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Molecular Species Delimitation of the Genus *Reishia* (Mollusca: Gastropoda) Along the Coasts of China and Korea

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Species of the predatory gastropod genus *Reishia* Kuroda and Habe, 1971 (Muricidae) inhabit intertidal rocky shores in East Asia. Due to their highly variable external shell morphology, the taxonomy of this genus at species-level is still in need of re-evaluation. Using DNA-based delimitation methods, we aimed to ascertain the number of species of *Reishia* along the coasts of China and adjacent Asian areas. Also, we looked for diagnostic traits using morphology-based statistical approaches. Our genetic data suggest that the studied individuals comprised two separate species of a *Reishia* complex in this region, in contrast to the previously proposed four or more taxa. This conclusion is further supported by statistical analyses of shell morphological characteristics. The morphospecies *R. bronni* (Dunker, 1860), *R. jubilaea* (Tan and Sigurdsson, 1990), and *R. luteostoma* (Holten, 1803) were assigned to a single taxon, indicating that they might be synonyms of the same species. The morphospecies *R. clavigera* (Küster, 1860) singly formed one group, suggesting that it is likely a valid name. The estimated divergence time of the two identified taxa indicates that speciation might have been associated with the sea level and temperature fluctuations during the Plio-Pleistocene period. Our study on *Reishia* species provides crucial information for further research on the ecology, evolutionary biology, and conservation of this genus.

Key words: East Asia, gastropod, integrative taxonomy, molecular identification, morphology

INTRODUCTION

The taxonomic classification and identification of species is a challenge in taxa that share morphological characteristics (Puillandre et al., 2012a). This taxonomic difficulty may be a serious constraint for biodiversity assessments and for research on species evolution. In addition to traditional morphology-based methods, DNA-based species delimitation is useful to estimate putative species boundaries (Roy et al., 2014). The single-gene DNA-based species delimitation approach can be applied without a priori identification of species; therefore, this method is useful for proposing taxonomic hypotheses (Goldstein and DeSalle, 2011; Puillandre et al., 2012a).

Members of the predatory gastropod genus *Reishia* (Muricidae) are economically and ecologically important. Due to their feeding ecology, they can cause significant damage to the oyster culture industry, which is an important branch of local economy (Brown and Richardson, 1988). Furthermore, *Reishia* individuals are regularly harvested in large numbers for commercial use. At the ecosystem level, *Reishia* individuals are thought to be important predators that determine the structuring of littoral communities (Taylor and Morton, 1996). *Reishia* species have also been used as effective bioindicators to assess human-induced pollution of marine environments with organotin compounds (Hung et al., 2001; Tang and Wang, 2009). The genus *Reishia* is of tropical origin and has diversified in the warm-temperate northern hemisphere (Claremont et al., 2013). This genus includes taxa that are endemic to the north-western Pacific area. Thus, *Reishia* represents an interesting model taxon for speciation studies. However, despite their economic, ecological, and evolutionary significance, the taxonomy of the genus *Reishia* at species level is still unclear (Tan and Liu, 2001).

Although *Reishia* was previously considered synonymous with *Thais*, it has been recognized by most authors as a single genus, as indicated by Claremont et al. (2013), including six species: *R. bitubercularis* (Lamarck, 1822), *R. bronni* (Dunker, 1860), *R. clavigera* (Küster, 1860), *R. jubilaea* (Tan and Sigurdsson, 1990), *R. keluo* (Tan and Liu, 2001), and *R. luteostoma* (Holten, 1803). Traditionally, identification of *Reishia* species is based on the external charac-

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teristics of shell and radula morphology. However, numerous instances of potential taxonomic misidentifications have remained unsolved (Tan and Liu, 2001) due to the highly variable external morphology of this genus and the lack of unambiguous morphological characteristics (Kool, 1993). For example, the taxonomy of *R. bitubercularis*, *R. bronni*, *R. jubilaea*, and *R. luteostoma* is unclear, and they might in fact belong to the same species (Tan and Sigurdsson, 1990). It has been suggested that *R. luteostoma* in Japan may represent a different species from that in Hong Kong, and *R. jubilaea* has been described as distinct from *R. luteostoma* (Tan and Sigurdsson, 1990). However, the low degree of genetic divergence (less than 0.9%) in their cytochrome *c* oxidase subunit I (COI) suggests that *R. luteostoma* in Japan, *R. luteostoma* in Hong Kong, and *R. jubilaea* may be conspecific, and divergence between the three entities and *R. bronni* was less than 2.4% (Claremont et al., 2013). Indeed, this confusing situation calls for a reassessment of the taxonomic status of nominal species included in the genus *Reishia*.

Unresolved taxonomies may lead to incomplete knowledge of species distribution (Cardoso et al., 2011). The distribution of *Reishia* ranges from tropical to temperate zones in the area of East Asia. The long span of the Chinese coastline provides optimal conditions for investigating the taxonomy of the genus *Reishia*. However, this species group has not been studied in depth, and the taxonomic status of the main *Reishia* lineages have been debated in China and adjacent Asian regions (Claremont et al., 2013). All the six species included in the genus *Reishia* have been reported from the South China Sea (Tan and Liu, 2001), albeit *R. bitubercularis*, *R. jubilaea*, and *R. keluo* have rarely been found.

Reishia species are widely distributed along the Chinese and adjacent Asian coastline. The following five species names, among others, have been used for East Asian *Reishia*:

(1) *Reishia bronni*: this is a well-known species found in Japan, Korea, and northern China. The shell is fusiform to biconical, with nodose spiral ribs and broad interspaces. The shell color is orange with dark dashes on axial ribs. The aperture is large, and the color is pale orange (Higo et al., 1999). It is characterized by large, off-white bulbous tubercles on the last whorl, according to the lectotype illustrated in Dunker (1861, plate 1, fig. 23). This species was originally named *Purpura bronni* Dunker, 1860, and its type locality contained in Japan. In China, *Purpura suppressus* Grabau and King, 1928 has been used as a synonym for *R. bronni*, but it has not been studied in depth. The typical habitat ranges from the intertidal to the sublittoral rocky bottom.

(2) *Reishia clavigera*: this is the most common species of this genus. This species was originally named *Purpura clavigera* Küster, 1860 and has also been referred to as *Purpura altispiralis* Grabau and King, 1928 and *Purpura alveolate* var. *pechiliensis* Grabau and King, 1928. Morphologically, this species is commonly characterized by black axial ribs with narrow interspaced white lines and a purplish-black aperture (Higo et al., 1999). The distribution of this species ranges from Singapore over Hong Kong and Taiwan to northern Japan (Tan and Liu, 2001). The typical habitat is an intertidal rocky shore.

