DNA barcoding and phylogeographic analysis of *Nipponacmea* limpets (Gastropoda: Lottiidae) in China

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ABSTRACT

To identify the *Nipponacmea* limpets along the coast of China, their taxonomy was investigated with three molecular markers (one mitochondrial gene, COI; two nuclear markers, 28S eDNA and H3). Three species (*N. radula*, *N. fuscoviridis* and *N. nigrans*) were found among 274 individuals collected from 14 sites. Intraspecific variation was far less than interspecific variation and obvious barcoding gaps existed. These results indicate that the three *Nipponacmea* species can be efficiently identified by DNA barcoding. The phylogeographic patterns of the three species were also analysed using COI sequences. There was clear biogeographic separation between the northern *N. radula* and the southern two species (*N. fuscoviridis* and *N. nigrans*), with the Yangtze River estuary as a barrier. In the southern *N. fuscoviridis*, there was a star-shaped haplotype network and the dominant haplotype was detected in all populations. In the northern *N. radula*, there were five main haplotypes; some adjacent populations showed no significant difference according to the pairwise Fst values. The southern *N. nigrans* showed two main haplotypes. The phylogeographic break between the *Nipponacmea* species is possibly due to the local and regional hydrographic conditions in the Yangtze River estuary, the large salt marsh in the river delta and difference of temperature between northern and southern China.

INTRODUCTION


In previous studies (e.g. Suter, 1907; Oliver, 1926; Powell, 1973; Ponder & Creese, 1980) taxonomic studies of limpets were commonly based on shell morphology and radular characters. However, the highly variable morphology commonly leads to the failure to recognize cryptic species of limpets (Sasaki, 1999; Nakano & Spencer, 2007; Johnson, Wareń & Vrijenhoek, 2008; Nakano et al., 2009). Therefore, it is desirable to test species identification of limpets with molecular markers.

DNA barcoding, identification using a short standard DNA sequence, is advocated as a method for species identification and classification (Hebert et al., 2003) and has been widely used for revealing cryptic species, discovering new species (Nakano & Spencer, 2007; Johnson, Wareń & Vrijenhoek, 2008; Baldwin & Weigl, 2012), delimiting species boundaries (Nieukerken, Mutanen & Doorenweerd, 2012), studying population genetic and phylogenetic patterns (Nakano & Ozawa, 2004; Nakano & Ozawa, 2007; Nakano et al., 2009) and detecting invasive species (Yancy et al., 2007; Ashton et al., 2008). The most frequently used barcoding marker for invertebrates is a fragment of the mitochondrial cytochrome c oxidase subunit I gene (COI) (Hebert, Ratnasingham & Dewaard, 2003). Although mitochondrial (mt) DNA has been widely used for identification and classification, it has been challenged because it is a single-gene identification system (Lipscomb, Platnick & Wheeler, 2003; Mallet & Willmott, 2003; Wheeler, 2005). Mitochondrial and nuclear genomes have different patterns of evolution and modes of inheritance, which can result in very different assessments of biodiversity (Rubinoff, 2005). In this case, researchers have proposed the concept of ‘integrative taxonomy’, which is defined as the science that aims to delimit the units of life’s diversity from multiple and complementary perspectives (phylogeography,
comparative morphology, population genetics, ecology, development, behaviour, etc.) (Dayrat, 2005). At the same time, some nuclear genes have been confirmed to be suitable for classification and identification by DNA barcoding, such as Internal Transcribed Spacer (ITS), 28S ribosomal DNA (28S rDNA) and Histone 3 (H3) (Nakano et al., 2009; Sengupta et al., 2009; Fernández et al., 2011).

Connectivity among populations is one of the central topics in marine biology. For most marine benthic species, genetic exchange occurs primarily during the pelagic larval stage (Scheltema, 1971), and the dispersal distance is related to regional hydrographic conditions and also to the dispersal ability of larvae (Avise et al., 1986). Habitats along different coastlines differ in their local nutrient concentrations, salinity, turbidity and chlorophyll concentration, which all have crucial impacts on larval dispersal (Ayre, Minchinton & Perrin, 2009; Nakano, Sasaki & Kase, 2010). The Yangtze River is the longest river in China, with a mainstream length of 6,300 km, and is defined as boundary of northern China and southern China. It has significant impacts on the phylogeographic pattern of rocky-shore species, including the limpets Cellana toruana (Dong et al., 2012) and Siphonaria japonica (J. Wang, unpubl.). Furthermore, the intertidal substrates near the Yangtze River Delta are muddy shores and are unsuitable for the settlement of rocky-shore species.

