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RESEARCH ARTICLE

Cryptic genetic diversity of *Neverita didyma* in the coast of China revealed by phylogeographic analysis: implications for management and conservation

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Abstract Revealing cryptic biodiversity and understanding the processes that promote lineage diversification will provide valuable insights into management and protection of exploitable species. *Neverita didyma* is one of the most common marine species along the coast of China and possesses highly economic and nutritional value. Despite being heavily harvested each year, the genetic diversity of this species has never been assessed in the coastal areas of China. Here, we analyzed the diversity of this species based on the barcode region of the mitochondrial gene Cytochrome Oxidase subunit I (*COI*) and utilized different species delineation approaches to infer evolutionarily significant units (ESUs). Three distinct ESUs, with high genetic distance among each, were identified. Divergence time estimates suggested that the high genetic distances were probably associated with historical isolation of the marginal seas during Pleistocene low sea level periods. The three ESUs did not map to distinct geographical distribution, possibly attributing to the repeated isolation in different refugia and random postglacial recolonization. Moreover, *N. didyma* in Haizhou Bay deserves priority protection due to its unique ESU. To improve management regulations in the marine realm, our research also stresses the need for more empirical studies on genetic diversity of commercially exploited species in coastal environments of China.

Keywords Evolutionarily significant units · Genetic conservation · Phylogeography · East Asia

Introduction

The presence of cryptic species and/or hidden genetic diversity poses a formidable challenge for the accurate assessment of biodiversity and conservation. The species is the taxonomic category most frequently used as conservation unit (Mace 2004). However, species classification may not reflect the underlying/cryptic genetic diversity worthy of conservation efforts, as it represents the potential of populations to evolve and adapt (Moritz 1994). With the help of molecular data, conservation geneticists investigated alternative intraspecific units of conservation allowing cryptic genetic diversity to be taken into account, for example the evolutionarily significant units (ESUs) and management units (MUs) (Torres-Cambas et al. 2017). As an effective tool to identify these units, molecular taxonomy coupled with phylogeography, apart from the pure discovery of hidden species diversity, can offer insights into the spatial structure of genetic diversity in understudied marine organisms, and the historical and ecological processes driving their present-day distribution (Leasi and Norenburg 2014). Practically, conservation genetics of marine species has so far been concerned primarily with this effective tool when designating conservation priorities (Moritz 2002).

Phylogeographic patterns and species diversity data can be effectively combined for integrated conservation planning at the level of communities and biogeographic subregions (Reilly et al. 2015). In the north-western Pacific, three primary factors influence the phylogeographic patterns of marine species: (1) population isolation and expansion during glacial cycles (Ni et al. 2014; Wang et al.

2016), (2) gene flow induced by ocean currents (Guo et al. 2015), and (3) the discharge of the Yangtze River outflow (Dong et al. 2012; Wang et al. 2015). Corresponding to these factors, Spalding et al. (2007) defined four marine ecoregions (Yellow Sea, East China Sea, Southern China, and Gulf of Tonkin) for regional protection management along the coastal and shelf waters of China. Nonetheless, genetic subdivision has been detected recently within the Yellow Sea ecoregion (Han et al. 2015; Ni et al. 2015), suggesting that the conservation units may be underestimated in this insufficiently explored area. Moreover, the present patterns of genetic structure in the marine realm is more likely a product of complex species-specific ecological processes. The ecoregion-based protection strategy may not be applicable to all cases, especially at the species level. Indeed, conservation biologists ought to consider the evolutionary processes operating within a species and then develop a criteria and strategy relevant to protecting the evolutionary processes and dinsuffici(, (ses. The ze mar)-19(specishelx)2er 9(t 999(18.899z1 rg/GS1 gs0 Tw(2015)Tj0MC40 0 rg/G

