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DEEP GENETIC DIVERGENCE WITHIN A "LIVING FOSSIL" BRACHIOPOD *LINGULA ANATINA*

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ABSTRACT—The geographic population patterns of *Lingula anatina* across the Indo-West Pacific region are analyzed based on mitochondrial COI and nuclear EF-1α gene sequences. Compared with the remarkable morphological stasis, genetic evidence of extant *Lingula* species displays deep genetic divergence. Three distinct COI lineages were detected for *L. anatina*, one of which is from Qinhuangdao (Hebei, China, Bohai Sea), the other two from Beihai (Guangxi, China, Gulf of Tonkin). Individuals from South Japan have a very close relationship with one of the two COI lineages found in Beihai, which is also supported by EF-1α results, suggesting a relatively recent migration between South China Sea and East China Sea. Genetic distances between the three lineages of *L. anatina* are rather high (8.9%, 8.6%, and 2.7%), and those between *L. anatina* and *L. adamsi* is much higher (44.5%), compared to other marine invertebrates. Both tectonic evolution and the repeated Quaternary glaciations have contributed to the complex phylogeographic pattern found in these recent *Lingula anatina* populations.

INTRODUCTION

LINGULA, a brachiopod genus of the family Lingulidae, is often considered to be one of the most ancient "living fossils" based on its supposed long-term morphological conservatism, its characteristic shell, which is referred to as "linguliform", and its remarkable survival tracing back to the Cambrian (Zhang et al., 2008; Stolk et al., 2009). Since appearing in the late Cambrian, the superfamily Linguloidea (Brachiopoda, Lingulata) expanded rapidly, and after Devonian times only the family Lingulidae persists (Emig, 2003; Peng et al., 2007). Lingulidae were apparently one of the few survivors of the several mass extinctions throughout the earth history. Today, the legacy of this group is represented by only two genera: *Lingula* Bruguière 1797 in Asia, Oceania and Africa; and *Glottidia* Dall 1870 on the American continent, occurring latitudinally limited within the 40° belt from temperate to equatorial areas (Emig, 1997; Zezina, 2010). Unlike their modern counterparts, the fossil lingulids have been found almost globally, from high-latitude, polar, cool-temperate environments to low-latitude tropical and subtropical warm-water settings (Peng et al., 2007). Yet the distribution of the lingulids appears rather similar when taking into account the paleolatitudinal positions in correlation with temperatures of water masses.

Despite their reputation for conservatism, lingulid taxa (both fossil and recent species) exhibit morphological variation and evolutionary changes, especially in their inner structures. These changes have led Emig (2003) to doubt the status of *Lingula* as being a "living fossil". Indeed, Emig (2003) restricted the genus *Lingula* to the era from Paleogene to the Present, and referred earlier members to be indeterminate lingulids if their taxonomic positions have not been reclassified. Likewise, Yegorov and Popov (1990), Biernat and Emig (1993) and Smirnova and Ushatinskaya (2001) have placed fossil lingulids in genera other than *Lingula*. In spite of these arguments about *Lingula*, it is still the most morphologically primitive member among the extant Brachiopoda (about 420 species) based on the general form of the organophosphate shells, the complex muscular system, the pedicle, the infaunal behavior, and the retention of the metabolic adaptations to osmotic stresses and low oxygen consumption (Emig, 2003).

The genetic, ecological, and evolutionary aspects of the lingulid radiation are thus of interest to both biologists and paleontologists. Although fossils of Lingulidae have been found worldwide, most of them lack inner structures and thus only record the morphological features of shell. However, the evolutionary history is imprinted in genes of modern organisms, and geographical patterns of genetic variation reflect both historical processes and present gene flow attributable to the biological characteristics of the organism under study. We can turn to the extant *Lingula* species, which have an extensive Indo-West Pacific distribution, to study the evolutionary history of this group from a molecular perspective.