(3) *Reishia luteostoma*: the shell of this species resembles that of *R. bronni*, but it is distinguished by a short spire, swollen body whorl, and conical black nodules (Higo et al., 1999). The original name of this species was *Buccinum luteostoma* Holten, 1803, and its type locality was not specified in the original description. It has also been referred to as *Purpura bronni* var. *suppressa* Grabau and King, 1928 (Coan et al., 2015) and *Purpura chusani* Souleyet, 1852, and it was previously assigned to the genus *Thaisella*. The type locality of *Purpura chusani* is Malacca. According to Higo et al. (1999), the typical habitat of this species is the rocky bottom from the sublittoral to 10 m depth. The distribution of this species has been disputed; according to Tan and Liu (2001), it is confined to the subtropical Chinese coasts, but it has also been recorded from northern to southern Hokkaido by Higo et al. (1999).

(4) *Reishia jubilaea*: the type locality of this species is in Singapore and West Malaysia within the Gulf of Thailand and the Strait of Malacca. According to its original description, *R. jubilaea* can easily be confused with *R. clavigera*, but is distinguishable by its low tubercles, smooth outer lip, and a pattern of brownish-black dashes on the spiral cords of the last whorl. Moreover, the taxonomic identity of *R. luteostoma* and *R. jubilaea* is unclear, as they may belong to the same species.

(5) *Reishia keluo*: this species resembles *R. bitubercularis*, *R. clavigera*, *R. jubilaea*, and *R. luteostoma* regarding general shell morphology according to its original description, but differs slightly from these in the morphology of radula and penis. The type locality is in Taiwan (Tan and Liu, 2001). It was also suggested to assign *R. bronni* as reported by Higo et al. (1999) to *R. keluo*, although this would require anatomical confirmation (Tan and Liu, 2001).

Among the five nominal species mentioned above, the morphology of the radula and penis of *R. keluo* and *R. jubilaea* are important characteristics to distinguish them from other taxa in this genus, according to the original descriptions (Tan and Sigurdsson, 1990; Tan and Liu, 2001). However, due to disparity in various factors (developmental stages of organisms, season, and environmental temperature), the radula characteristics may vary in size, shape, and number, even within the same species (Huang et al., 2013; Fujioka, 1985). Therefore, previous delimitation of *Reishia* species based on the morphology of the radula and penis should be treated with caution.

Due to the uncertain taxonomic status of species assigned to the genus *Reishia* using morphological characters, molecular analyses are required to infer both species boundaries and species relationships within the genus *Reishia*. In this study, we aimed to ascertain the number of species within *Reishia* complex using DNA-based delimitation methods and look for diagnostic traits using morphology-based statistical approaches along the coasts of China and Korea. We then estimated the time of divergence of these species to infer the potential cause of speciation events of sister species.

MATERIALS AND METHODS

Sample collection

We collected *Reishia* individuals from 14 sites distributed across the coasts of China and Korea, from October 2015 to July 2016 (Fig. 1, Table 1). Samples were hand collected and preserved

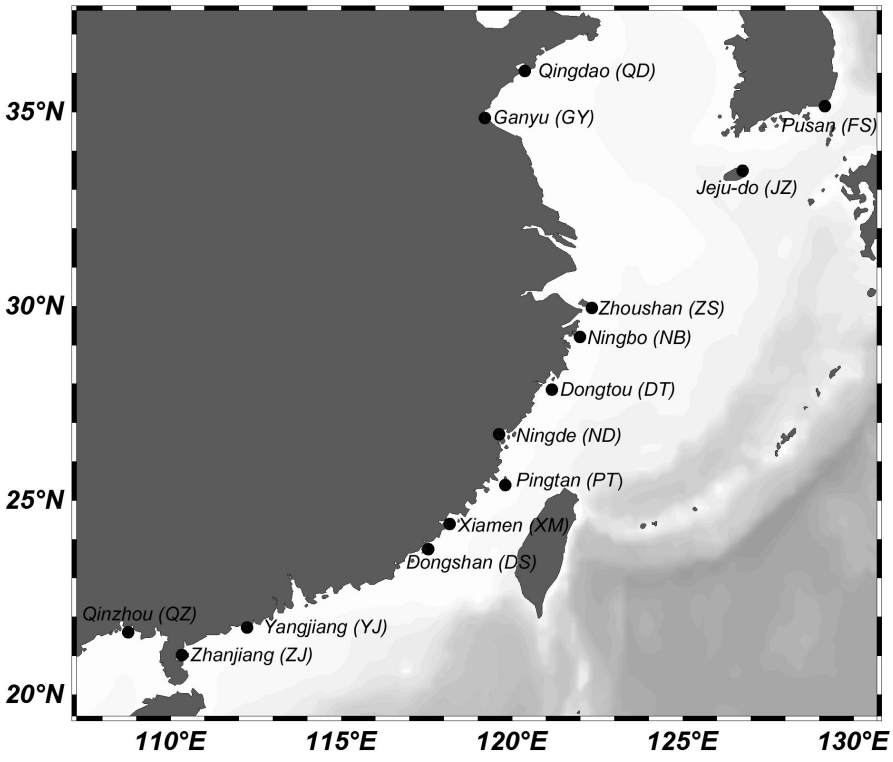

Fig. 1. Sampling localities of *Reishia* specimens in this study. Refer to Table 1 for detailed information of sampling stations.

Table 1. Sampling localities and numbers of samples.

Locality	Country	Coordinates	N
Dongshan	China	23.75° N, 117.54° E	17
Dongtou	China	27.86° N, 121.16° E	8
Pusan	Korea	35.15° N, 129.15° E	5
Ganyu	China	34.86° N, 119.20° E	5
Jeju-do	Korea	33.50° N, 126.75° E	1
Ningbo	China	29.22° N, 121.98° E	5
Ningde	China	26.71° N, 119.61° E	2
Pingtan	China	25.41° N, 119.80° E	11
Qingdao	China	36.06° N, 120.36° E	11
Qinzhou	China	21.62° N, 108.76° E	10
Xiamen	China	24.40° N, 118.17° E	9
Yangjiang	China	21.74° N, 112.23° E	17
Zhanjiang	China	21.03° N, 110.32° E	2
Zhoushan	China	29.96° N, 122.33° E	4

N = number of samples

in 95% ethanol. As morphological characterization of these taxonomic units may be misleading, we selected individual specimens in order to cover a wide range of morphological variation. A total of 107 individuals were used for the subsequent analyses. The DNA sequences and the morphometric analyses were performed on the same individuals.

