Mitochondrial Genetic Variation of Pen Shell, *Atrina pectinata* in Korea and Japan

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ABSTRACT

In the northwestern Pacific region, the pen shell (*Atrina pectinata*) is a widely distributed bivalve and economically important in fisheries. Recently, stock of this species has been greatly reduced due to overexploitation and marine pollution, which arouses interest in conservation. Studies on genetic and taxonomic entities of pen shells have not been tried in Korea, which makes difficult to take measures for effective conservation of this marine resource. In this study, we investigated mitochondrial genetic polymorphism of pen shells collected from 4 locations in Korea and Japan using cytochrome c oxidase I (COI) gene sequences. A total of 39 haplotypes were identified among 86 individuals of pen shell. Although only 5 haplotypes were shared, no significant genetic differentiation was observed between Korean and Japanese populations. These results suggest that pen shell populations of these regions share an ancestral population which might have experienced expansion during the Pleistocene, but gene flow must have been highly restricted after expansion.

Keywords: pen shell, mitochondrial genetic variation, COI, Korea, Japan

INTRODUCTION

Riding ocean current during their planktonic larval stage, most of marine animals have potential for wide dispersion. Genetic structure of marine populations could be greatly influenced by such high dispersion ability which is directly connected with high level of gene flow (Bohonak, 1999). Many factors such as marine topology, sea temperature and duration of larval stages influence on population genetic structure as well. Genetic structure of population provides valuable information to understand geographic distribution of species and genetic diversities of marine organisms. Aside from genetic structure, history of population such as range expansion, colonization and fragmentation would also give insight into marine biodiversity. Population history will be closely related with geological changes. Hence, historical factors in Korean and Japanese seas may be more influential on genetic variation of marine organisms than any other regions because this region has experienced great geological changes during the Pleistocene.

Geographic distribution of genetic polymorphism of marine populations, therefore, should be understood not only from historical perspective but also in view of recurrent factors such as gene flow forming genetic structure of populations. Gene flow has been thought to be prevalent between Korean and Japanese seas owing to their geographical proximity. However, many studies tell different stories. Branches of Kuroshio Current flowing between Korean Peninsula and Japanese Islands may have played as genetic barriers to disturb gene flow (Yin et al., 2009; Sakaguchi et al., 2011; Shen et al., 2011). They have caused genetic and species divergence between Korean and Japanese seas. In addition, these seas have experienced common geological events, which also influenced geographic distribution of marine biodiversity.

The pen shell (*Atrina pectinata*) is a sedentary suspension feeder. This species is a large bivalve which is economically important in the northwestern Pacific regions (Fu et al., 2010). It is used as raw material to make decorative crafts as well (Rao and Dorairaj, 1971). Recently, population of this marine

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resource, however, has been greatly reduced due to over-exploitation and marine pollution that results in lowering dissolved oxygen and decreasing the area of sandy bottom, i.e. its habitat (Hong et al., 2002; Suzuki et al., 2007). This situation arouses interest in conservation of pen shell populations.

Now taxonomic status of pen shells has been actively studied. Its species integrity has been doubt owing to its extreme variation in some characteristics such as degree of inflation, sculpture, and coloration of shell (Rao and Dorairaj, 1971). Scaly form and non-scaly form of pen shells in Japan have been called as Zube-type and Ken-type, respectively of which genetic characteristics have been investigated (Yokogawa, 1996). Recently pen shells occurring in China turned out to be a species complex composed of several cryptic species through the investigation of mitochondrial and nuclear DNA polymorphisms (Liu et al., 2011). However, pen shells of Korea have been discussed in-depth in view of genetic or taxonomic entities in relation with pen shells occurring from neighboring countries. This makes it difficult to take measures for effective conservation of this marine resource in this country.

We investigated mitochondrial genetic polymorphism of individuals collected from 3 locations in Korea and 1 location from Japan using cytochrome c oxidase I (COI) gene sequences. COI gene is adequate for our study because it has genetic polymorphisms enough to identify species as well as to investigate intraspecific genetic variation (Hebert et al., 2003; Kim et al., 2016). Then we briefly discussed taxonomic status of pen shells in northwestern Pacific region based on genetic polymorphism.