Compared with the remarkable morphological stasis in the fossil record, genetic evidence of extant *Lingula* and *Glottidia* species demonstrates significant differentiation among populations. Previous molecular studies confirmed that the history of lingulid divergence is very deep. The mean divergence between lingulids and articulate brachiopods was dated to 547 Ma, and the divergence between *Lingula* and *Glottidia* was estimated to be 191 Ma based on seven nuclear housekeeping genes (Sperling et al., 2011). Interestingly, the mitochondrial genome structure of *Lingula anatina* exhibits a number of unusual features, such as its large genome size, elongated genes, divergent gene sequences, and unique gene order (Endo et al., 2005). Endo et al. (2001) studied geographic population structure in *Lingula* from Japan and Hong Kong based on a short 200 bp segment of the mitochondrial cytochrome oxidase 1 gene (COI) and found distinct differentiation among them, contradicting the standing hypothesis that populations of *L. anatina* are panmictic and share a single homogeneous gene pool over the entire Indo-West Pacific region (Hammond and Poiner, 1984). The heterogeneity of *L. anatina* was further supported by Reyment et al. (2007) based on longer intron region of the elongation factor 1 alpha (EF-1α).

In this study, *L. anatina* samples from Qinhuangdao and Beihai, are studied based on longer mitochondrial COI and

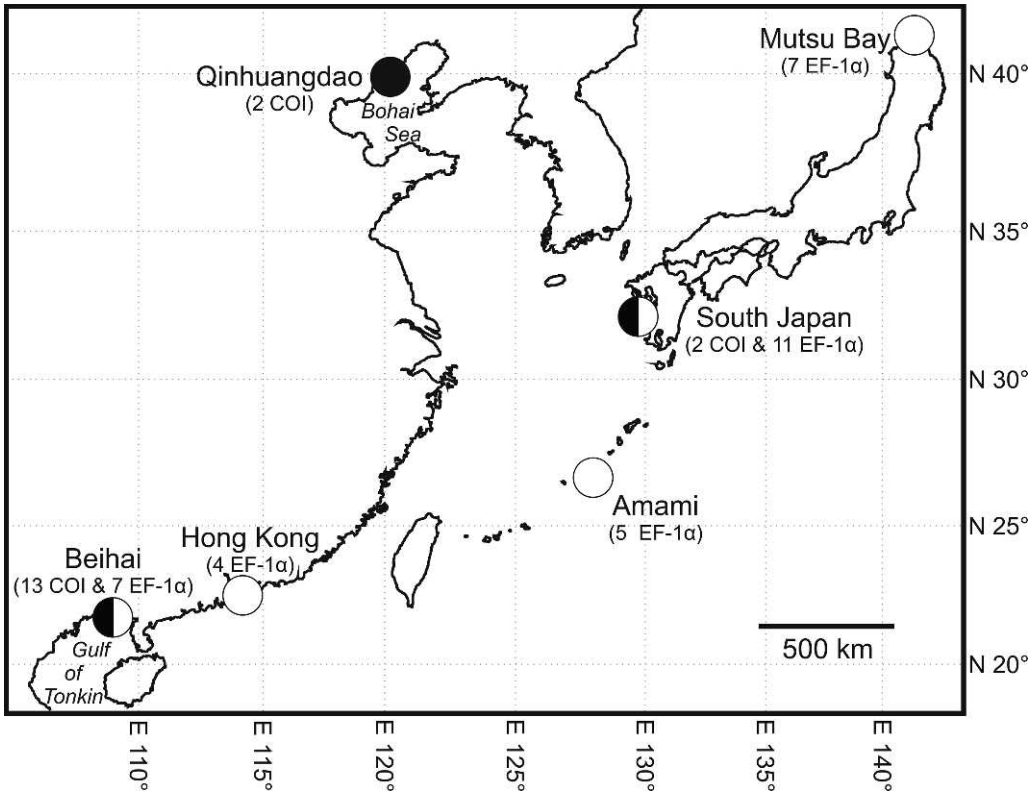

FIGURE 1—Sample localities of *Lingula anatina* analyzed in this study. Black color represents COI gene sequences available and white color represents EF-1α.

nuclear EF-1α sequences, aiming to shed more light on the evolution of this intriguing organism.