DNA extraction and sequencing

Genomic DNA extraction followed the cetyltrimethyl-ammonium bromide (CTAB) protocol (von der Schulenburg et al., 2001). The primer pairs COIF and COI-MUR reported by Claremont et al. (2011) were used for PCR amplification and sequencing of fragments of the mitochondrial COI. PCR amplification conditions were the same as in Williams et al. (2010). Seven previously published sequences (GenBank accession numbers: HE584368–584372, FR695721–695722; Supplementary Table S1) were used as references for subsequent delimitation analyses.

All COI fragments were sequenced in two directions. Forward and reverse sequences of individual loci were trimmed, assembled, and merged into consensus sequences using DNA Baser Sequence Assembler ver. 4 (2013, Heracle BioSoft, www.DnaBaser.com).

Molecular species delimitation

An alignment was produced using CLUSTAL_X 1.81 with default settings (Thompson et al., 1997).

Two datasets were used as input for subsequent analyses: the preliminary dataset included all sequences of the *Reishia* complex (sequences produced in the current study and previously published sequences), and the main dataset from which the sequence FR695721 from *R. bitubercularis* was excluded.

Distance-based and tree-based approaches can infer different group assignments, potentially under- or overestimating the number of putative species groups (Dellicour and Flot, 2018). Thus, the Automatic Barcode Gap Discovery (ABGD) method (Puillandre et al., 2012b), the Generalized Mixed Yule Coalescent (GMYC) method (Pons et al., 2006), and the Bayesian Poisson Tree Process (bPTP) approach (Zhang et al., 2013) were employed to propose primary species hypotheses (PSHs) and elucidate congruence (Roy et al., 2014).

First, we used the ABGD method (Puillandre et al., 2012b) on the preliminary dataset via an online server (http://www.abi.snv.jussieu.fr/public/abgd/), with default settings. The ABGD method uses the ordered ranked genetic distances between COI fragments to identify gaps in the distribution of low intraspecific distances to higher interspecific distances. These variations in genetic distances can be used to assign individuals to different taxonomic groups (Puillandre et al., 2012b).

Before the tree-based delimitation analyses, the phylogenetic relationships among all haplotypes were examined using Bayesian inference (BI) and maximum-likelihood (ML) reconstruction methods implemented in BEAST ver. 1.8.2 (Drummond et al., 2012) and RAxML ver. 8.2.4 (Stamatakis, 2014), respectively. Prior to the phylogenetic reconstruction, the best-fitting model of nucleotide substitution was determined using JMODELTEST ver. 2.1.1 (Darriba et al., 2012) with Akaike information criterion. The model HKY + G was selected as the best fit for subsequent analyses. Branch supports were assessed using 1000 bootstrap replicates for ML trees. For the BEAST analysis, the Yule process of speciation and an uncorrelated log-normal relaxed-clock model were used as a tree prior. Two independent MCMC runs of 200 million generations were performed with tree sampling every 5000 generations. The first 10% generations were discarded as burn-in. Convergence and effective sample size (ESS) of estimated parameters were examined using TRACER ver. 1.6 (Rambaut et al., 2014). After that, the phylogenies were used for GMYC and bPTP analyses.

Then, as recommended by Tang et al. (2014), we implemented the GMYC method (Pons et al., 2006) with a BEAST tree, and the bPTP model (Zhang et al., 2013) with a RAxML gene tree for the main dataset, both operated using an online server (http://species.h-its.org). Half a million Markov chain Monte Carlo (MCMC) generations were used for the bPTP analyses. The GMYC method (Pons et al., 2006) separately models the fit of Yule and coalescent processes to an ultrametric tree to define a transition from species-level to population-level processes. The bPTP approach (Zhang et al., 2013) models speciation and coalescent events relative to the number of substitutions rather than time and uses heuristic algorithms to identify the most likely classification of branches into population- and species-level processes. These approaches produce an objective clade-specific threshold to delimit PSHs.

To assess the genetic divergence between the distinct PSHs, Kimura 2-parameter (K2P) genetic distances between and within PSHs were calculated using MEGA ver. 6 (Tamura et al., 2013).

Morphological analyses

We examined shell morphology of all confirmed 107 *Reishia* specimens to look for diagnostic morphological characters by which to identify PSHs to be detected by molecular analyses. The following shell parameters for all specimens were recorded: shell height (SH), shell width (SW), apical angle (AA), aperture length (AL), aperture width (AW), number of spiral ribs on the body whorl (N1), number of crenulations on the spiral ribs below the suture near the aperture (N2), color of the aperture (CA), consecutiveness of nodules (CN), and acuteness of nodules (AN).

To summarize the shell characteristics contributing most significantly to morphological differences, a principal component analysis (PCA) was conducted using five continuous variables (SH, SW, AA, AL, and AW) and two discrete variables (N1 and N2). A linear discriminant analysis (LDA) was then performed on the first two principal components (PC1 and PC2) of the PCA against PSH A and PSH B. The dataset containing all individuals of the current study was split into a training set (54 individuals) and a test set (53 individuals), and an LDA model was trained using the training set. To test the accuracy of the LDA model, the test set was used to produce the percentage of correct predictions. To test whether the independent grouping variable (PSH A and PSH B) would simultaneously explain a statistically significant amount of variance in the dependent variables (measured shell parameters), a multivariate analysis of variance (MANOVA) and a one-way analysis of variance (ANOVA) were conducted on two PC scores (PC1 and PC2) and five continuous variables (SH, SW, AL, AW, and AA) against the PSHs.

To test the accuracy of three descriptive characters (CA, CN, and AN) for categorizing the shells, a regression analysis against the main genetic groups (PSH A and PSH B) was performed on

Table 2. Two molecular clock calibrations used to estimate the divergence time of the distinct primary species hypotheses (PSHs). All units are per site per million years.