MATERIALS AND METHODS

Sample collection

Samples used in this study were collected from Ise Bay (ISE) in Japan and from 3 locations in Korea: Deukryang (DEK), Yeosu (YES), and Ocheon (OCH). Sampling locations are denoted in Fig. 1A. Samples were collected directly by the authors using SCUBA-diving or were purchased by nearby fishery market. When samples were purchased, we confirmed the collection location. Sequences were obtained from a total of 82 individuals in this study (Table 1). We also used four sequences retrieved from GenBank database: two sequences from Ohmi Bay in Japan (Zube-type: AB059421, Ken-type: AB059423) and two sequences from Ariake Bay in Japan (Zube-type: AB059422, Ken-type: AB059424).

DNA extraction, polymerase chain reaction, and sequencing

DNAs were extracted from adductor muscle tissue using QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA). Partial region of COI was amplified by the primers, the LCO1490 and HCO2190 (Folmer et al., 1994). Total of 50 μL of polymerase chain reaction (PCR) mixture was composed of $1 \times$ *Taq* polymerase buffer, 1 mM dNTP mixture, 2.5 mM $MgCl_2$, 0.5 μM of each primer, 2.5–250 pg of genomic DNA, and
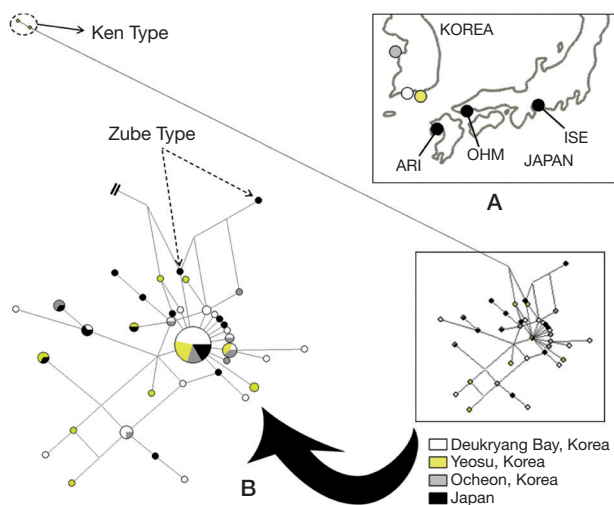
Fig. 1. A map denoting the sampling locations (A) and a haplotype network diagram (B). In the network diagram, size of the circles denotes the number of individuals that contain the haplotype. The proportions of individual sequences belonging to sampling locations are represented in the pie chart. ARI, Ariake Bay, Japan; OHM, Ohmi Bay, Japan; ISE, Ise Bay, Japan.

Table 1. Geographical and sample information of the locations included in this study

Location	Coordinates	No. of samples
Deukryang, South Korea (DEK)	34°37′47.02″N, 124°1′57.95″E	33
Yeosu, South Korea (YES)	34°43′34.32″N, 127°43′42.43″E	13
Ocheon, South Korea (OCH)	36°26′20.71″N, 126°31′10.5″E	19
Ariake Bay, Japan (ARI)	32°58′33.78″N,130°14′30.95″E	2(1)[a]
Ohmi Bay, Japan (OHM)	34°1′2.89″N, 131°29′26.03″E	2(1)[a]
Ise Bay, Japan (ISE)	34°56′36.94″N, 136°48′51.31″E	17
	Total	86(2)[a]

[a]Numbers in parentheses represent the number of "Ken-type" specimens.

0.04 unit/μL of *Taq* DNA polymerase (Promega, Madison, WI, USA). PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with the following cycle conditions: 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 2 min, followed by a final elongation at 72°C for 10 min. The PCR products were isolated from 1% agarose gel and purified with a Dyne PCR Purification Kit (Dynebio, Seoul, Korea). The PCR products from each individual were sequenced with both amplification primers using the Big-Dye Terminator Cycle Sequencing Ready Kit (Applied Biosystems) according to the manufacturer's instructions. The sequences were then analyzed using an ABI PRISM 3730 Automated Genetic Analyzer (Applied Biosystems).