According to available records from mainland China, only one species in the genus Nipponacmea, N. schrenckii, has been recorded (as Notoacmea schrenckii, Huang, 2008; Liu, 2008). However, previous studies showed that there were nine different species of Nipponacmea in Japan (Sasaki & Okutani, 1993; Nakano & Ozawa, 2007). Therefore, there may be more than one Nipponacmea species in mainland China. In the present study, an integrative method comprised of comparative morphology, phylogenetic and phylogeographical analysis was used for Nipponacmea identification along the coast of China.

**MATERIAL AND METHODS**

Sample collection and identification

In total, 274 individuals from the genus Nipponacmea were collected from 14 sites along the coast of China (Fig. 1; sequences from TW were provided by T. Nakano). Five populations were sampled from the Yellow Sea (YS), seven from the East China Sea (ECS) and two from the South China Sea (SCS). After collection, the specimens were stored in 95% ethanol for further analysis. The identifications of Nipponacmea species were based on BLAST searches and morphological characters. BLAST searches were used to match our sequences with named sequences in GenBank. We also examined the animal coloration and configuration of the radula sac of the Nipponacmea species as described in taxonomic works from Japan (Sasaki & Okutani, 1993; T. Nakano, personal communication).

**DNA extraction, PCR amplification and DNA sequencing**

COI: Genomic DNA was extracted from foot muscle tissue using methods described by Dong et al. (2012). Partial sequences of mitochondrial COI were amplified using the universal primers LCO1490 and HCO2198 (Folmer et al., 1994). The PCR followed the standard profile with a 3 min initial denaturation at 95°C, followed by 35 cycles of 95°C for 60 s, 40°C for 60 s and 72°C 60 s, and a final extension phase at 72°C for 10 min. To confirm that amplifications were successful, 5 μl aliquots of PCR reaction were visualized by agarose-gel electrophoresis. The successful amplification was then purified with the QIAquick Gel Purification Kit (QIAGEN, Germany). Purified DNA was quantified using agarose-gel electrophoresis and sequenced in an ABI3700 (Applied Biosystems, USA) automated sequencer.

H3 and 28S: Preliminary mtDNA analysis of Nipponacmea populations revealed three phylogenetic clades (see Results). In order to further confirm the results from the mitochondrial gene, H3 and 28S rDNA sequences of five individuals from each clade were analysed (H3: QD1-5, XM1-5, ZS1-3, ZS5 and ZS6; 28S: LGY21-25, CW1-5, ZS1-3, ZS5 and ZS6). A 1,480-bp fragment of 28S rDNA was amplified with the following primers: LSU5 (F) (Littlewood, Curini-Galletti & Herniou, 2000) and LSU1600 (R) (Williams, Reid & Littlewood, 2003). The cycling parameters for 28S were an initial denaturation step at 95°C for 2 min, followed by 35 cycles of 95°C for 60 s, 50°C for 60 s and 72°C for 90 s, and a final extension phase at 72°C for 10 min. The H3 fragment was amplified using the forward primer H3aF and reverse primer H3aR (Colgan et al., 1998). The cycling parameters for 28S were an initial denaturation step at 95°C for 2 min, followed by 35 cycles of 95°C for 60 s, 50°C for 60 s and 72°C 90 s, and a final extension phase at 72°C for 10 min.