Molecular clock	Sequence divergence rates	Substitution rates specified in BEAST Prior distribution = normal
Hellberg et al. (1999)	Mean = 2.4% SD = 0.5%	Mean = 1.2% SD = 0.25%
Mcgovern et al. (2010)	Mean = 1.52% SD = 0.2%	Mean = 0.76% SD = 0.1%

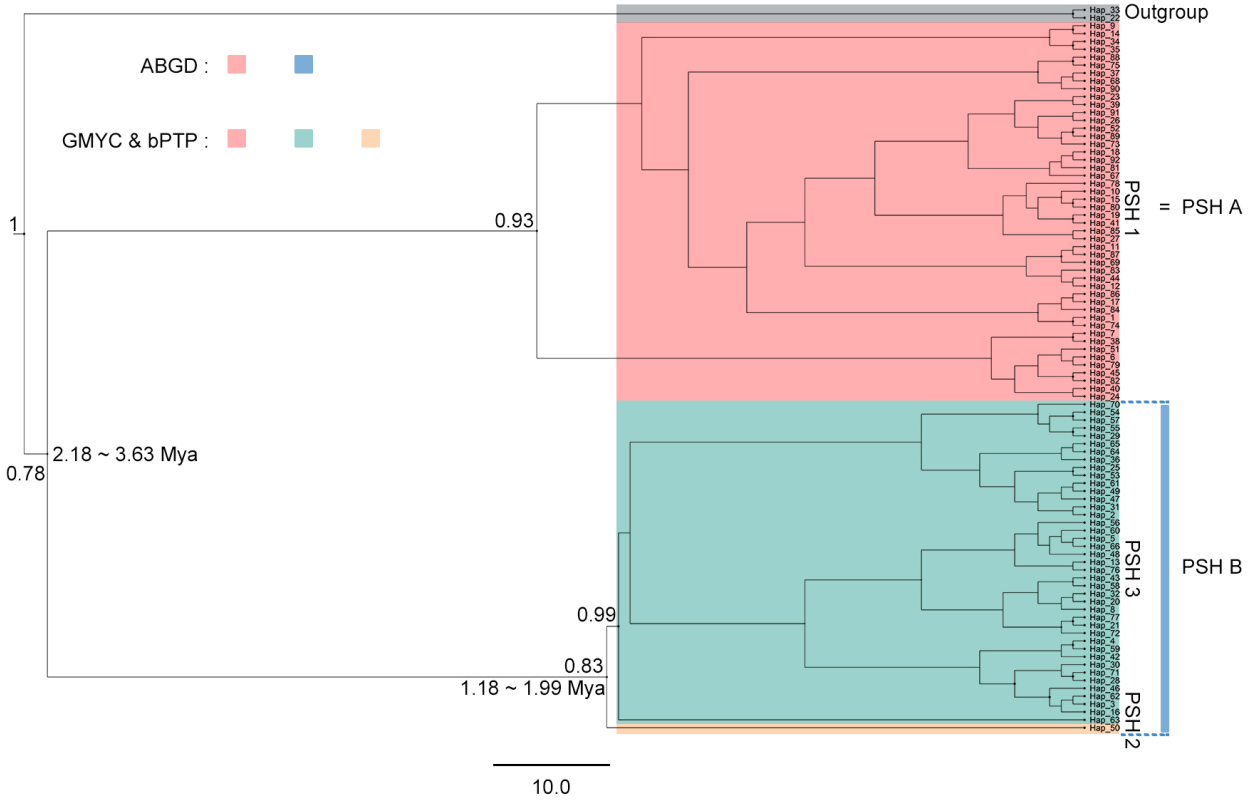

Fig. 2. Results of the three delimitation methods (ABGD, GMYC and PTP) and BEAST divergence time estimation of the distinct primary species hypotheses (PSHs) using a calibrated molecular clock method. Node age intervals and Bayesian posterior probabilities (values ≥ 0.7 shown) are shown at nodes. Timescales in million years before present (Mya). The names of samples are displayed in Table S1 corresponding to the tip labels.

PC1, PC2, CA, CN, and AN. The measured characteristics included both dichotomous categorical and continuous variables; therefore, a logistic regression model was used to explain the relationships between measured characteristics and genetic groups. The dataset containing all individuals was the same split as in LDA. The training data was used to fit a logistic regression model, which was then evaluated using the test data.

All statistical analyses were performed on R ver. 3.4.3 program (Team RC, accessed online 10 May 2018).

Divergence time estimates

To investigate the timing of the diversification events, we performed divergence time estimates. Given that no appropriate fossil was available for genus *Reishia*, we employed a calibrated molecular clock method to estimate the divergence times of the main clades using BEAST ver. 1.8.2 with the main data set. As *Rapana* Schumacher, 1817 (Gastropoda: Muricidae) was the most closely related genus with *Reishia* in the phylogenetic study of Rapaninae (Gastropoda: Muricidae) (Claremont et al., 2012), sequences of *Rapana bezoar* (Linnaeus, 1767) (GenBank accession number FN677421; Tab. S1) and *Rapana rapiformis* (Born, 1778) (GenBank Accession number HE584366; Supplementary Table S1) available in GenBank were included as outgroups. HKY + G was selected as the best-fitting model using JMODELTEST ver. 2.1.1. Prior settings were used as detailed previously. For the lack of calibrations for the genus *Reishia* COI gene, sequence divergence rates of 1.52% and 2.4% per million years, as calibrated for genus *Nucella* Röding, 1798 (Gastropoda: Muricidae) (Mcgovern et al., 2010) and two trochid species (Hellberg and Vacquier, 1999), were used to assess the time range of key nodes. Detailed prior settings of the molecular clock are shown in Table 2.

RESULTS

The preliminary dataset comprised 114 sequences, and the main dataset comprised 113 sequences. The length of the COI fragment alignment was 633 base pairs; 208 positions were variable, of which 136 were parsimony-informative. All sequences were deposited in GenBank under the accession numbers MG149595–MG149701.

The ABGD method used on the preliminary dataset led to partitions with three PSHs, when extreme a priori thresholds were excluded. The *R. bitubercularis* sequence downloaded from GenBank produced a single PSH. The downloaded *R. clavigera* sequence together with 53 sequences produced in the current study corresponded to a single PSH (PSH B; Fig. 2). Remarkably, sequences that were obtained from GenBank as "*R. luteostoma*" collected in Japan and Hong Kong, together with "*R. bronni*" from Japan and "*R. jubilaea*" collected in Singapore, were assigned to a further PSH (PSH A; Fig. 2) with 54 of the sequences generated in this study. The sequences produced in the present study were thus divided into two separate PSHs (PSH A and PSH B; Fig. 2) in the ABGD analysis.