MATERIALS AND METHODS

Thirteen individuals were collected from Beihai (Guangxi, China) and then preserved in 99 percent ethanol and stored at –20°C for DNA extraction. Genomic DNA was isolated from pedicle tissue by proteinase K digestion followed by the standard phenol chloroform method. Sequences of PCR primers are coxL-5′-AAgAgTgTgACTggCATTAgggT-3′ and coxH-5′-TTgATgggTgACTgTgCTTC-3′ for mitochondrial COI genes; EF-H-5′-gAT ATT gCT CTg Tgg AAR TTY GAR AC-3′ and EF-G-5′-gTC TgA CCA TTC TTT gAD ATN CCN gCY TC-3′ for EF-1α genes (Endo et al., 2001).

PCR was carried out in 50 μL volumes containing 1.25 U Taq DNA polymerase, 20 ng template DNA, 200 nmol/L forward and reverse primers, 200 mmol/L of each dNTPs, 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$. The PCR amplification was carried out in a Biometra thermal cycler under the following conditions: 3 min initial denaturation at 94°C, and 40 cycles of 45 s at 94°C for denaturation, 45 s at 55°C for annealing, and 45 s at 72°C for extension, and a final extension at 72°C for 10 minutes. All sets of PCR included a negative control reaction tube containing all reagents, except template DNA. PCR products were purified and then used as the template DNA for cycle sequencing reactions, and sequencing was conducted on an ABI 3730 automatic sequencer using the same primers as for PCR amplification.

Sequences were edited and aligned using Dnastar software (DNASTAR Inc., Madison, WI, U.S.A.). Molecular diversity indices such as number of haplotypes, polymorphic sites, transitions, transversions, and indels were obtained using the program ARLEQUIN (ver. 2.000) (Schneider et al., 2000). Haplotype diversity (h), nucleotide diversity (π), and the mean number of pairwise differences and their corresponding variances were calculated following Nei (1987) as implemented in ARLEQUIN and DnaSP 4.0 (Rozas et al., 2003). Genetic distances were generated for phylogenetic reconstruction with MEGA 5.0 using the model of Tamura and Nei (Tamura et al., 2011).

Maximum likelihood and UPGMA trees of the haplotypes and EF-1α were constructed in MEGA 5.0. Maximum parsimony networks of haplotypes were constructed by using median-network approach (Bandelt et al., 1995).

RESULTS

A 621-bp segment of the mitochondrial COI gene was sequenced for 13 individuals of *Lingula anatina* from Beihai. With two sequences from Qinhuangdao and two from South Japan (extracted from GenBank), the total 17 individuals revealed 62 polymorphic sites and eight haplotypes (Fig. 1, Table 1). Maximum likelihood tree was constructed based on haplotypes and identified three distinct lineages (H1 and H2 are labeled as A; H3 through H6 as B; H7 and H8 as C; Figs. 2, 3). There were obviously geographical differences in haplotype frequencies of the three lineages. Lineage A contains two individuals from Qinhuangdao, Lineage B contains nine individuals from Beihai, and Lineage C contains four from Beihai and two from South Japan. There is only one nucleotide substitution between H7 and H8 within Lineage C. Net average genetic distances between A/B is 8.6 percent, B/C 2.7 percent, and A/C 8.9 percent, respectively.

A maximum likelihood tree was constructed based on the 351 bp COI sequence datasets that were available for both *Lingula anatina* and *L. adamsi* (Fig. 4). *Lingula adamsi* and *L. shantungensis* were extracted from GenBank, and actually the

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TABLE 1—Distribution of haplotypes among localities. Number of individuals (n), haplotype diversity (h), nucleotide diversity (π).