Data analysis

Bidirectional sequences were aligned and visually checked using the SEQUENCE NAVIGATOR ver. 1.0.1 software (PE-Applied Biosystems). After multiple alignments of sequences performed by CLUSTAL X 1.83 (Thompson et al., 1997), the data were edited and were exported to NEXUS and PHYLIP formats for further analyses using SE-AL Sequence Alignment Editor ver. 2.0a11 (Rambaut, 2002). COLLAPSE 1.2 (available from: http://darwin.virgo.es) were used for determining haplotypes and calculating their frequencies. Median joining network diagram, denoting genetic distance among haplotypes, were drawn with NETWORK 4.6 (Fluxus Technology Ltd., Clare, UK). Summary statistics representing genetic diversity such as gene diversity, nucleotide diversity, θ_S, and θ_π were calculated using DnaSP 5.10 (Librado and Rozas, 2009) and ARLEQUIN 3.11 (Excoffier et al., 2005). Hierarchical structure of genetic variation was investigated with analysis of molecular variance (AMOVA) using the software ARLEQUIN. Mean genetic distance between populations or species were calculated using MEGA 4 (Tamura et al., 2007). AMOVA and calculation of mean genetic distance among groups were performed based on p-distance between sequences. The statistical significance of AMOVA was then evaluated over 10,000 random permutations. Mantel's test was conducted using ARLEQUIN to evaluate any possible significant relationships between the geographic distance and the population genetic distance (Slatkin's linearized $F_{ST} = F_{ST}/1 - F_{ST}$). The p-value was determined via permutation with 10,000 repeats. Recent demographic changes were inferred from mismatch distribution and neutrality tests based on Tajima's D and Fu's F_S with the ARLEQUIN 3.11. The differences between the expected and observed mismatch patterns were tested by calculating the sum of squared deviation.

RESULTS

A total of 39 haplotypes were identified among 86 individuals of pen shell from Korea and Japan including sequences retrieved from GenBank (Table 2). We got GenBank accession number for 37 haplotypes of "Zube-type" (GenBank accession numbers, MF378606-MF378642). We obtained 28 haplotypes among 65 individuals from Korea and 16 haplotypes (including two haplotypes of "Ken-type") among 21 individuals from Japan. Of these haplotypes, Hap 6 represents the highest frequency both in Korea (25 from 39 individuals) and Japan (5 from 19 individuals). Five haplotypes were commonly observed in Korea and Japan, which

Table 2. Haplotypes and their geographic distribution

Location	Haplotype																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
DEK	1	1				14			1	3			1	1					1	
YES	1					4	1			1	1								2	2
OCH			1	2		7		2				1						1	2	
ARI																				
OHM																				
ISE				1	1	5									1	1	1	1		1
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38 (K)	39 (K)	S
DEK			1	1	1	1	2		1		1					1	1			33
YES									1											13
OCH								1				1		1						19
ARI															1				1	2
OHM										1								1		2
ISE	1	1				2							1							17

DEK, Deukryang, South Korea; YES, Yeosu, South Korea; OCH, Ocheon, South Korea; ARI, Ariake Bay, Japan; OHM, Ohmi Bay, Japan; ISE, Ise Bay, Japan; K, haplotype of "Ken-type"; S, sum of sequences.

Table 3. Summary statistics showing genetic variation of sampling locations (excluding Ken-type)

Location	No. of individuals	No. of haplotypes	Gene diversity	θ_S	Tajima's D (p-value)
			Nucleotide diversity (π)	θ_π	Fu's Fs (p-value)
DEK	33	17	0.8201±0.0672	5.17433±1.88434	−1.62939 (0.03800)
			0.0043±0.0026	2.66667±1.62109	−9.82988 (0.00000)
YES	13	8	0.8974±0.0666	3.86696±1.77829	−1.13195 (0.14200)
			0.0045±0.0029	2.79487±1.77354	−2.45205 (0.06000)
OCH	19	10	0.8596±0.0696	4.86394±1.98930	−1.22864 (0.11200)
			0.0053±0.0032	3.28655±1.97619	−2.58574 (0.07600)
ISE	17	12	0.9191±0.0571	5.02850±2.09398	−1.19050 (0.11600)
			0.0055±0.0033	3.39706±2.04626	−4.37543 (0.00900)
Korea[a]	65	28	0.8447±0.0431	6.53472±2.04147	−1.79715 (0.01200)
			0.0047±0.0028	2.88558±1.70336	−19.18751 (0.00000)
Japan[b]	19	13	0.9064±0.0596	5.72228±2.28508	−1.37809 (0.07600)
			0.0060±0.0035	3.67252±2.17180	−5.88647 (0.00100)
Total[b]	84	37	0.8666±0.0351	7.59686±2.22744	−1.89323 (0.00400)
			0.0050±0.0029	3.06483±1.78460	−26.37950 (0.00000)

DEK, Deukryang, South Korea; YES, Yeosu, South Korea; OCH, Ocheon, South Korea; ISE, Ise Bay, Japan.
[a] Individuals of DEK, YES, and OCH were pooled.
[b] Individuals of ARI and OHM were included.