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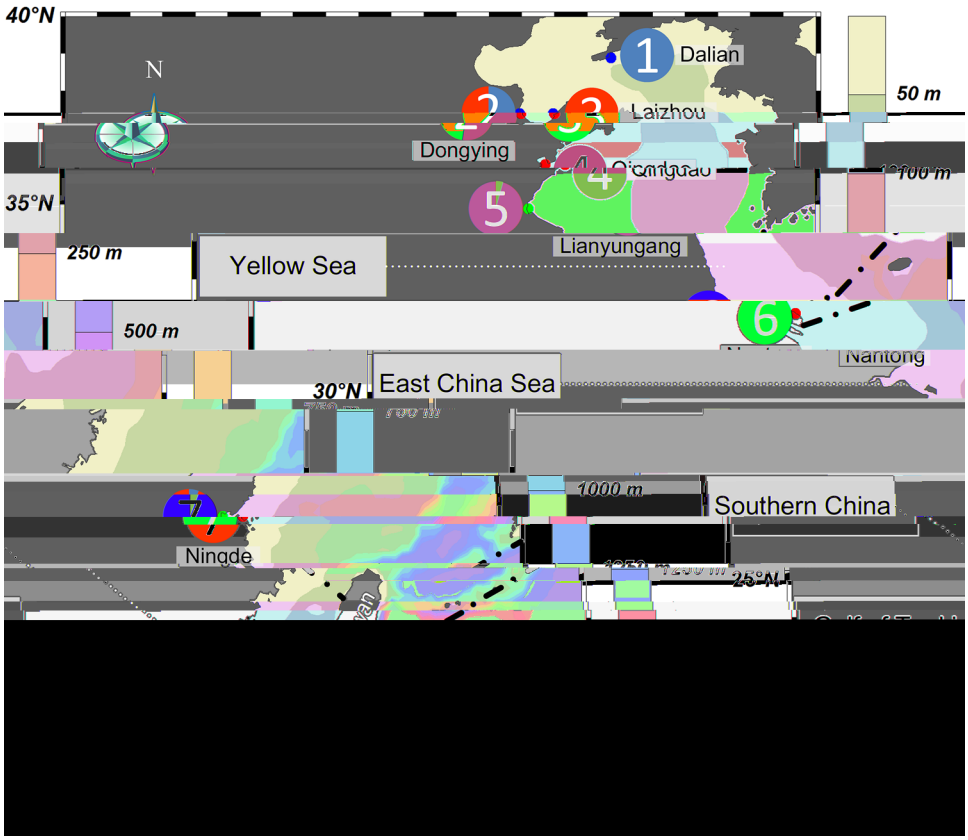

Fig. 1 Map of China showing the study sampling locations and the distribution of the three ESUs for *Neverita didyma*. The *di erent colors* in the *pie charts* correspond to the three ESUs (see Fig. 2 for ESUs identification), and the *labels* within *grey boxes* on the *left* represent the ecoregions Spalding defined. (Color figure online)

ESUs delimitation

The relatively conserved morphology of *N. didyma* presents substantial challenges when applying morphological taxonomy methods for ESUs delimitation. As an alternative approach, DNA barcoding and single-locus coalescent-based methods were employed to assess cryptic diversity. ABGD method uses the ordered, ranked genetic distances from *COI* fragments to distinguish significant shifts from low intraspecific distances to higher interspecific distances. These observed transitions in genetic distances assigns sampled individuals into separate groups (Puillandre et al. 2012). The GMYC method (Pons et al. 2006) separately models the fit of Yule and coalescent processes to an ultrametric tree to define the transition from species-level to population-level processes. The PTP (Zhang et al. 2013) models speciation and coalescent events relative to numbers 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 provide objective, clade-specific threshold with which to delimit ESUs of diversity.

First, we used the Automatic Barcode Gap Discovery (ABGD) method (Puillandre et al. 2012) through the online server (http://www.abi.snv.jussieu.fr/public/abgd/) with the default settings. Second, as recommended by Tang et al. (2014), we implemented the Generalized Mixed Yule Coalescent (GMYC) method (Pons et al. 2006) with a BEAST tree, and the Poisson Tree Process (PTP) model (Zhang et al. 2013) with a RAxML gene tree, both operated through the online server (http://species.h-its.org). A half million MCMC generations were set for the bPTP analyses.

Prior to the delimitation analyses, phylogenetic relationships among all haplotypes were inferred, using Bayesian inference (BI) and maximum-likelihood (ML) reconstruction methods implemented in BEAST v1.8.2 (Drummond et al. 2012) and RAxML v8.2.4 (Stamatakis 2014), respectively. Prior to the phylogenetic reconstruction, the best-fit model of nucleotide substitution was determined by JMODELTEST v2.1.1 (Darriba et al. 2012) under the Akaike Information Criterion (AIC). GTR+G was selected as the most appropriate model for subsequent analyses. Branch supports were assessed using 1000 bootstrap replicates for ML trees. For the BEAST analysis, the Yule process of speciation and uncorrelated log-normal relaxed clock model were used as the tree prior. Two independent Markov-chain Monte Carlo (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 checked in TRACER v1.6.

After that, the phylogenies were then subjected to GMYC and bPTP analysis.

Haplotype network and population genetics analyses

To investigate shallow relationships among closely related haplotypes, a median-joining network was constructed in NETWORK v4.6.1.3 (Bandelt et al. 1999) for the *COI* marker.