Haplo-types	GenBank Accession No.	Qinhuangdao, China, n=2, π=0.003, h=1	South Japan, n=2, π=0, h=0	Beihai, China, n=13, π=0.014, h=0.731
H1	GU056040	1		
H2	GU056041	1		
H3				1
H4				1
H5				6
H6				1
H7				4
H8	AB026520/AB178773		2	

two of them are conspecific from the viewpoint of both morphological and genetic variation (Sato et al., 2004), which is also supported by this study (Fig. 4). Both *L. anatina* and *L. adamsi* are monophyletic. The net mean genetic distance between *L. anatina* and *L. adamsi* is 44.5 percent, which is much higher than those within *L. anatina*.

The 462 bp EF-1α sequence dataset of *Lingula anatina* contains seven alleles from Beihai (retrieved in this study), four from Hong Kong, five from Amami (Japan), 11 from South Japan, and seven from Mutsu Bay (Japan) (Reyment et al., 2007) (Fig. 1). Distinct geographic differentiation was found among these five populations, except between the populations from Beihai and South Japan, which have a close relationship and are sister populations to the one from Mutsu Bay. However, alleles from Hong Kong and Amami, which are geographically located between Beihai and South Japan, are clustered together, forming a sister group to Mutsu-Beihai-South Japan clade (Fig. 5).

DISCUSSION

Phylogeographic pattern of Lingula anatina.—The results of this study revealed distinct phylogeographic differentiation of *Lingula anatina* across Indo-Western Pacific region, and allowed us to infer some of the related geological history and ecological factors affecting this species. A possible explanation for the extensive COI divergence of Qinhuangdao (Bohai Sea) from South Japan and Beihai may be long-term geographic isolation. Bohai Sea is a semi-enclosed continental sea basin, which is separated from South Yellow Sea basins by a submerged central uplift. The Japanese Islands have been independent of the Asian continent since the Sea of Japan started expanding 20 million years ago (Barnes, 2003). After that, many sea basins (including Bohai Sea and Yellow Sea) were generated and repeatedly experienced Quaternary glaciations (Yi et al., 2003). The Japanese Islands were connected with the main continent by land during the last glacial cycle (tens of thousands to 100,000 years ago) because the sea level fell by 120 m exposing connecting landforms (Bloom and Park, 1985; Pirazzoli, 1991).

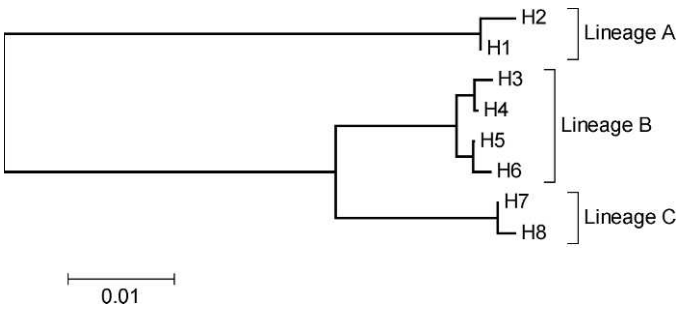

FIGURE 2—Maximum likelihood tree based on 621 bp COI sequences of *Lingula anatina*. Scale bar indicates genetic distances.