**Phylogenetic analyses**


For analysis of COI sequences the outgroup consisted of Patelloidea conulus (Dunker, 1961) (GenBank: AB196507) and P. pygmaea
Sequences of each individual were aligned with CLUSTAL X v. 1.81 (Thompson et al., 1997), and individual consensus sequences were retrieved with both alignment and manual checks. Their authenticity and homology with the targeted genes were evaluated with a BLAST search in the NCBI genetic database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The accuracy of COI was confirmed by translating the nucleotide data into amino acid sequences. The phylogenetic relationships among species were inferred with Neighbor-Joining (NJ) and Bayesian methods. NJ trees were generated using MEGA4 (Tamura et al., 2007) under the Kimura-2-parameter (K2P) model of substitution with 1,000 replicates (Kimura, 1980). Levels of K2P distances were estimated both within and between the species defined by phylogenetic analysis. Sequence divergence was also calculated using the K2P model in MEGA4 (Tamura et al., 2007). MrBayes was run with the following settings: the maximum-likelihood model employed six substitution types (nst = 6); rate variation across sites was modelled using a gamma distribution, with a proportion of the sites being invariant (rate = invgamma); Markov chain Monte Carlo (MCMC) search was run with four chains for 1,000,000 generations, with trees being sampled every 100 generations and the first 2,500 trees were discarded as burnin.

Population genetic analysis

Estimates of molecular diversity and molecular genetic diversity values (\(\pi\), nucleotide diversity; \(h\), gene diversity; \(F_s\), values, and haplotype frequencies) were performed using ARLEQUIN v. 3.5 (Excoffier & Lischer, 2010). For each species, the models that best fitted our observed patterns of mtDNA COI sequence divergence were selected. The best-fit model was evaluated using jModelTest (Posada, 2008) under Bayesian information criteria (BIC). The GTR model was selected for all the three species.

The hierarchical distribution of genetic variation among populations was estimated using an analysis of molecular variance (AMOVA) (Excoffier, Smouse & Quattro, 1992) in ARLEQUIN v. 3.1.1 (Excoffier, Laval & Schneider, 2005). Historical demographic expansions were examined with Tajima’s D test (Tajima, 1989) and Fu’s Fs (Fu, 1996). Historic demographic expansions were also investigated by examining frequency distributions of pairwise differences between sequences (mismatch distribution) (Excoffier, Smouse & Quattro, 1992). Neutrality and mismatch distribution tests were also performed in ARLEQUIN.

To test whether the genetic variation among populations fitted the isolation-by-distance (IBD) model and Bayesian coalescent inference, Mantel tests and Bayesian skyline plots were performed.
conducted. The Mantel test (with 10,000 randomizations) was implemented in the web-based program IBDWS v. 3.14 (Jensen, Bohonak & Kelley, 2005). For each species, statistical parsimony networks were constructed in TCS v. 1.18 (Clement, Posada & Crandall, 2000) to determine the relationships among haplotypes. Bayesian skyline plots (Drummond et al., 2006) were constructed using BEAST v. 1.5.3 (Drummond & Rambaut, 2007) under a strict molecular clock to estimate the change in population size with time. Because fossils are scarce, it is difficult to estimate divergence times of limpets (Koufopanou et al., 1999). In this study, the divergence rate of COI was taken as 0.85–1.15% per Myr, as calculated from cowries (Kirkendale & Meyer, 2004). Each dataset was run for 50 million generations under an HKY nucleotide substitution model with individual parameters estimated from the data, a constant skyline model with 10 groups, and uniform priors. The chain was sampled every 1,000 generations and the first 50,000 generations were discarded as burn-in. The analysis of each dataset was run three times to ensure consistency and the results from the three replicate runs were combined and resampled with LogCombiner v. 1.5.3 (Rambaut & Drummond, 2007). Trace plots were inspected to assess convergence, mixing and stationarity of the MCMC process in Tracer v. 1.5 (Rambaut & Drummond, 2007) and the effective sample sizes were also checked and confirmed to be ≥200.

RESULTS

Identification of Nipponacmea species

The 274 COI sequences were aligned through BLAST on NCBI, and showed high identity to Nipponacmea fuscoviridis, N. radula and N. nigrans. Morphological characters of the animal coloration and radula sac also support these identifications. The cephalic tentacles and mantle margin between the pallial

Table 2. Mean Kimura two-parameter (K2P) sequence divergence of COI, H3 genes and 28S rDNA between species.