Table 4. Results of analysis of molecular variance (AMOVA)

Group assignment	Source of variation	F-value[a]	Percentage of variation
(DEK), (YES), (OCH), (ISE)	Among populations	−0.00534	−0.53
	Within populations		100.53
[(DEK), (YES), (OCH)], (ISE)	Among groups	0.00953	0.95
	Among populations within groups	−0.00978	−0.97
	Within populations	−0.00016	100.02
[(DEK), (OCH)], [(YES), (ISE)]	Among groups	0.01427	1.43
	Among populations within groups	−0.01477	−1.46
	Within populations	−0.00029	100.03
(DEK+OCH+YES)[b], (ISE)	Among populations	0.00597	0.60
	Within populations		99.40

DEK, Deukryang, South Korea; YES, Yeosu, South Korea; OCH, Ocheon, South Korea; ISE, Ise Bay, Japan.
[a] No significant F-value was detected.
[b] Individuals of DEK, YES, and OCH were pooled together.

was small portion compared to total number of haplotypes observed. All haplotypes occurred in this study belong to Zube-type. And genetic distance between haplotypes ranged 0.16%−1.79%. Sequences observed in this study shows considerable genetic distance with Ken-type haplotypes (Fig. 1B). Mean genetic distance between these two groups was 7.9%, and mean genetic distances between 4 locations investigated in this study were ranging 0.44%−0.55%.

Gene diversity and nucleotide diversity of locations were ranging 0.8201−0.9191 and 0.0043−0.0055, respectively (Table 3). θ_S and θ_π values were ranging 3.86696−5.17433 and 2.6667−3.39706, respectively. ISE was genetically most diverse among locations except the θ_S value.

Mean F_{ST} value among locations is 0.00953, which indicates populations studied are nearly panmictic. From the results of AMOVA, there seems to be a little genetic divergence between DEK+OCH+YES and ISE, and between DEK+OCH and YES+ISE, but none of which was statistically significant (Table 4). But the result of Mantel's test evidenced that there may be non-random association (p=0.037) between mitochondrial genetic divergences and geographic distances between locations (Fig. 2).

Observed mismatch distribution pattern was not significantly different from one that expected under population expansion (Fig. 3). Tajima's D value of ISE was not different from randomly generated values, but both of Tajima's D and Fu's F_S values were significant in Korea and Korea+Japan populations (Table 3). This means that Korean populations might have experienced recent population expansion.
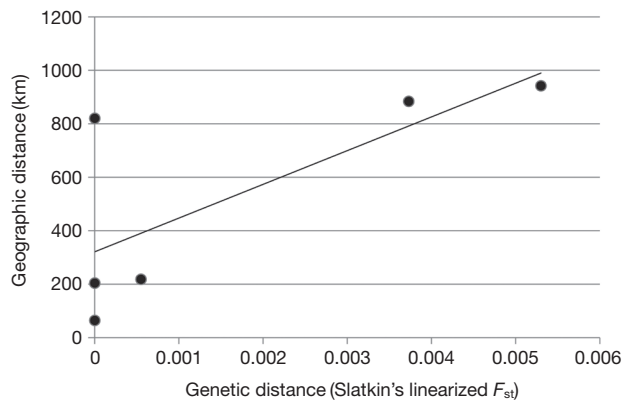
Fig. 2. A plot showing the relationship between geographic distance and genetic distance (Slatkin's linearized F_{ST}) ($R^2 = 0.733302$, p=0.037).

DISCUSSION

The result that the same haplotype is the most frequent in both Korean and Japanese locations indicates that pen shell populations of these regions share a common ancestral population. As the evolutionary rate of mitochondrial COI gene is ranging 0.7%–2.4% per million years (Liu et al., 2011), genetic distance among haplotypes (0.16%–1.79%) observed in this study suggest pen shell populations in Korean and Japanese seas have lasted for about one million years. It implies, therefore, that present pen shell population would have expanded in these regions roughly after Last Glacial Maximum. Expansion of population is also supported by the results of neutrality test and mismatch distribution analysis.

Having originated in tropical regions, pen shells may have expanded their ranges from southern to northern region during the late Cenozoic. This conforms to the results of Liu et al. (2011). They found that most of hidden genetic lineages of pen shell occur in southern region, the South China Sea. Haplotypes belonging to the Lineage 1 was widely distributed over central to northern China seas which are close to Korean and Japanese seas. Moreover, the Lineage 1 of Liu et al. (2011) seems to correspond to Zube-type pen shell as they share common haplotypes. It is likely that the Lineage 2, a sister group of Lineage 1 is equated with Ken-type pen shell occurring in Japanese seas, and it is more southerly distributed than Lineage 1 to South China Sea. The other lineages outside the Lineage 1 and 2 occur only in South China Sea. These results implicate that serial speciation have resulted in species of *Atrina pectinata* complex as they have expanded northward.