The GMYC and bPTP methods produced identical results for the main dataset, delineating three distinct PSHs (PSH 1, PSH 2, and PSH 3; Fig. 2). An individual collected at Pingtan (PT-I; Fig. 3g) belonging to PSH B in the ABGD analysis was assigned to a separate PSH (PSH 2; Fig. 2). The remaining individuals in PSH A formed PSH 1 (Fig. 2) in the GMYC and bPTP analyses. The composition of PSH 3 was identical to that of PSH B. Representative shell forms in different PSHs are shown in Fig. 3.

Morphological species *R. bronni* (Fig. 3a–c), *R. luteostoma* (Fig. 3d), and *R. jubilaea* (Fig. 3e) are included in

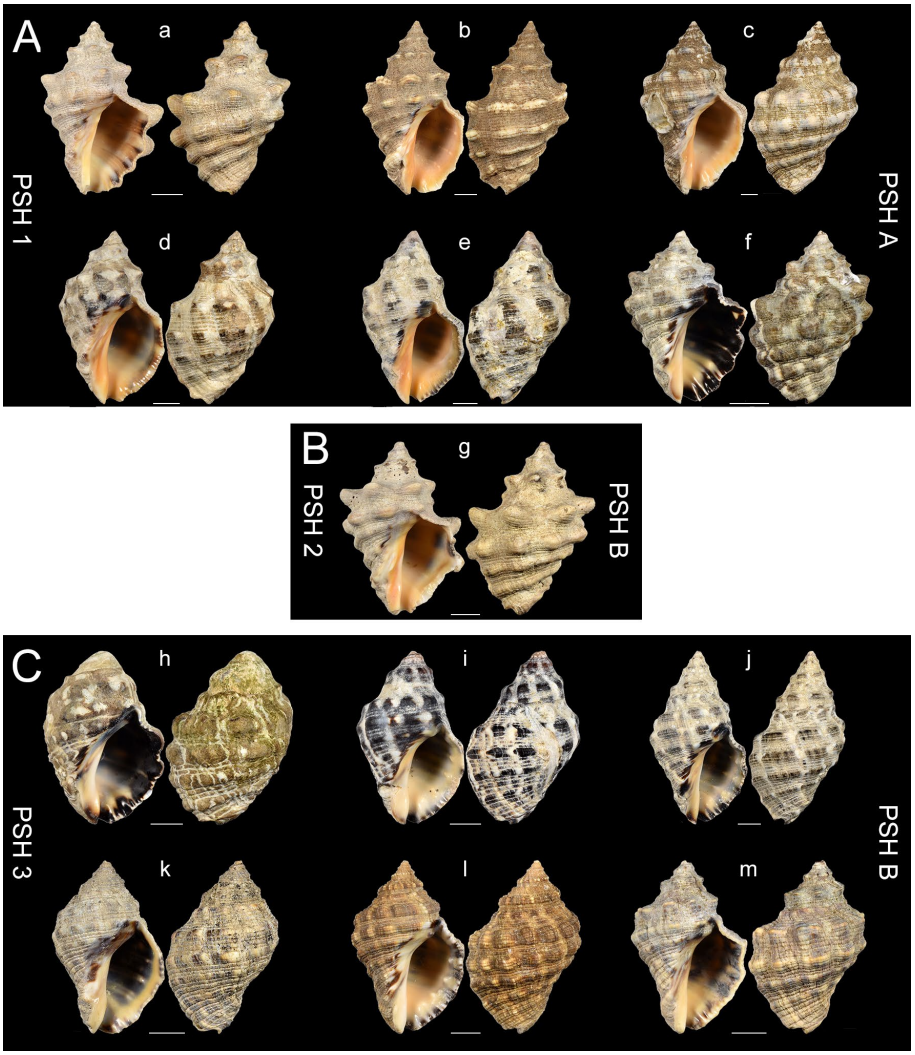

Fig. 3. Representative shell forms in different primary species hypotheses (PSHs). **(A)** Representative shell forms (a–f) in PSH 1 (PSH A). **(B)** Representative shell form (g) in PSH 2 (PSH B). **(C)** Representative shell forms (h–m) in PSH 3 (PSH B). Scale bars = 5 mm; a = ZS-A1; b = NB-D; c = QD-A1; d = DS-F1; e = YJ-M1; f = FS-C2; g = PT-I; h = FS-C1; i = XM-F1; j = YJ-B1; k = GY-B1; l = QD-I; m = QZ-E2.

Table 3. Summary of the principle components analyses in morphological analysis. Contributions of each of the seven characters and percentage of total variance are given to seven principal components (PCs).

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Shell height	0.48	−0.079	0.139	−0.112	0.375	−0.147	0.754
Shell width	0.482	−0.105	−0.094	−0.09	−0.027	0.849	−0.136
Apical angle	−0.169	−0.439	−0.859	−0.031	0.14	−0.03	0.14
Aperture length	0.471	−0.191	−0.008	−0.125	0.427	−0.391	−0.625
Aperture width	0.457	−0.196	−0.103	0.02	−0.802	−0.311	0.048
N1	−0.265	−0.506	0.321	−0.747	−0.103	0.041	0.005
N2	−0.088	−0.677	0.348	0.636	0.058	0.074	0.002
Proportion of Variance	0.578	0.216	0.114	0.063	0.019	0.006	0.004
Cumulative proportion	0.578	0.794	0.908	0.971	0.989	0.996	1

N1 = the number of spiral ribs on the body whorl; N2 = the number of crenulations on the spiral ribs below the suture near the aperture