<table>
<thead>
<tr>
<th></th>
<th>N. radula</th>
<th>N. fuscoviridis</th>
<th>N. nigrans</th>
</tr>
</thead>
<tbody>
<tr>
<td>COI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. radula</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. fuscoviridis</td>
<td>25.7%</td>
<td>30.2%</td>
<td></td>
</tr>
<tr>
<td>N. nigrans</td>
<td>29.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. radula</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. fuscoviridis</td>
<td>1.7%</td>
<td>2.4%</td>
<td></td>
</tr>
<tr>
<td>N. nigrans</td>
<td>3.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. radula</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. fuscoviridis</td>
<td>0.2%</td>
<td>0.2%</td>
<td></td>
</tr>
<tr>
<td>N. nigrans</td>
<td>0.2%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Phylogenetic trees generated from H3 and 28S sequences for Nipponacmea species using Bayesian analyses, with bootstrap values above each branch.

Figure 4. Frequency distribution of K2P distances for Nipponacmea in COI phylogeny. There was an obvious barcoding gap of genetic divergence between intraspecific and interspecific variation.
tentacles of *N. fuscoviridis* were black in colour, and the rest pale yellow, lacking black pigmentation. In *N. radula* and *N. nigrans*, the mantle margin, head and lateral sides of the foot, except the dorsal area, were faintly blackish. The configuration of the radula sac of *N. fuscoviridis* and *N. radula* is quite different from that of *N. nigrans*. The radula sac of *N. fuscoviridis* was very long, that of *N. radula* moderately long, and that of *N. nigrans* of medium length. There were no obvious differences between the radular dentition of the three species.

**Molecular phylogeny**

Using the two species of *Patelloida* as outgroup, the phylogenetic tree for *Nipponacmea* based on COI sequences showed three well supported clades, representing *N. radula*, *N. fuscoviridis* and *N. nigrans* (Fig. 2). Among the 274 individuals, 85, 152 and 37 individuals were allocated to *N. radula*, *N. fuscoviridis* and *N. nigrans*, respectively (Table 1). All individuals of *N. radula* were collected from northern China, and all *N. fuscoviridis* and *N. nigrans* from southern China. The molecular diversity values based on COI for the three species are shown in Table 1. After classification of individuals using COI, 28S rDNA and H3 were also sequenced from five individuals of each species. Tree topologies were not identical for each gene, but the recovered tip clades (i.e. putative species) were the same in each tree, with the exception of the placement of one individual (Fig. 3). Individual ZS2 was allocated to *N. nigrans* using COI, but to *N. fuscoviridis* using the nuclear genes. Repeated DNA extraction and sequencing were carried out to confirm this result and therefore excluded possible technical errors. K2P sequence divergence for COI between clusters was 25.0–31.2% (Table 2) and within clusters 0.1–0.3%.

There were five main haplotypes (H1–H5) in *N. radula*. Adjacent collection sites showed similar haplotype compositions (Fig. 5). The *F*$_{ST}$ values (Table 3) also showed that there was no significant difference (*P* > 0.05) between some adjacent sites (DL and WH; QD and RZ).

For *N. fuscoviridis*, there was a dominant haplotype that was found at all collection sites in southern China. The *F*$_{ST}$ values showed that most of the populations have no significant difference, although the *F*$_{ST}$ value of population TW was significantly different from the others (Table 4). *Nipponacmea nigrans* was found at three sites (one individual at NA; 13 individuals at XP; 23 individuals at ZS) and there were two main haplotypes.

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**Table 3.** Matrix of *F*$_{ST}$ values for *Nipponacmea radula*. See Figure 1 for locality abbreviations.

<table>
<thead>
<tr>
<th></th>
<th>DL</th>
<th>LYG</th>
<th>QD</th>
<th>RZ</th>
<th>WH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DL</strong></td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>LYG</td>
<td>0.68764</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>QD</td>
<td>0.61358</td>
<td>0.43355</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>RZ</td>
<td>0.61237</td>
<td>0.42514</td>
<td>−0.02221</td>
<td>0.00000</td>
<td>*</td>
</tr>
<tr>
<td>WH</td>
<td>0.13514</td>
<td>0.55519</td>
<td>0.42852</td>
<td>0.44335</td>
<td>0.00000</td>
</tr>
</tbody>
</table>

*Indicates significant difference at *P* < 0.05.
The Mantel test indicated a significant relationship between \( F_{st} \) values and geographic distances for \( N. \) radula \( (P = 0.004) \) and \( N. \) nigrans \( (P = 0.016) \).