Genetic differentiation between Korean and Japanese pen shell populations is not significant as a result of AMOVA even though they share only a small number of haplotypes.
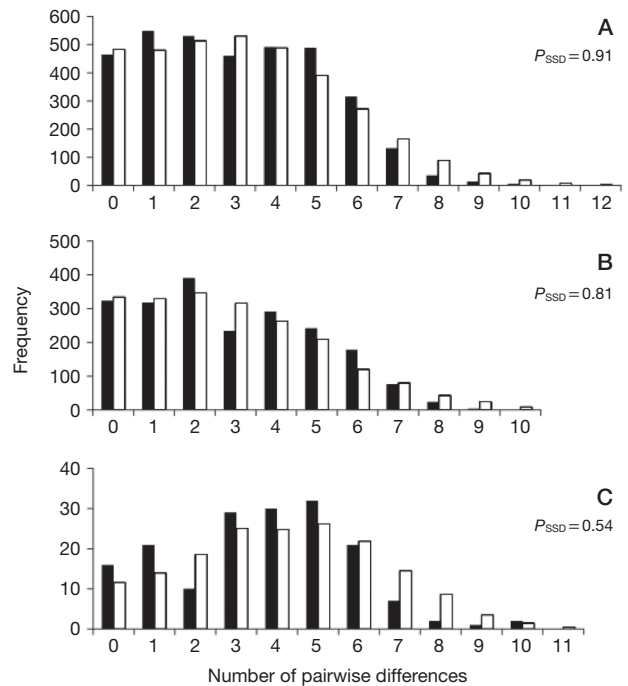
Fig. 3. The observed mismatch distributions (solid bars) and expected distributions (open bars) simulated under the sudden expansion model of total population (A), Korea population (B), and Japan population (C) fitted to the observed distributions. SSD, sum of squared deviation.

There are two possible explanations for this result. Firstly, gene flow between two regions after range expansion of pen shell to Korean and Japanese seas might have been restricted, but opportunistic gene flow might have occurred and have formed such geographic distributional pattern of haplotypes. This could be backed up by the result that some terminal haplotypes in network diagram are shared by both regions (Fig. 1B). Secondly, this pattern might be the result of incomplete lineage sorting of ancestral polymorphism instead of opportunistic gene flow. Incomplete lineage sorting has very similar effect with gene flow on genetic polymorphism of diverging populations, which complicates study on population history (Pinho et al., 2008). These two hypotheses cannot be tested in this study due to insufficient data. For this analysis, not only multiple genetic markers are required to test hypotheses, but also samples from wider range including ones from northern China which surely share common ancestral population with ones from Korean and Japanese seas. At all events, both hypotheses are based on the assumption that gene flow between Korean and Japanese seas may be highly restricted. It turns out that marine environment is less open to larval migration than to be expected owing to several factors that restrict gene flow. Actually branches of Kuroshio Current such as East Korea Current and Tsushima

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Current are thought to have played genetic barriers between Korean and Japanese seas. This was evidenced by studies on diverse marine organisms in this region (Yin et al., 2009; Sakaguchi et al., 2011; Shen et al., 2011).

It is highly probable that Zube-type and Ken-type pen shells are discrete species. At present, Zube-type and Ken-type pen shells are thought as *A. pectinata japonica* and *A. p. lischkeana*, respectively. Many malacologists, however, argued that these two types of pen shells are separate species based on various evidences (Yokogawa, 1996). Mean genetic distance of COI gene between Zube-type and Ken-type is about 7.9%, which corresponds to that of between species based on genetic distance between oyster species (2.55%–29.29%) (Lam and Morton, 2003) and between *Lepetodrilus* limpet species (3.01%–31.25%) (Johnson et al., 2008). Liu et al. (2011) also considered genetic lineages including the Lineage 1 (Zube-type) and Lineage 2 (Ken-type) that represent such high genetic divergence as separate cryptic species. Present study together with Liu et al. (2011), therefore, strongly support dividing genetic lineages of pen shells into separate species, and suggest taxonomic revision on this group.

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REFERENCES

Bohonak AJ, 1999. Dispersal, gene flow, and population structure. Quarterly Review of Biology, 74:21-45. https://doi.org/10.1086/392950

Excoffier L, Laval G, Schneider S, 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online, 1:47-50.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R, 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3:294-299.