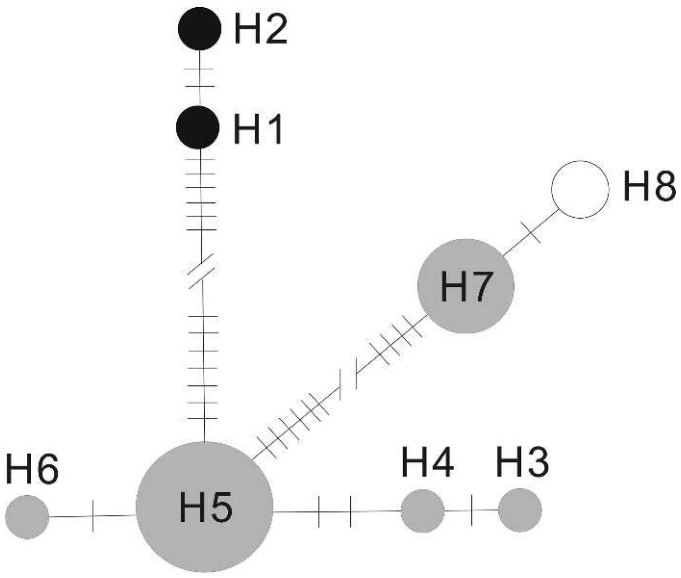

FIGURE 3—Maximum parsimony networks showing genetic relationships within *Lingula anatina* based on 621 bp COI sequences. The sizes of the circles are proportional to the frequencies of those haplotypes. Haplotypes are marked by names that correspond to Table 1. Perpendicular tick marks on the lines joining haplotypes represent the number of nucleotide substitutions. Black color represents samples from Qinhuangdao, gray color represents Beihai, and white color represents South Japan. The number of nucleotide substitutions between H5 and H1 is 50, H5 and H7 is 17.

As a result, most of the regional sea life is expected to have retreated southwards during glaciations and expanded northward during interglacial periods. Such geographic patterns were found in many marine fish species, but with less genetic diversity than *Lingula*. For example, the net average genetic distances between three major lineages of *Chelon haematocheilus* (marine fish) were 1.55 percent, 2.35 percent, and 2.41 percent, far more spatially restricted than predicted by the potential dispersal capabilities of this species, indicating population isolation during Pleistocene glaciations (Liu et al., 2007). In a very recent study, two distinct lineages were detected for *Ammodytes personatus*, with a net average genetic distance of 3.63 percent, which might have been divergent in the Sea of Japan and Pacific coastal waters of Japanese Islands (Han et al., 2012). Both of these studies of fish are based on the mitochondrial control region, and the sequence divergence rate of which is usually a little higher than COI genes (Page and Hughes, 2010). Whereas *Lingula*, which has a sessile and infaunal lifestyle and spends only a few weeks in its planktonic larval form, has much lower motility and dispersal probability than fish.

The two distinct COI lineages of *Lingula anatina* found in Beihai probably represent two different historical populations, although we have not identified any morphological difference or geographic isolation between the two lineages. The genetic isolation and high genetic distance among the sympatric populations (without intermediate haplotypes) may indicate that they had been isolated over a long period of time, and then made second contact. The close relationship between South Japan population with one of the two clades of Beihai suggests that there might be a very recent migration between South China Sea and East China Sea, possibly during or after the last glacial maximum based on a difference of only one single nucleotide substitution. Apart from the evidence from COI genes, the results from EF-1α genes also support a much closer genetic relationship between South Japan and Beihai, than alleles from Hong Kong and Amami, which are geographically located between Beihai