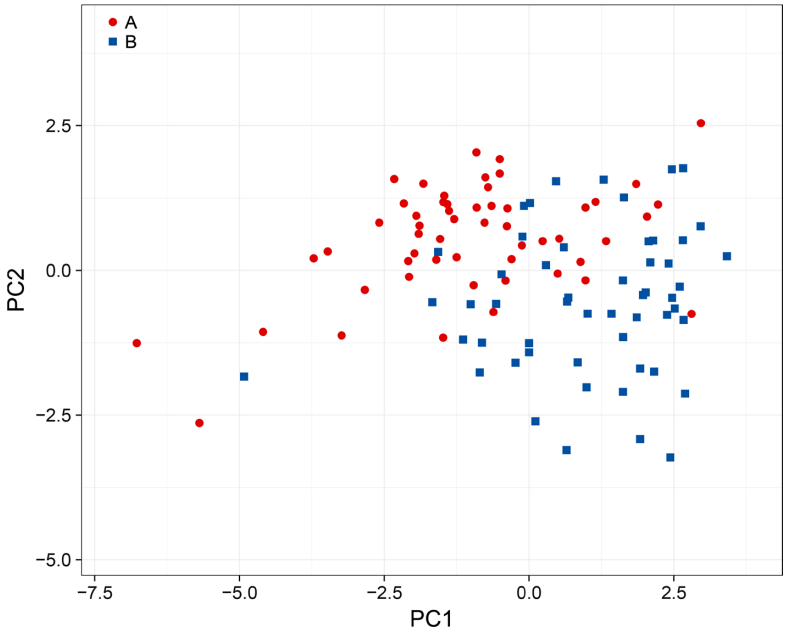

Fig. 4. Shell shape variation of *Reishia* species as a function of the first two principal components (PCs), based on the seven measured characters.

PSH A (1). Various forms of morphological species *R. clavigera* (Fig. 3h–m) were included in PSH B (3). The individual FS-C2 (Fig. 3f), which possessed characters belonging to both *R. clavigera* and *R. luteostoma*, was allocated to PSH A (1). Notably, the individual PT-I (Fig. 3g), which should be identified as *R. bronni* morphologically, was also assigned to PSH B (3).

The average pairwise genetic distances (K2P) among PSHs were maximal between PSH 1 and PSH 3 (7.2%). The minimum distance value among PSHs was found between PSH 1 and PSH 2 (2.4%). The distance between PSH 2 and PSH 3 was 5.5%; genetic distances within PSH 1 and PSH 3 were 0.9%, each.

Regarding the morphological analysis, the PCA successfully transformed the original seven shell parameters to seven components that explained 100% of the variation. The contributions of each of the seven shell parameters to the seven principal components (PCs) are shown in Table 3. The first two PCs explained 79.4% of the total variance (axis 1: 57.8%; axis 2: 21.6%). Ordinations of the first two PCs revealed a segregation pattern between PSH A and PSH B (Fig. 4), although a slight overlap was detected. The LDA on the first two PCs produced a correct classification for 79.6% of the training data set and for 81.1% of the test data set. Results of the MANOVA and one-way ANOVA showed that both PC scores (PC1 and PC2) and the five continuous variables SH, SW, AL, AW, and AA differed significantly ($P < 0.01$) between PSH A and PSH B.

In the logistic regression analysis, PC1 and PC2 differed significantly ($P < 0.01$), and CA was significant ($P = 0.01732$) in the logistic regression model. However, the remaining two shell characteristics, CN and AN, were not statistically significant ($P > 0.05$). The classification accuracy was 84.9%, based on the test dataset.

Figure 2 shows the divergence time estimates based on COI gene variation, using calibration rates of 1.52% and 2.4% per million years. The estimated time of divergence of PSH A (1) and PSH B (2+3) was approximately 3.63 and 2.18 million years ago (Mya). The time of divergence of PSH 1 and PSH 2 was approximately 1.99–1.18 Mya, based on different calibration rates.

DISCUSSION

As emphasized by Dayrat (2004), there is a critical need for rigorous delineation of species, not only for producing accurate species inventories but also for precisely addressing questions pertaining to evolutionary biology, ecology, and conservation biology. In the case of the variable *Reishia* taxa, the combination of morphology and molecular delimitation methods based on the barcode gene COI provide support for species identification and thus lay the groundwork for further research on ecology, evolutionary biology, and conservation biology.

Morphological variation and species delimitation

Morphological analyses revealed significant variations between PSH A and PSH B, which were confirmed by the separation pattern observed in a PCA (Fig. 4) and by the significant differences in shell characteristics using an ANOVA. However, a slight overlap in the PCA (Fig. 4) also suggested that the boundaries between the two PSHs are not entirely resolved, which was further supported by the moderate classification accuracy rate of the LDA and the logistic regression analysis (81.1% and 84.9%, respectively). The addition of CA improved classification precision regarding PSH A and PSH B, indicating that CA is an important classification characteristic of the two groups. Our results thus confirm that shell characteristics are insufficient for the identification of *Reishia* species.

Classical morphology-based species delimitation and identification in the genus *Reishia* are complicated for non-specialists and are also challenging for expert taxonomists. Our DNA-based delimitation results revealed that at least two *Reishia* PSHs co-occur along the coasts of China and Korea. These PSHs were further supported by the COI variations, of which the mean K2P divergences between distinct PSHs were consistently above intraspecific divergences (> 2%). If two or more evolutionarily significant units remain distinct in sympatry, it can be assumed that the respective groups do not interbreed, and thus they can be considered distinct biological species (Coyne and Orr, 2004). Regarding the distribution of the sampled *Reishia* individuals, PSH 1 and PSH 3 (PSH B) were sympatric; we therefore suggest that they are distinct species. Two of the putative PSHs have distinctive morphological features in CA (yellow aperture of PSH 1 and black aperture of PSH 3).

Owing to the absence of definitive morphological characteristics to distinguish *Reishia* species, exploratory single-gene methods for species delimitation may provide an effective complementary way to resolve the taxonomy of the genus *Reishia*.

Time of speciation

The time of divergence of PSH A and PSH B was estimated at 3.6252–2.1839 Mya, which corresponds to the late Pliocene to early Pleistocene. This is consistent with the fossil-based divergence time between *R. clavigera* and *R. bronni* estimated by Claremont et al. (2013); however, the present study produced an estimate with a wider range. One possible interpretation is that the COI sequence divergence rates used in this study were not specifically calibrated for *Reishia* species. However, speciation events during this period have also been demonstrated in a range of marine organisms in the north-western Pacific area (Wang et al., 2008; Shen et al., 2011; Ren et al., 2016).

Sea-level and temperature fluctuations during the Plio-Pleistocene period are assumed to play a major role in creating the marine species diversity of the north-western Pacific that is found today (Shen et al., 2011), and the emergence of the Dongshan land bridge was proposed to represent a biogeographic barrier in this period (Zhao et al., 2017). Regarding *Reishia* taxa, the geographical distribution provides no clues to investigate the cause of their divergence, and sympatry of the two species was likely a result of post-glacial migration due to their potentials for long-distance dispersal (Guo et al., 2015). Furthermore, sympatric PSH 1 and PSH 3 occupy distinct tidal levels on the shore, implying potential competitive effects on ecological traits. Thus, it is not easy to determine whether isolation due to barriers or ecological separation elicited the speciation process.