High haplotype diversity \( (h) \) and nucleotide diversity \( (\pi) \) were found in \( N. \) radula and \( N. \) nigrans, and relatively low \( h \) and \( \pi \) in \( N. \) fuscoviridis \( (Table \text{ 5}) \), Tajima’s \( D \) and Fu’s \( F_s \) were used to test for neutrality \( (Table \text{ 5}) \). All the \( D \) and \( F_s \) values were negative. The northern species \( N. \) radula had insignificant negative values. In contrast, the southern species \( (N. \) fuscoviridis and \( N. \) nigrans) showed significant negative values, indicating significant population expansion. Mismatch distributions were unimodal for \( N. \) fuscoviridis and \( N. \) nigrans, and closely fitted the distribution under the sudden expansion model \( (Fig. \text{ 6}) \).

Bayesian skyline plots showed exponential growth in effective population size \( (Fig. \text{ 6}) \). \( Nipponacmea \) nigrans experienced population expansion before 240 Kyr, but population expansion time is not clear for \( N. \) fuscoviridis and \( N. \) radula, probably due to the small number of populations and haplotypes available.

**DISCUSSION**

**DNA barcoding and phylogenetic analyses**

Three \( Nipponacmea \) species \( (N. \) radula, \( N. \) fuscoviridis and \( N. \) nigrans) were identified along the coast of China. The interspecific variations of the molecular markers COI and H3 were notably higher than the intraspecific variations, so that obvious ‘barcoding gaps’ existed. Although this gap did not exist in 28S rDNA, there was no overlap between intraspecific and interspecific variation. Hebert et al. \( (2004) \) proposed a standard screening threshold of sequence difference \( (10 \times \) average intraspecific difference) that could be used to distinguish sister species. The present results indicate that DNA barcoding depending on both barcoding gap and the threshold approach can effectively discriminate among these \( Nipponacmea \) species.

The nuclear and mitochondrial markers of one individual from Zhoushan \( (ZS2) \), in the middle of the China coastline, showed conflicting results. Using COI, ZS2 was identified as \( N. \) fuscoviridis, but using nuclear 28S and H3, however, it was identified as \( N. \) nigrans. Both \( N. \) fuscoviridis and \( N. \) nigrans occur in Zoushan, so this result is possibly due to introgressive hybridization. The phenomenon of introgressive hybridization has been widely reported in plants and is also found in fish and molluscs \( (Gardner, 1995; \) Radchenko, 2004; Vonlanthen et al., 2012). Mitochondrial markers are useful and effective for identification by DNA barcoding, but there is still considerable evidence for processes such as incomplete lineage sorting and introgressive hybridization that confound the results \( (Renoult et al., 2009; Liu, Kong & Zheng, 2011) \). Therefore, for species identification, recognition and discovery it is necessary to integrate the mitochondrial marker with other information, including nuclear genes and morphological traits. Further study using more specimens is necessary for confirmation of introgressive hybridization in \( Nipponacmea \) species.

**Barriers to the distribution of \( Nipponacmea \) in China**

The three \( Nipponacmea \) limpets showed different distribution patterns. \( Nipponacmea \) radula was found in the Yellow Sea and the other species \( (N. \) fuscoviridis and \( N. \) nigrans) in the East China Sea and South China Sea. The clear separation indicates the existence of a phylogeographic break, with the Yangtze River as the boundary between the northern and southern regions. Significant differences in the distribution of haplotypes of \( Cellana torea \) have been found to the north and south of the Yangtze River \( (Dong et al., 2012) \). However, some studies on species in this area have failed to detect a genetic break at the Yangtze River \( (Atrinapectinata, Liu et al., 2011; Cyclina sinensis, Ni et al., 2012) \). Thus the Yangtze River seems to affect some but not all molluscs, due to the differentiation in biological characteristics and habitat specificity. The reason for the existence of barrier to the distribution of \( Nipponacmea \) limpets is likely to be related to the Yangtze River discharge, to substrate availability and temperature difference.