To assess the genetic divergence among the distinct ESUs, distances between and within ESUs were calculated in MEGA 6 (Tamura et al. 2013). Genetic diversity was analyzed using DNASP v5 (Librado and Rozas 2009).

Divergence time estimates

Estimation of the divergence times between the ESUs was conducted in BEAST v1.8.2 on the *COI* gene data using a calibrated molecular clock method. GTR+G was selected as the most appropriate model by JMODELTEST v2.1.1. Detailed prior settings were used as previously mentioned. Given the absence of a clear fossil or geological record, *COI* sequence divergence rates 1.52 and 2.4% per million years, calibrated for genus *Nucella* (McGovern et al. 2010) and two trochid species (Hellberg and Vacquier 1999) respectively, were used to assess conservatively a range of dates for key nodes. Note that sequence divergence rate = substitution rate × 2 (Wilke et al. 2013). Detailed prior settings for each molecular clock are shown in Table 1.

Results

The 426-bp *COI* fragment (with no indels) showed a high level of polymorphism, with a total of 75 haplotypes for 168 individuals. Among these, 58 haplotypes were unique. Shared haplotypes among different localities distributed mainly in Dongying (DY), Qingdao (QD), Dalian (DL), Nantong (NT) and Ningde (ND). Notably, individuals from Beihai (BH) and Laizhou (LZ) shared an identical haplotype (Fig. 3) despite being separated by more than 3300 km. Individuals in Lianyungang (LYZ) did not share any haplotype with samples in other sites.

The ABGD, GMYC, and bPTP methods generated identical results, delineating three distinct ESUs (G1, G2, and G3; Figs. 2, 3). These ESUs did not map to distinct geographical distributions. The G1 clade contained individuals from BH, ND, NT, LZ, and DY; whereas, G2 clade comprised individuals from ND, LYZ, QD, DY, and DL. In comparison, the G3 clade contained haplotypes found only in LYZ. The median-joining network recovered the three well-defined ESUs. Two subnetworks were displayed in the G1 clade; two distinct lineages were also revealed in this clade in the phylogenetic analyses.

Fig. 2 BEAST divergence time estimation of the three ESUs of *Neverita didyma* using a calibrated molecular clock method. Node age intervals are shown at nodes. Timescales in million years before present (Ma)

The pairwise genetic distances (uncorrected p-distance) were maximal between G1 and G2 (8.6%). Minimum distance value was found between G1 and G3 (5.8%). Distance between G2 and G3 was 6.8%. Genetic distances within each ESUs were 1.3% (G1), 0.8% (G2) and 0.4% (G3), respectively.

Figure 2 shows the *N. didyma* phylogeny based on *COI* gene sequence variation, using a calibration rate ranging from 1.52 to 2.4% per million years. The divergence date estimates revealed relatively recent split times among the three main clades. The initial divergence between Clade G1+G3 and Clade G2 was estimated at approximately 1.442–2.382 million years ago (Ma), from the early Pleistocene to mid-Pleistocene. Estimated divergence age between Clade G1 and Clade G3 ranges from 1.167 to 2.004 Ma. The most recent split between the two distinct lineages within G1 dates to 0.896–1.555 Ma corresponding to the middle Pleistocene.

Discussion

ESUs delimitation

Our results showed three distinct ESUs of *N. didyma* within the Yellow Sea ecoregion, suggesting cryptic diversity that until now, has gone unrecognized. Degree of phenotypic variation for *N. didyma* in the Yellow Sea ecoregion did not satisfy the divergence level of subspecies (Sun et al. 2012). However, morphological differentiation does not always correlate with genetic diversification. All three species delimitation approaches yielded identical results, providing

Table 1 Two molecular clock calibrations used to estimate the divergence time of the distinct *Neverita didyma* lineages

Molecular clock	Sequence divergence rates		Substitution rates specified in BEAST prior distribution = normal	
Hellberg and Vacquier (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%