Fu H, Wu Y, Gong Y, Jiang S, Xiong Y, Qiao H, Cheng L, Tong J, Li M, 2010. Isolation and characterization of 15 polymorphic microsatellite markers for comb pen shell (*Atrina pectinata*). Aquaculture Research, 41:e703-e705. https://doi.org/10.1111/j.1365-2109.2010.02494.x

Hebert PDN, Cywinska A, Ball SL, deWaard JR, 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society B, 270:313-321. https://doi.org/10.1098/rspb.2002.2218

Hong SH, Lee JB, Im YJ, Hwang HJ, Yeon IJ, Park YC, Ko TS, Ma CW, Park KY, 2002. Population ecology of pen shell, *Atrina pectinata*, in the western waters of Korea. I. Geographic distribution and biomass. Journal of the Korean Society of Fisheries Resources, 5:12-23.

Johnson SB, Warén A, Vrijenhoek RC, 2008. DNA barcoding of *Lepetodrilus* limpets reveals cryptic species. Journal of Shellfish Research, 27:43-51. https://doi.org/10.2983/0730-8000(2008)27%5B43:DBOLLR%5D2.0.CO;2

Kim Y, Lee J, Kim H, Jung J, 2016. Comparison of population genetic structure of two seashore-dwelling animal species, periwinkle *Littorina brevicula* and acorn barnacle *Fistulobalanus albicostatus* from Korea. Animal Systematics, Evolution and Diversity, 32:105-111. https://doi.org/10.5635/ASED.2016.32.2.105

Lam K, Morton B, 2003. Mitochondrial DNA and morphological identification of a new species of *Crassostrea* (Bivalvia: Ostreidae) cultured for centuries in the Peral River Delta, Hong Kong, China. Aquaculture, 228:1-13. https://doi.org/10.1016/S0044-8486(03)00215-1

Librado P, Rozas J, 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25:1451-1452. https://doi.org/10.1093/bioinformatics/btp187

Liu J, Li Q, Kong L, Zheng X, 2011. Cryptic diversity in the pen shell *Atrina pectinata* (Bivalvia: Pinnidae): high divergence and hybridization revealed by molecular and morphological data. Molecular Ecology, 20:4332-4345. https://doi.org/10.1111/j.1365-294X.2011.05275.x

Pinho C, Harris DJ, Ferrand N, 2008. Non-equilibrium estimates of gene flow inferred from nuclear genealogies suggest that Iberian and North American wall lizards (*Podarcis* spp.) are an assemblage of incipient species. BMC Evolutionary Biology, 8:63. https://doi.org/10.1186/1471-2148-8-63

Rambaut A, 2002. SE-AL Sequence Alignment Editor, v2.0a11. University of Oxford, Oxford.

Rao KS, Dorairaj K, 1971. On the occurrence of the fan shell *Atrina* (*Servatrina*) *pectinata pectinata* (Linnaeus) off Mandapam in Palk Bay. Journal of the Marine Biological Association of India, 13:139-142.

Sakaguchi SO, Ueda H, Ohtsuka S, Soh HY, Yoon YH, 2011. Zoogeography of planktonic brackish-water calanoid copepods in western Japan with comparison with neighboring Korean fauna. Plankton and Benthos Research, 6:18-25. https://doi.org/10.3800/pbr.6.18

Shen KN, Jamandre BW, Hsu CC, Tzeng WN, Durand JD, 2011. Plio-Pleistocene sea level and temperature fluctuations in the northwestern Pacific promoted speciation in the globally-distributed flathead mullet *Mugil cephalus*. BMC Evolutionary Biology, 11:83. https://doi.org/10.1186/1471-2148-11-83

Suzuki K, Kiyomoto S, Koshiishi Y, 2007. Observation on behavior of the large suspension feeding bivalve *Atrina pectinata lisckeana* under natural conditions. Bulletin of

Fisheries Research Agency, 19:17-25.

Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 24:1596-1599. https://doi.org/10.1093/molbev/msm092

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, 24:4876-4882.

Yin W, Fu C, Guo L, He Q, Li J, Jin B, Wu Q, Li B, 2009. Species delimitation and historical biogeography in the genus *Helice* (Brachyura: Varunidae) in the northwestern Pacific. Zoological Science, 26:467-475. https://doi.org/10.2108/zsj.26.467

Yokogawa K, 1996. Genetic divergence in two forms of pen shell *Atrina pectinata*. Japanese Journal of Malacology, 55:25-39.

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