The Yangtze River discharge causes a decrease in the salinity of offshore sea water, and also can affect the nutrient concentration, phytoplankton biomass and hydrodynamic conditions \( (Jiang, Zhang & Wang, 2006; Ayre, Miichinton & Perrin, 2009; Nakano, Sasaki & Kase, 2010; Lin et al., 2011) \). Substrate type is a major factor in the overall composition of benthic communities \( (Al Bakri, Bebehanani & Khuarabiet, 1997) \). The intertidal substrate near the Yangtze River estuary is salt marsh, and cannot provide a suitable substrate for the settlement of rocky-shore species.

Temperature has long been suggested as an important factor regulating early development \( (Costlow & Bookhout, 1971; Anger, 1991) \), length of pelagic life \( (Houde, 1989) \) and mortality of larvae \( (Lutz et al., 1980; Pepin, 1991) \) of marine organisms. Different ambient temperatures lead to substantial among-

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**Table 4. Matrix of \( F_{st} \) values for \( Nipponacmea \) fuscoviridis. See Figure 1 for locality abbreviations.**

<table>
<thead>
<tr>
<th></th>
<th>CW</th>
<th>HK</th>
<th>NA</th>
<th>TW</th>
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<th>X</th>
<th>XM</th>
<th>XP</th>
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<th>ZS</th>
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<tbody>
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<td></td>
<td></td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>TW</td>
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<td>0.4490</td>
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<td>*</td>
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<td></td>
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<tr>
<td>WZ</td>
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<td>0.0699</td>
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<td>0.0000</td>
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<tr>
<td>XM</td>
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<td>0.0416</td>
<td>0.0334</td>
<td>0.2609</td>
<td>0.0906</td>
<td>0.0000</td>
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<tr>
<td>XP</td>
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<td>0.0600</td>
<td>0.0400</td>
<td>0.4516</td>
<td>0.0060</td>
<td>0.0561</td>
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<tr>
<td>ZH</td>
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<td>0.0203</td>
<td>0.0238</td>
<td>0.4409</td>
<td>0.0688</td>
<td>0.0050</td>
<td>0.0392</td>
<td>0.0000</td>
<td>*</td>
<td></td>
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<tr>
<td>ZS</td>
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<td>0.1386</td>
<td>0.0760</td>
<td>0.3666</td>
<td>0.0292</td>
<td>0.0749</td>
<td>0.0166</td>
<td>0.1135</td>
<td>0.0000</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

*Indicates significant difference at \( P < 0.05 \).

**Table 5. Genetic diversity values based on COI. \( Nipponacmea \) radula, \( N. \) fuscoviridis and \( N. \) nigrans.**

<table>
<thead>
<tr>
<th></th>
<th>( h )</th>
<th>( \pi )</th>
<th>( D )</th>
<th>( F_s )</th>
<th>( \tau )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( N. ) radula</td>
<td>0.813</td>
<td>0.0022</td>
<td>-0.423 (ns)</td>
<td>-2.267 (ns)</td>
<td>1.809</td>
</tr>
<tr>
<td>( N. ) fuscoviridis</td>
<td>0.516</td>
<td>0.00199</td>
<td>-1.820**</td>
<td>-10.8358**</td>
<td>0.018</td>
</tr>
<tr>
<td>( N. ) nigrans</td>
<td>0.865</td>
<td>0.002823</td>
<td>-1.934**</td>
<td>-16.089**</td>
<td>2.198</td>
</tr>
</tbody>
</table>

\( h \), haplotype diversity; \( \pi \), nucleotide diversity; \( D \), Tajima’s \( F_s \); \( F_s \); \( \tau \), mismatch parameter; ns, non-significant.