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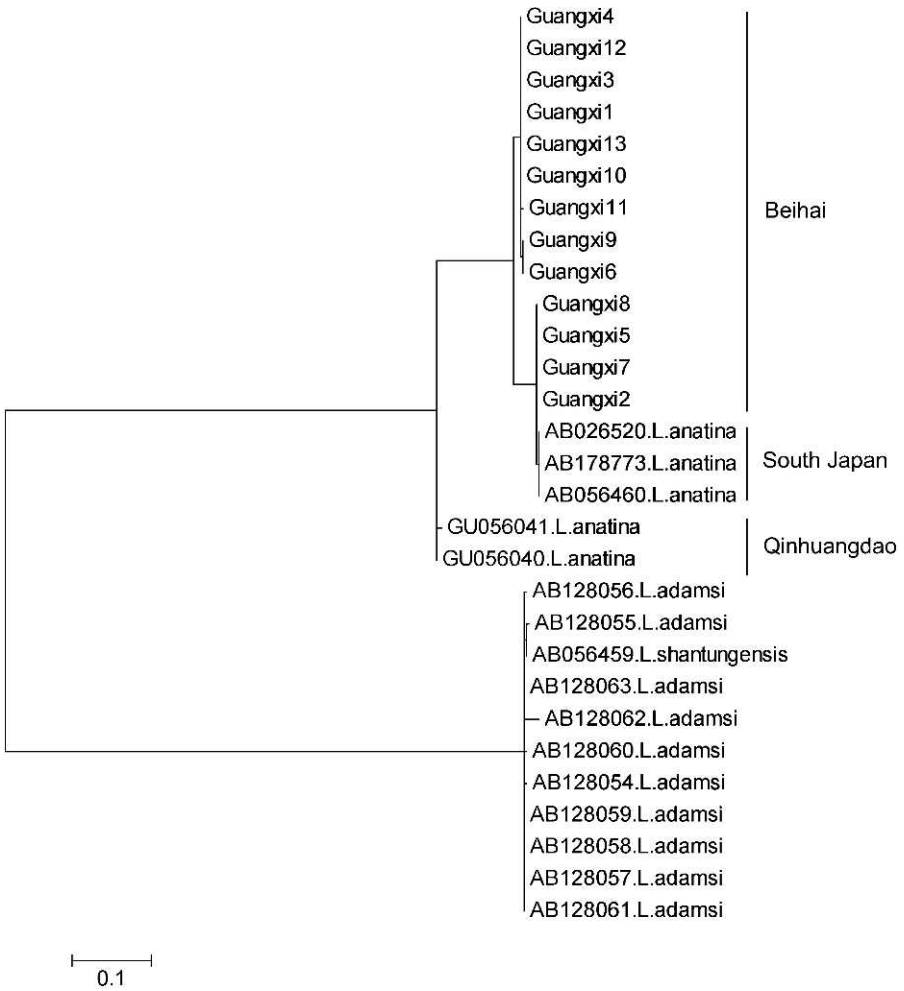

FIGURE 4—Maximum likelihood tree based on 351 bp COI sequences datasets of *Lingula anatina* and *Lingula adamsi*. Cited sequences are preceded by their accession number in GenBank, and the Guangxi sequences were obtained from Beihai population in this study.

and South Japan (Fig. 1). However, EF-1α sequences of *L. anatina* from Beihai do not indicate any distinct lineages. This kind of incongruence between mitochondrial (mtDNA) and nuclear (nuDNA) measures of divergence has been previously demonstrated in numerous organisms (Avise, 2004). Because mtDNA has a smaller effective population size than nuDNA, it is more sensitive to population bottlenecks and any subsequent expansion events than the nuclear genome (Avise, 2004). Fluctuating population sizes during repeated Quaternary glaciations may probably decouple the evolutionary relationships between mtDNA and nuDNA.

Unfortunately, we were not able to obtain COI sequences of *Lingula anatina* from Hong Kong, which is geographically between South Japan and Beihai. The only two COI sequences of *L. anatina* from Hong Kong in GenBank (with accession numbers AB056461 and AB056462, respectively) are questionable. The authors who sequenced those genes suggested that the two sequences might be pseudo-genes or introduced by introgression from other species (Endo et al., 2001) and therefore they cannot be used to analyze the phylogeographic structure of *L. anatina*. Notwithstanding this, the EF-1α genes of individuals from Hong Kong and other areas can provide complementary evidence, as mentioned above.