Although COI data has been used extensively as a tool for inferring the evolutionary and demographic past of both populations and species (Shen et al., 2011; Zhao et al., 2017), mitochondrial DNA markers are often under selection and evolve under unusual evolutionary rules compared to other genomes (Ballare and Whitlock, 2004). To reduce the risk of incorrect inferences made from a single marker, further molecular and ecological research using multiple markers is required for better understanding of the speciation process of *Reishia* species.

Taxonomic status

It is noteworthy that the taxa referred to as *R. bronni*, *R. jubilaea*, and *R. luteostoma* were assigned to one single taxon (PSH 1 or PSH A) by our molecular analyses. In some cases, due to age-related or environmental factors, intraspecific variation in shell morphology in *Reishia*, such as color and sculpture, may even exceed interspecific variation (Tan and Sigurdsson, 1990). Characteristics of specimens examined in this study (Fig. 3) supported this observation. Thus, a 'morphological' species may in fact represent different biological species, according to molecular analyses (Fig. 3a and Fig. 3g). *Rapana venosa* (Valenciennes, 1846), a species in a genus closely related to *Reishia*, comprises two forms that vary substantially regarding the shape and degree of nodular sculpture; however, insignificant differences were detected between the two forms in a previous molecular study (Yang et al., 2005). It is possible that this is similar in *R. bronni, R. jubilaea*, and *R. luteostoma*, which are currently considered different biological species because their shell morphologies differ mainly in the shape of nodules.

Reishia bitubercularis (Lamarck, 1822) reportedly occurred in the South China Sea (Tan and Liu, 2001); however, the ABGD results of the present study suggested that there is no *R. bitubercularis* in our *Reishia* specimens. The existence of this species in China should be confirmed by further sampling; therefore, it was not included in the discussion of the present results.

According to Puillandre et al. (2012b), ABGD and GMYC methods are problematic when species are represented by only few specimens. Only one individual was assigned to PSH 2; thus, it is difficult to assign its taxonomic status, and it would be necessary to increase the sample size to ascertain the phylogenetic relationship of this individual with the other two PSHs. The ABGD and GMYC analyses suggest that *R. bronni*, *R. jubilaea,* and *R. luteostoma* should be placed in the same taxonomic species. The type locality of *R. luteostoma* might be "South Sea and Coast of China", according to Kuroda et al. (1971), and the type locality of *R. bronni* appears to be Nagasaki, Japan (Higo et al., 1999). Additionally, *Buccinum luteostoma* Holten, 1803 was described earlier than *Purpura bronni* Dunker, 1860. Following the Principle of Priority, we hypothesize that *Reishia luteostoma* can be considered as the valid name for PSH 1 (A). Grabau and King (1928) described a taxon *Purpura bronni* var. *suppressa* in Peitaiho, northern China, and it was specified as a synonymized name of *R. luteostoma* (Coan et al., 2015). Our results support that "*R. bronni*" along the coasts of China and Korea is likely another form of *R. luteostoma*. On the other hand, *R. clavigera* is likely the proper name for PSH 3.

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COMPETING INTERESTS

The authors have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

DZ performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables; QL conceived and designed the study; LK collected samples; TS analyzed the data. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs190153)

Supplementary Table S1. Original data of measured shell characters and information of COI sequences available in NCBI.

Supplementary Table S2. Name of samples corresponding to the haplotypes.

REFERENCES

Ballard J, Whitlock M (2004) The incomplete natural history of mitochondria. Mol Ecol 13: 729–744

Born I (1778) Index rerum Naturalium Musei Caesarei Vindobonensis, Pars Prima, Testacea. Officina Krausiana, Vindobona

Brown K, Richardson T (1988) Foraging ecology of the southern oyster drill *Thais haemastoma* (Gray): constraints on prey choice. J Exp Mar Biol Ecol 114: 123–141

Cardoso P, Erwin T, Borges P, New T (2011) The seven impediments in invertebrate conservation and how to overcome them. Biol Conserv 144: 2647–2655

Claremont M, Vermeij G, Williams S, Reid D (2013) Global phylogeny and new classification of the Rapaninae (Gastropoda: Muricidae), dominant molluscan predators on tropical rocky seashores. Mol Phylogenet Evol 66: 91–102

Claremont M, Williams S, Barraclough T, Reid D (2011) The geographic scale of speciation in a marine snail with high dispersal potential. J Biogeogr 38: 1016–1032

Coan E, Lutaenko K, Zhang J, Sun Q (2015) The molluscan taxa of AW Grabau & SG King (1928) and their types. Malacologia 58: 179–224

Coyne J, Orr A (2004) Speciation. Sinauer Associates, Sunderland

Darriba D, Taboada G, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nat Methods 9: 772–772

Dayrat B (2004) Towards integrative taxonomy. Biol J Linn Soc 85: 407–415

Dellicour S, Flot J (2018) The hitchhiker's guide to single-locus species delimitation. Mol Ecol Resour 18: 1234–1246

Drummond A, Suchard M, Xie D, Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol 29: 1969–1973

Dunker W (1860) Neue japanische Mollusken. Malakozoologische Blätter

Dunker G (1861) Mollusca Japonica descripta et tabulis tribus iconum illustrata. Schweizerbart, Stuttgart

Fujioka Y (1985) Systematic evaluation of radular characters in Thaidinae (Gastropoda: Muricidae). J Sci Hiroshima Univ 1: 255–287

Goldstein P, Desalle R (2011) Integrating DNA barcode data and taxonomic practice: determination, discovery, and description. BioEssays 33: 135–147

Grabau AW, King SG (1928) Shells of Peitaiho. Peking Laboratory of Natural History, Beijing

Guo X, Zhao D, Jung D, Li Q, Kong L, Ni G, et al. (2015) Phylogeography of the rock shell *Thais clavigera* (Mollusca): evidence for long-distance dispersal in the Northwestern Pacific. PLOS ONE 10: e0129715

Hellberg M, Vacquier V (1999) Rapid evolution of fertilization selectivity and lysin cDNA sequences in teguline gastropods. Mol Biol Evol 16: 839–848