\( *P < 0.05, **P < 0.01, ***P < 0.001. \)
species variation in life-history traits, population dynamics and ecosystem processes. For example, genetic differentiation was found in cod from northeastern and southwestern Iceland where water temperatures are different, and tagged individuals released in the southwestern region seldom migrated to the northern region (Pampoulie et al., 2006). Santos et al. (2006) also found that no gene flow existed in Macrodon ancylopon between tropical and subtropical clades that had homogeneous morphological features.

The difference of surface seawater temperature (SST) between the Yellow Sea, East China Sea and South China Sea is relatively small in summer (YS: 24–27°C; ECS and SCS: 26–29°C). In winter, however, differences are striking. The SST of the Yellow Sea drops below freezing, while in the East China Sea SST is 10–20°C, and in the South China Sea SST remains above 15°C throughout the year (Qiao, 2012). Temperature might therefore be a key factor limiting the northward dispersal of the southern species, even though the East China Sea Coastal Current flows from southwest to northeast along the coast in summer and provides conditions for northward dispersal (Fig. 1). However, the two southern species (N. nigrans and N. fuscoviridis) can also be found in higher latitudinal regions in Japan (e.g. Hokkaido) (Sasaki & Okutani, 1993; Sasaki & Nakano, 2007). Therefore, comparative physiological studies should be carried out to study the difference of thermal tolerance among different populations and to elucidate the effect of temperature on the distribution of N. nigrans and N. fuscoviridis.

Phylogeography of Nipponacmea limpets

The phylogeographic pattern of the northern species N. radula is one of isolation by distance. The pairwise $F_{st}$ values were significant between most populations except the two sets of neighbouring populations (DL and WH; QD and RZ). The Mantel test indicated a significant relationship between $F_{st}$ values and geographic distance ($P = 0.004$), and the network and haplotype frequencies also supported isolation by distance (Fig. 4).

The haplotype diversity and nucleotide diversity were relatively low in N. fuscoviridis. In addition, the significant negative Tajima’s $D$ ($P < 0.01$) and Fu’s $F$ values ($P < 0.001$) in N. fuscoviridis indicated that significant population expansion has occurred. Based on the $F_{st}$ values, there were no significant differences among most populations, indicating the existence of strong gene flow among populations in the East China Sea and

**Figure 6.** Bayesian skyline (A–C) and mismatch distribution (D–F) for Nipponacmea species, showing observed pairwise difference (bars) and the expected mismatch distributions (line) under the sudden expansion model for the COI gene. A, D, N. radula. B, E, N. fuscoviridis. C, F, N. nigrans.
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South China Sea. Phylogeographic research on pen shells has also shown strong gene flow between the East China Sea and South China Sea (Liu et al., 2011). However, the TW population of *N. fuscoviridis* was significantly different from the other populations in the South China Sea. The larval dispersal of this population is likely mainly affected by the Kuroshio Current, while other populations in mainland China are mainly affected by the China Coastal Current and Taiwan Warm Current (Fig. 1).

The difference in phylogeographic patterns between *N. radula* and *N. fuscoviridis* could partly be due to the difference in ocean circulation between the Yellow Sea and the other marginal seas (East China Sea and South China Sea). Ocean currents are a crucial factor affecting the larval dispersal and biogeographic distribution of marine organisms (Chan, Hsu & Southward, 2008). According to published records, the ocean current along the northern China coast in summer (the half year from May to November) forms a nearly closed cycle, because of the density circulation of the Yellow Sea Cold Water Mass, which may limit the southward dispersal of larvae of northern species. In summer, under the impact of the monsoon, the East China Sea Coastal Current basically flows from southwest to northeast along the coast (Guan, 1994). The well mixed seawater in summer in the East China Sea could be an important cause of the strong gene flow in *N. fuscoviridis* (Fig. 4B).

Overall, we have demonstrated the existence of one northern species (*N. radula*) and two southern species (*N. fuscoviridis* and *N. nigrous*) in the genus *Nipponacmea* along the coast of China. Ocean currents, freshwater discharge, substrate and temperature are probably important for the distribution of *Nipponacmea* limpets along this coast. Ongoing global warming will gradually increase the air and ocean temperature in northern China along the coast (Guan, 1994). The well mixed seawater in summer in the East China Sea could be an important cause of the strong gene flow in *N. fuscoviridis* (Fig. 4B).

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