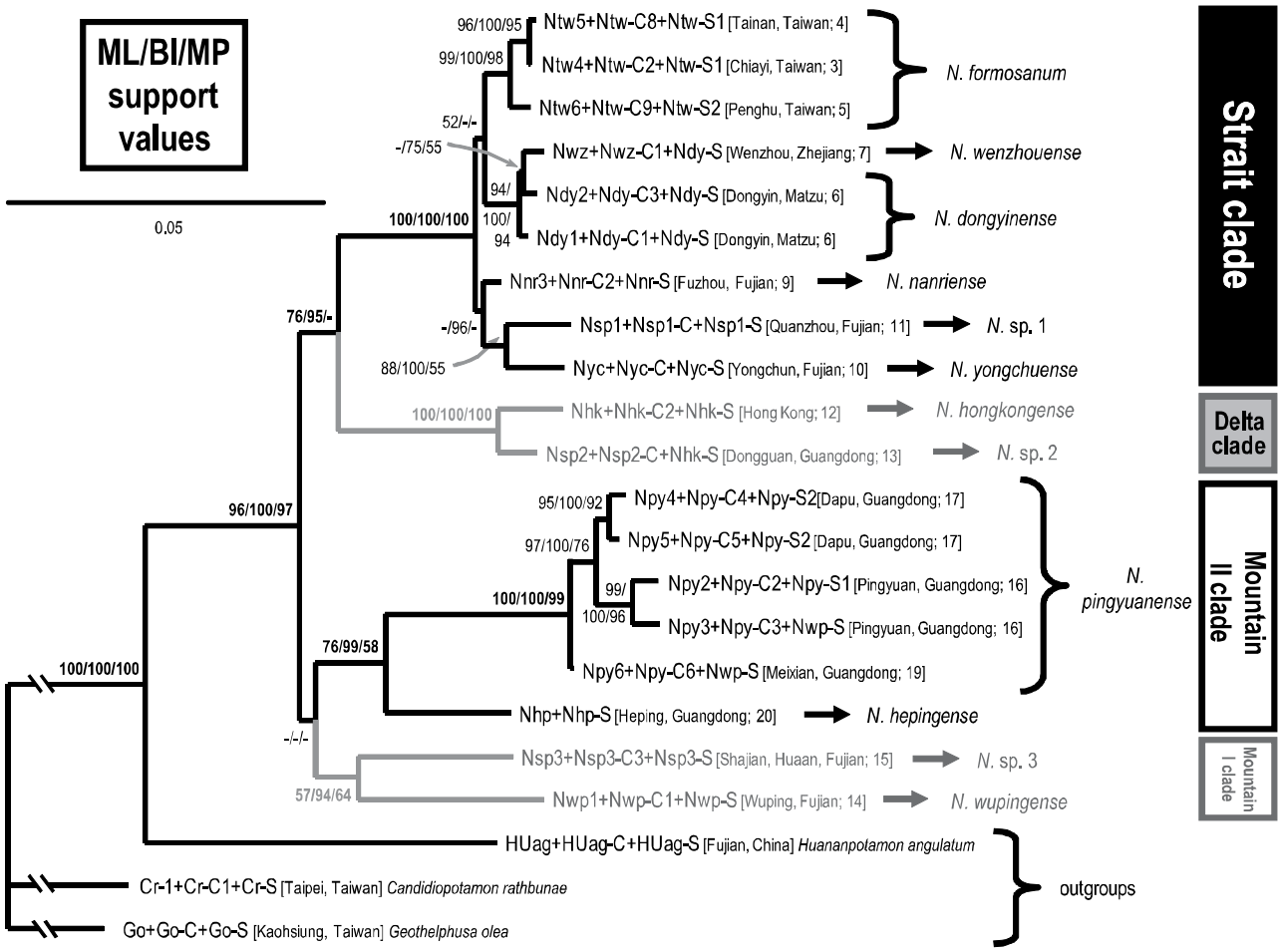

Fig. 2. A maximum likelihood (ML) tree of the genus *Nanhaipotamon* from Taiwan, Hong Kong and the Chinese coastal provinces (Guangdong, Fujian and Zhejiang), and outgroups, based on the combined 16S rRNA, COI and 28S rRNA genes. Support values (⩾50%) for ML, Bayesian inference (BI) and maximum parsimony (MP) are represented at the nodes. Locality names and the corresponding numbers in Fig. 1 are parenthesized behind haplotypes. For abbreviations and haplotypes, see Table 1.

discarded as the burnin (determined by the average standard deviation of split frequency values below the recommended 0.01; Ronquist et al., 2005). Partitioned maximum likelihood (ML) analysis was conducted in RAxML (v. 7.2.6, Stamatakis, 2006) for the two combined datasets. The model GTR + G (i.e. GTRGAMMA) was used for all subsets with 100 runs, and found the best ML tree by comparing the likelihood scores. The robustness of the ML tree was evaluated by 1000 bootstrap pseudoreplicates under the model GTRGAMMA. A consensus MP tree was constructed using PAUP* with 2000 bootstrap replications of a simple heuristic search, tree bisection-reconnection (TBR) branch-swapping, and 100 random-addition sequence replications. Gaps in MP tree construction were treated as missing. All characters were equally weighted.