Higo S, Callomon P, Goto Y (1999) Catalogue and Bibliography of the Marine Shell-Bearing Mollusca of Japan. Elle Scientific, Osaka

Huang J, Ding S, Wang D, Ke C (2013) The radula research of 13 *Thais* species in China coast. Mar Sci 37: 72–77 (in Chinese with English abstract)

Hung T, Hsu W, Mang P, Chuang A (2001) Organotins and imposex in the rock shell, *Thais clavigera*, from oyster mariculture areas in Taiwan. Environ Pollut 112: 145–152

King S, Grabau A (1928) Shells of Peitaiho. Peking and Peking Leader Press, Beijing

Kool S (1993) Phylogenetic analysis of the Rapaninae (Neogastropoda, Muricidae). Malacologia 35: 155–259

Kuroda T, Habe T, Oyama K (1971) The sea shells of Sagami Bay: collected by His Majesty the Emperor of Japan. Maruzen

Küster H (1860) Die Schliessschnecken und die verwandten Gattungen (Clausilia, Balea, Cylindrella, Megaspira): in Abbildungen nach der Natur mit Beschreibungen. Bauer & Raspe

Lamarck J (1822) Histoire naturelle des animaux sans vertébres. Paris

Linnaeus C (1767) Systema naturae per regna tria naturae: secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Ed. 12. 1. Salvius, Holmiae, 533–1327

Mcgovern T, Keever C, Saski C, Hart M, Marko P (2010) Divergence genetics analysis reveals historical population genetic processes leading to contrasting phylogeographic patterns in co-distributed species. Mol Ecol 19: 5043–5060

Pons J, Barraclough T, Gomez-zurita J, Cardoso A, Duran D, Hazell S, et al. (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Syst Biol 55: 595–609

Puillandre N, Lambert A, Brouillet S, Achaz G (2012b) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Mol Ecol 21: 1864–1877

Puillandre N, Modica M, Zhang Y, Sirovich L, Boisselier M, Cruaud C, et al. (2012a) Large-scale species delimitation method for hyperdiverse groups. Mol Ecol 21: 2671–2691

Rambaut A, Suchard D, Drummond A (2014) Tracer 1.6. URL: http://beast.bio.ed.ac.uk/tracer

Ren J, Hou Z, Wang H, Sun M, Liu X, Liu B, et al. (2016) Intraspecific variation in mitogenomes of five *Crassostrea* species provides insight into oyster diversification and speciation. Mar Biotechnol 18: 242–254

Röding P (1798) Museum Boltenianum sive Catalogus cimeliorum e tribus regnis naturæ quæ olim collegerat Joa. Fried Bolten, M. D. p. d. per XL. annos proto physicus Hamburgensis. Pars secunda continens Conchylia sive Testacea univalvia, bivalvia & multivalvia. Hamburg

Roy V, Constantino R, Chassany V, Giusti-Miller S, Diouf M, Mora P, et al. (2014) Species delimitation and phylogeny in the genus *Nasutitermes* (Termitidae: Nasutitermitinae) in French Guiana. Mol Ecol 23: 902–920

Schumacher C (1817) Essai d'un nouveau système des habitations des vers testacés. Mr. le directeur Schultz

Shen K, Jamandre B, Hsu C, Tzeng W, Durand J (2011) Plio-Pleistocene sea level and temperature fluctuations in the northwestern Pacific promoted speciation in the globally-distributed flathead mullet *Mugil cephalus*. BMC Evol Biol 11: 83

Souleyet L, Eydoux J (1852) Voyage autour du monde exécuté pendant les années 1836 et 1837 sur la corvette la Bonite commandée par M. Vaillant. Paris

Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30: 1312–1313

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30: 2725–2729

Tan K, Liu L (2001) Description of a new species of *Thais* (Mollusca: Neogastropoda: Muricidae) from Taiwan, based on morphological and allozyme analyses. Zool Sci 18: 1275–1289

Tan K, Sigurdsson J (1990) A new species of *Thais* (Gastropoda: Muricidae) from Singapore and peninsular Malaysia. Raffles Bull Zool 38: 205–212

Tang C, Wang W (2009) Organotin accumulation in oysters and rock shells under field conditions. J Environ Monit 11: 1601

Tang C, Humphreys A, Fontaneto D, Barraclough T (2014) Effects of phylogenetic reconstruction method on the robustness of species delimitation using single-locus data. Methods Ecol Evol 5: 1086–1094

Taylor J, Morton B (1996) The diets of predatory gastropods in the Cape D'Aguilar Marine Reserve, Hong Kong. Asian Mar Biol 13: 141–166

Team RC (2017) R: A language and environment for statistical computing. Computer program, Vienna, Austria. http://www.r-project.org Accessed online 10 May 2018

Thompson J, Gibson T, Plewniak F, Jeanmougin F, Higgins D (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882

Valenciennes A (1846) Atlas de Zoologie. Mollusques. In: A. du Petit-Thouars, Voyage autour du monde sur la frégate la Venus pendant les années 1836–1839. Vol 4, pg 22, pl 7, fig. 1

von der Schulenburg J, Hancock J, Pagnamenta A, Sloggett J, Majerus M, Hurst G (2001) Extreme length and length variation in the first ribosomal internal transcribed spacer of ladybird beetles (Coleoptera: Coccinellidae). Mol Biol Evol 18: 648–660

Wang C, Li C, Li S (2008) Mitochondrial DNA-inferred population structure and demographic history of the mitten crab (*Eriocheir sensu stricto*) found along the coast of mainland China. Mol Ecol 17: 3515–3527

Williams S, Donald K, Spencer H, Nakano T (2010) Molecular systematics of the marine gastropod families Trochidae and Calliostomatidae (Mollusca: Superfamily Trochoidea). Mol Phylogenet Evol 54: 783–809

Yang J, Zheng X, Li Q, Wang R, Song Z, You B (2005) Quantitative study of phynotypic genetic diversity of *Rapana Venosa* in China's coastwaters. Oceanol Limnol Sin 37: 385–392 (in Chinese with English abstract)

Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29: 2869–2876

Zhao D, Li Q, Kong L, Yu H (2017) Cryptic diversity of marine gastropod *Monodonta labio* (Trochidae): did the early Pleistocene glacial isolation and sea surface temperature gradient jointly drive diversification of sister species and/or subspecies in the Northwestern Pacific? Mar Ecol (Berl) 38: e12443

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