No taxon-specific estimations of the rate of divergence in COI genes have been made for Brachiopoda, but we can infer something about divergence times based on other invertebrate organisms. Per-million-years divergence rates range from 1.4 percent in the shrimp *Alpheus* (Knowlton and Weigt, 1998), 1.59 percent in seven echinoid taxon pairs (Hickerson et al., 2006), 1.8–2.2 percent in sea urchins (Bermingham and Lessios, 1993, whole mtDNA), 2.3 percent on a variety of recently diverged arthropod taxa (Brower, 1994), 2.8 percent in the sea urchin *Tripneustes* (Lessios et al., 2003), and 3.1 percent in the barnacle *Chthalamus* (Wares, 2001) and the sea urchin *Eucidaris* (Lessios et al., 1999). Among these, 2.3 percent is an often-used intermediate value, and was applied to roughly date biogeographic events in bryozoans (Schwaninger, 2008). If we use this value to coarsely estimate the date of divergence of the three COI linages of *Lingula anatina*, the divergence time between lineages B and C should be approximately one million years ago, and the divergences between A/B and between A/C should be about four million years ago. The one million year date is in the middle of the Pleistocene, since which time there have been more than 20 glacial cycles, and the four million year date is in the Pliocene prior to the onset of Quaternary glacial cycles. The complexity of tectonic evolution and the repeated Quaternary glaciations are therefore likely to have contributed to the phylogeographic pattern found in *L. anatina*.

Taxonomic consideration on Lingula.—DNA barcoding has recently attracted much attention as a standardized tool for molecular taxonomy and identification (Hebert et al., 2003,

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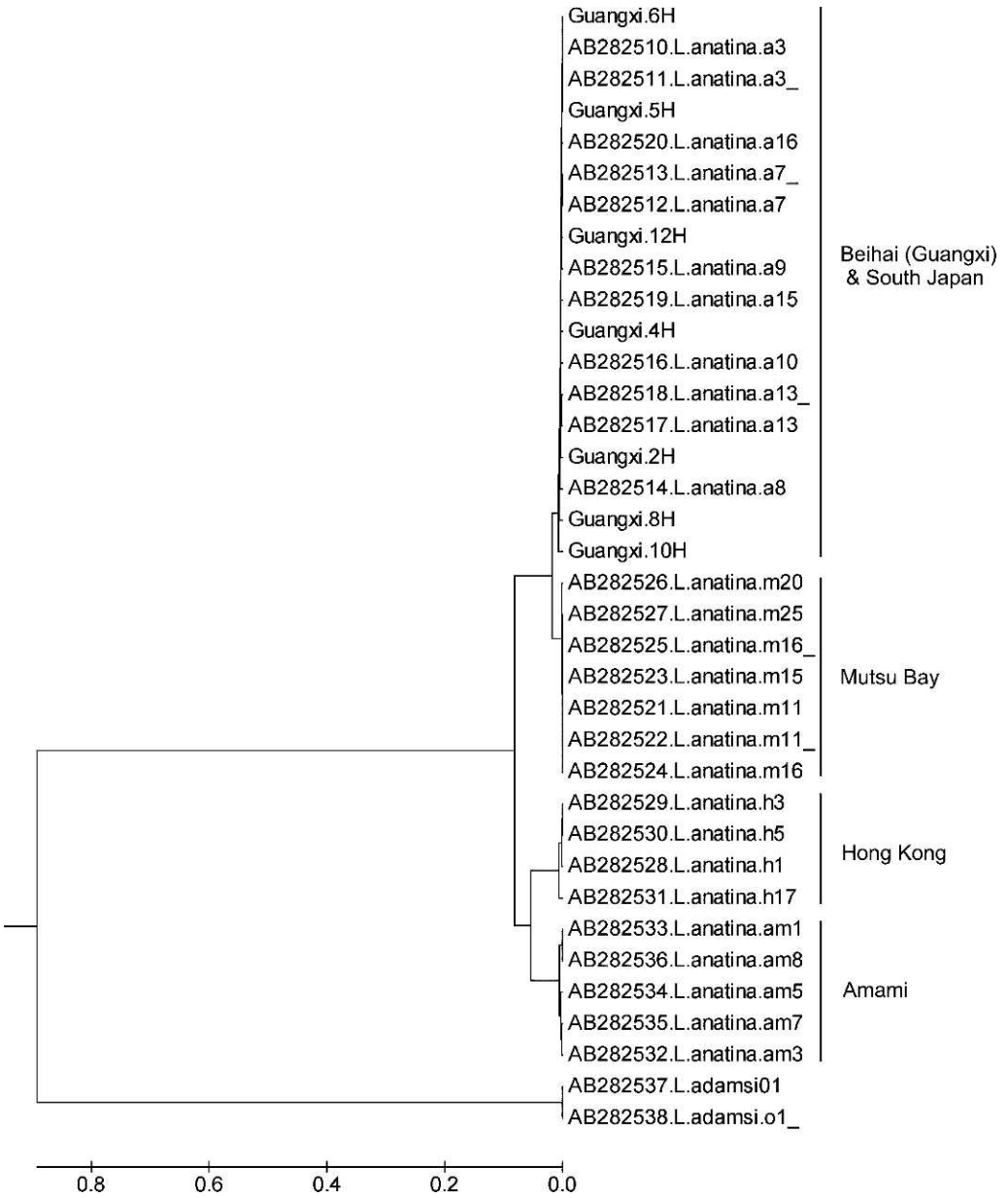