The haplotype network of the nuclear 28S for *Nanhaipotamon* (Table 1) was constructed by TCS (v. 1.20, Clement et al., 2000), with the treatment of gaps as missing states. Phylogenetic reconstructions identified a subclade of *N. formosanum* composed of very closely related haplotypes which included individuals collected from the main island of Taiwan and Penghu Islands (Pescadores) located in the center of Taiwan Strait. To examine the relationships of these haplotypes in detail, a gene genealogy of combined 16S and COI was constructed using TCS.

2.4. Divergence time estimation

The divergence times among species were estimated from the combined 16S and COI, based on the program BEAST (v. 1.61, Drummond and Rambaut, 2007) under the uncorrelated lognormal model (Drummond et al., 2006). Three calibration points were used (see below). On the basis of a study of potamid phylogeny by Shih et al. (2009), the divergence time between *Nanhaipotamon* and *Huananpotamon* was estimated as 6.72 ± 1.39 mya and was set as the calibration point 1 in our study (with normal prior distribution), which is consistent with the divergence time 6.33 ± 0.79 mya (p-distance and standard deviation between the two genera are 5.57% and 0.7%, respectively) estimated from the 16S sequences of this study if the substitution rates of 0.88% per million years (established for terrestrial *Sesarma* (Sesarmidae); see Schubart et al., 1998) was applied. A preliminary analysis of the mitochondrial phylogeny indicated that most clades of *Nanhaipotamon* converging in the region around southwestern Fujian and northeastern Guangdong (Fig. 1). This suggests that this area may be the center of origin for the genus (Fig. 1) (Cain, 1944; Lomolino et al., 2005). Geologically, this area has been quite stable and no major orogenic processes were reported during the Cenozoic (Yi, 1996; Zhou and Li, 2000). However, there are some records of Cenozoic basaltic rocks dated to 4.96 mya (Xu and Xie, 2005) and as such, we set 5 mya as calibration point 2 for the cladogenesis of *Nanhaipotamon*, with 1 million years of deviation (with normal prior distribution). During the glacial periods, Taiwan I. was connected with China by a landbridge (Boggs et al., 1979; Voris, 2000; Shih et al., 2007), and the end of glaciation, which resulted in a sea separating Taiwan and China, was set as point 3. Due to repeated glaciation events, we set 2 mya as the upper limit when the