All units are per site per million years

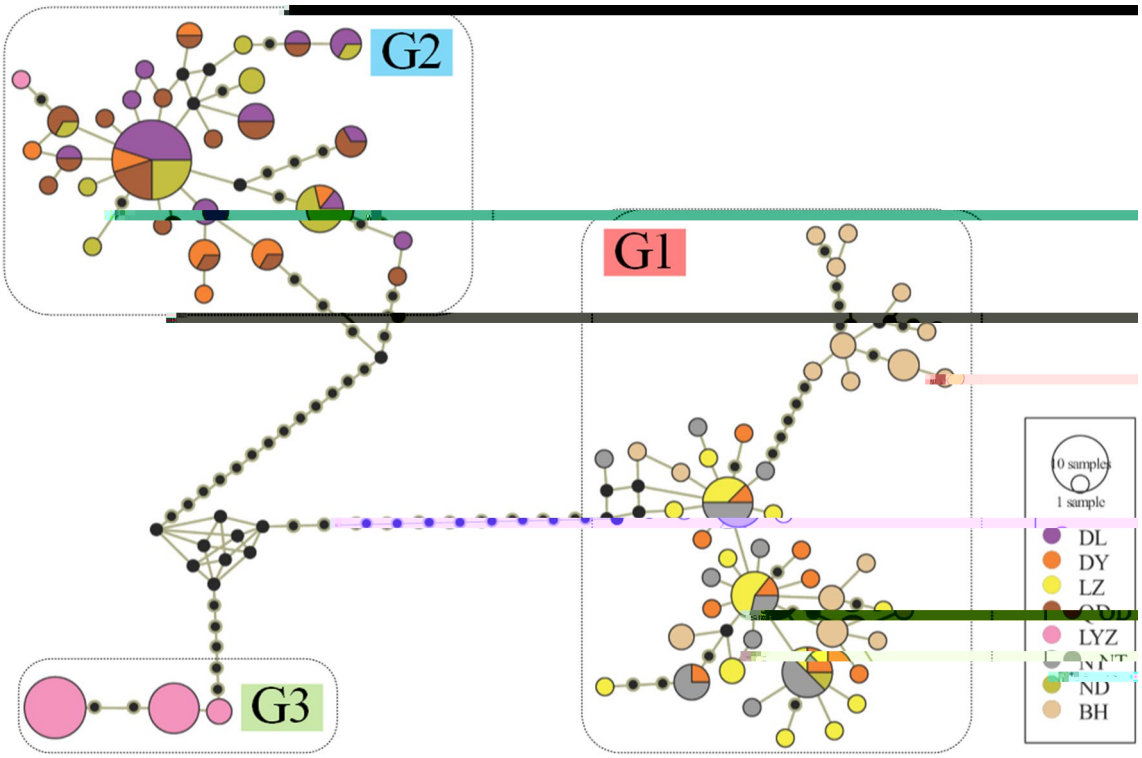

Fig. 3 Median-joining networks of *Neverita didyma* for *CO1*. The *pie charts* represent the haplotypes, and *di erent colors* correspond to sampling localities (DL: 1, DY: 2, LZ: 3, QD: 4, LYZ: 5, NT: 6, ND: 7, BH: 8; see Fig. 1). *Lines* joining haplotypes refer to one base pair mutation and the *black dots* along the *lines* are missing haplotypes (not sampled or extinct). *Circle sizes* are proportional to haplotype frequencies. (Color figure online)

strong evidence for this cryptic genetic diversity. Whether these ESUs represent distinct species remains unclear.

The p-distances among the ESUs ranged from 5.8 to 8.6%. In contrast, individuals within each ESU differed by only 0.4–1.3%. Previous study showed that interspecific divergences ranged from 5.5 to 11.1% in genetic p-distances within *Polinices*, a genus closely related to *N. didyma* (Zhang 2003). Intra-specifically, p-distances varied, depending on species; *Polinices cumingianus, Polinices mellosus* and *Polinices uber* had low intra-specific divergence ranging from 0.1 to 1.3%, even between individuals of the same species collected from widely separated localities. In comparison, individuals of *P. sp. 1* differed 8.6–9.1% in p-distance. The genetic divergence within the *P. sp. 1* species was similar or even higher than that between other taxonomically distinct *Polinices* species (Huelsken et al. 2012). Given such a wide range of divergences among species, we have not specified the taxonomic status of the three distinct ESUs detected in *N. didyma* through the divergence of p-distances at this time.

Indeed, the application of barcoding and single-gene based approaches to species delimitation has been a controversial topic. Some caveats of single-gene approaches include the presence of pseudogenes (Bensasson et al. 2001), introgression (Ballard and Whitlock 2004) or incomplete lineage sorting (Funk and Omland 2003). To ascertain the taxonomic status of the divergent cryptic lineages in *N. didyma*, one or more nuclear unlinked genes will be required (Corl and Ellegren 2013). Nonetheless, for the purpose of setting conservation priorities and recognizing

the bulk of undescribed biodiversity, the *COI* identification system provides a reliable, cost-effective and accessible solution to the issue of species identification, and can serve as an effective approach of providing information for sustainable management of marine species. For example, the *COI* identification system has disentangled the actual diversity of meiofauna (Leasi and Norenburg 2014).