FIGURE 5—UPGMA tree based on 462 bp EF-1α sequence datasets of *Lingula anatina*. Abbreviations: a=Ariake Sea (South Japan); m=Mutsu Bay; am=Amami; h=Hong Kong. The Guangxi sequences were obtained from Beihai population in this study. Cited sequences are preceded by their accession number in GenBank.

2004a, 2004b; Holmes et al., 2009; Feng et al., 2011). According to a recent study (Sun et al., 2012), the average genetic distances within species, genera and families of Caenogastropoda based on COI genes were 0.44 percent, 13.96 percent, and 22.27 percent, respectively. For marine mollusks, the pairwise intraspecific genetic distances range from 0–1.9 percent, while pairwise interspecific genetic distances range from 14–77 percent, more than an order of magnitude higher than intraspecific distances (Mikkelsen et al., 2007). Compared to these invertebrates, the intraspecific genetic distances among the three *Lingula anatina* lineages are rather high, while the interspecific distance between *L. anatina* and *L. adamsi* is much higher (44.5%), translating an ancient divergence time (~20 Ma) between them based on the above mentioned 2.3 percent rate.

The pronounced morphological stability displayed by many "living fossil" species often for millions of years, contrasts sharply with the deep genetic changes documented in many extant species. Some previous studies on "living fossils" such as the coelacanth (Holder et al., 1999), the horseshoe crab (Avise et al., 1994), tuatara (Hay et al., 2008), and tadpole shrimp (Vanschoenwinkel et al., 2012) have suggested a substantial and high nucleotide diversity in these phylogenetically distinct species, suggesting rates of neutral molecular and phenotypic evolution are decoupled, and morphological stasis does not arise from a lack of genetic variability. Rapidly emerging evidence indicates that a lack of readily observable phenotypic change (morphological stasis) during the evolutionary history of a lineage does not necessarily imply genetic stasis. Recent species, which are virtually identical to fossils in terms of their morphology, often represent very divergent genetic lineages.

The persistence of linguliform shell of lingulids is generally interpreted by many paleontologists as indicating morphological stability through the whole of the Phanerozoic record.

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Consequently, many Paleozoic species have been referred to *Lingula*, although both the shell and the internal structures have evolved since the Palaeozoic (Biernat and Emig, 1993; Emig, 2003). Since ancient divergence from its only sister genus *Glottidia* about 191 Ma, *Lingula* shows much recent phylogeographic differentiation. Molecular genetic evidence of extant lingulids reveals a complex, albeit morphologically cryptic, evolutionary history. Our data support the view that *Lingula* is not an unchanging genus, as the "living fossil" epithet suggests, but an organism that is as responsive to changing climates and environments as any other. Nevertheless, many more samples should be necessarily collected from other localities for further investigation of this intriguing organism.

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