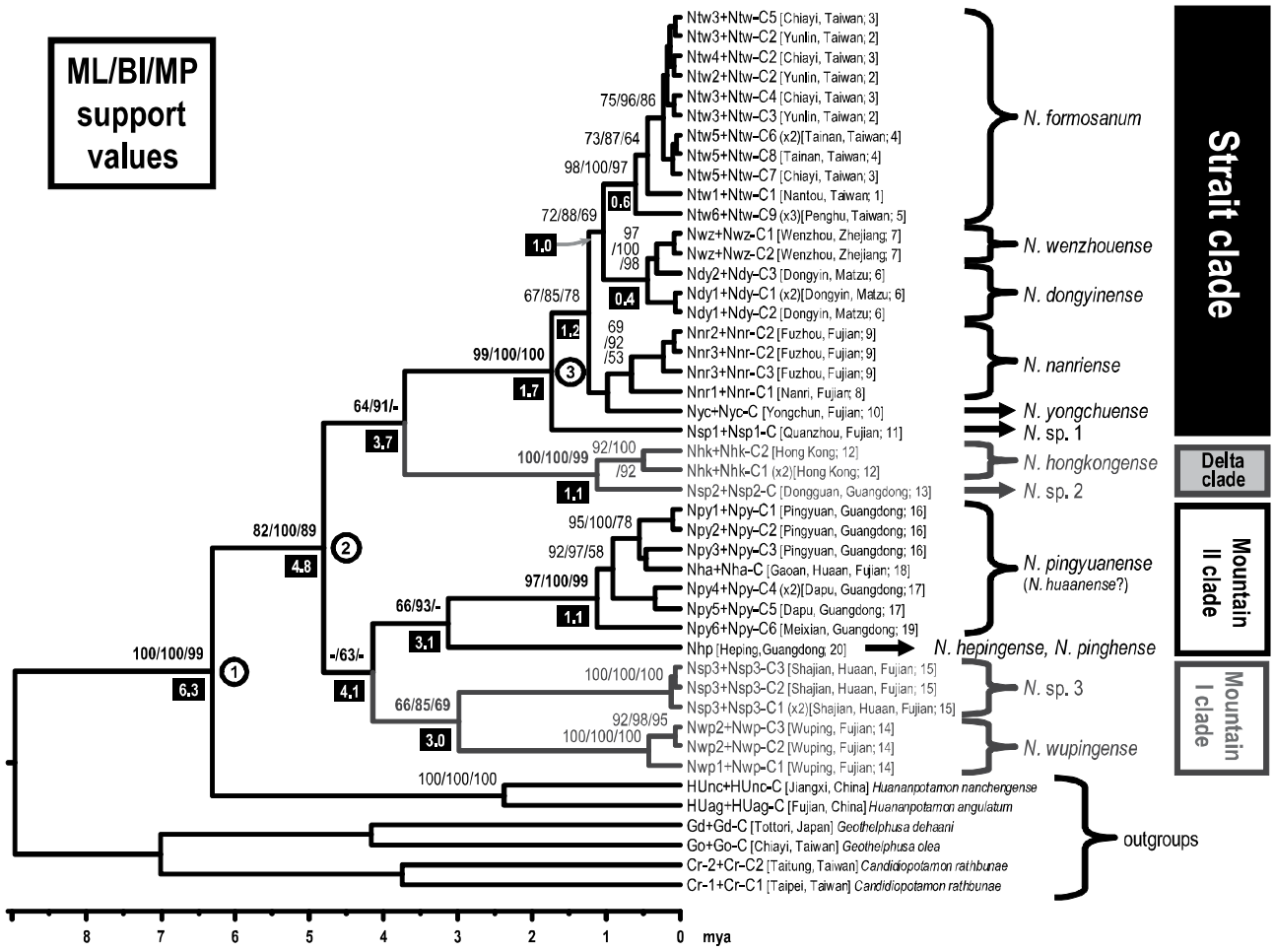

Fig. 3. A chronogram of the genus *Nanhaipotamon* from Taiwan, Hong Kong and the Chinese coastal provinces (Guangdong, Fujian and Zhejiang), and outgroups, based on the combined 16S rRNA and COI genes. Support values (⩾50%) for maximum likelihood (ML), Bayesian inference (BI) and maximum parsimony (MP) are represented above the nodes. Calibration point 1 was set for the divergence time between *Nanhaipotamon* and *Huananpotamon*; calibration point 2 was set for the cladogenesis of *Nanhaipotamon*; and calibration point 3 was set for the glacial periods in Taiwan Strait. The divergence times estimated are shown in reverse color below the main nodes. Locality names and the corresponding numbers in Fig. 1 are parenthesized behind haplotypes. For abbreviations and haplotypes, see Table 1.

end of the first glaciation with −100 m sea level after 5 mya, and 0.01 mya as the lower limit (with uniform prior distribution) which corresponds with the end of the Last Glacial Maximum (Haq et al., 1987; Woodruff, 2003). A Yule speciation process was conducted for the speciation within *Nanhaipotamon*. We used a GTR + I + R model with the parameters obtained from MrModeltest for each gene. Twelve independent MCMC chains were run for 10 million generations sampled every 1000 generations. The convergence of the 12 combined chains was determined by the ESS (>200 as recommended) for each parameter in Tracer after appropriate burnin cutoff (default 10% of sampled trees). Trees in the 12 chains were combined using LogCombiner (v. 1.6.1, distributed as part of the BEAST package) and were assessed using TreeAnnotator (v. 1.6.1, distributed as part of the BEAST package). A chronogram was constructed by FigTree (v. 1.3.1, Rambaut, 2009).

3. Results

3.1. Sequence diversity

Within *Nanhaipotamon*, a 552 bp segment (excluding the primer regions) of the 16S from all 47 specimens was amplified and aligned; of which 79 positions were variable and 58 were parsimony informative. *N. hepingense* and *N. pinghense* share the same sequence of 16S. Among the total number of sequences, 28 different haplotypes were distinguished (Table 1). The studied segment of 16S was AT rich (72.1%) (T: 36.5%, A: 35.6%, G: 17.9%, C: 10.1%). For COI, a 658 bp segment was compared from 45 specimens, resulting in 35 different haplotypes. We failed to obtain COI sequences from specimens of *N. hepingense* and *N. pinghense*, and the missing data were designated as a '?' in the alignment. The COI segment was AT rich (65.4%) (T: 37.6%, A: 27.8%, G: 16.1%, C: 18.4%). In this gene, 166 positions were variable and 145 were parsimony informative. A 707 bp segment of the 28S was compared from 19 specimens, and 12 different haplotypes were obtained. The segment of 28S was GC rich (65.3%) (T: 19.0%, A: 15.7%, G: 34.9%, C: 30.4%), with 24 positions variable and 9 parsimony informative.