Interpreting phylogeographic patterns

Determining divergence times among the distinct clades is crucial for evaluating potential diversification mechanisms and historical biogeographic events. These information can provide an improved view of conservation for a particular species (Villanova et al. 2017). The estimated divergences among the three ESUs of *N. didyma* date back to the mid Pleistocene to early Pleistocene, a time frame that is congruent with the hypothesis of isolation by Dongshan land bridge (Zhao et al. 2017).

In this study, the estimated divergence time between clade G1+G3 and clade G2 corresponds to the initiation of large-scale ice sheets in Eastern Asia region, dated at 2.5–2.3 Ma. In this epoch, sea level fell by as much as 50–60 m and the Dongshan land bridge emerged, separating the populations in the enclosed ancient East China Sea (AECS) and the semi-closed ancient South China Sea (ASCS) (Cronin et al. 1994). Comparable biogeographic events may have taken place during the time of divergence between clade G1 and clade G3. Similarly, estimated divergence between clade A+B and clade C has been dated to 1.983 Ma (Zhao et al. 2017). Nonetheless, no direct records demonstrate that the Dongshan land bridge emerged during the period of 1.165–2.004 Ma. The coalescence time of subgroups within G1 dates back to 0.896–1.5547 Ma, a time frame that is congruent with the Middle Pleistocene Transition (MPT) (Pisias and Moore 1981). During this epoch, 40–50 m of sea level decrease was detected from 0.9 to 1 Ma (Sosdian and Rosenthal 2009; Kitamura 2015). Vicariance between the deep trough in the Qiongzhou Strait and the ASCS following the emergence of the Qingzhou Strait landmass, which is 40 m below present sea level, may explain the observed substructure within G1. Given the absence of a clear fossil record, the time frame was calculated under the 'time-dependent mutation rate' hypothesis. As this hypothesis is still controversial (Bandelt 2008) and applying selected rates that may or may not apply to this particular species, conclusions abstracted here should be treated with caution.

Conservation implications

How to delineate and prioritize conservation units below the level of taxonomically recognized species has long been a debatable topic. Generally, biogeographic regions that have sustained the longest periods of isolation will represent the maximum species diversity and phylogeographic diversity (Moritz 2002). However, this recommended rule may not be appropriate in all circumstances. The admixture of genetic lineages following the recolonization from separate refugia often poses difficulty in determining conservation units and stresses the need for species-specific treatment (Fraser and Bernatchez 2001). In this study, the genetic diversity of the populations that recolonized the Yellow Sea ecoregion should be composed of subsets of that in the source refugial populations. Hence, ecoregion-based protection strategy is inappropriate for *N. didyma*.

Knowledge of the distribution of genetic diversity allows the identification of conservation units. Within *N. didyma*, the geographical distribution of the three ESUs was not ecoregion-based. Both ESU G1 and ESU G2 show wide overlapping geographical ranges, and if one stock is overharvested, it can be replenished by migrants from elsewhere. By contrast, ESU G3 was found only in LYZ among all sampling localities. Under the ESU paradigm, the unique population LYZ is the primary focus of conservation concerns.

Knowing which places are the most important to conserve is central to marine spatial management (Crowder and Norse 2008). LYZ is located in Haizhou Bay, an area known for its unique genetic diversity of marine species. Understanding the evolutionary history and tectonic structure in this area will help unravel the factors leading to the unique genetic diversity. Yuntai Mountain, situated in LYZ, was an island or seamounts prior to 300 years ago (Wang et al. 1980). Presumably, it would have modified ocean currents and provided shallower substrates than the surrounding muddy abyssal plains. Similar to our findings, remarkable genetic differentiation was revealed between LYZ and northern populations based on mitochondrial data for the surf clam *Mactra chinensis* (Ni et al. 2015). A genetic discontinuity was also detected in Haizhou Bay for the Asian paddle crab, *Charybdis japonica* (Han et al. 2015). Together, these findings suggest that this region may be biologically unique, and therefore, necessary to protect from anthropogenic activities that threaten the extirpation of local populations and genetic diversity.

Indeed, perhaps the most important insight that marine ecologists can share with managers is that some places have much greater importance than others for particular species, ecosystems or processes, and hence for humans (Crowder and Norse 2008). The results of our study in China's coastal areas also emphasize the need for more empirical studies on genetic diversity of commercially exploited species in coastal environments. The information will provide valuable insights into the sustainable development of fisheries and biodiversity conservation strategies.

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