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Research paper

Hidden genetic history of the Japanese sand dollar *Peronella* (Echinoidea: Laganidae) revealed by nuclear intron sequences

Megumi Endo[a], Mamiko Hirose[b,1], Masanao Honda[a], Hiroyuki Koga[a], Yoshiaki Morino[a], Masato Kiyomoto[b], Hiroshi Wada[a,*]

[a] Faculty of Life and Environmental Sciences, University of Tsukuba, Japan
[b] Tateyama Marine Biological Station, Ochanomizu University, Japan

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ABSTRACT

The marine environment around Japan experienced significant changes during the Cenozoic Era. In this study, we report findings suggesting that this dynamic history left behind traces in the genome of the Japanese sand dollar species *Peronella japonica* and *P. rubra*. Although mitochondrial Cytochrome C Oxidase I sequences did not indicate fragmentation of the current local populations of *P. japonica* around Japan, two different types of intron sequence were found in the *Alx1* locus. We inferred that past fragmentation of the populations account for the presence of two types of nuclear sequences as alleles in the *Alx1* intron of *P. japonica*. It is likely that the split populations have intermixed in recent times; hence, we did not detect polymorphisms in the sequences reflecting the current localization of the species. In addition, we found two allelic sequences of the*Alx1* intron in the sister species *P. rubra*. The divergence times of the two types of *Alx1* intron sequences were estimated at approximately 14.9 and 4.0 million years ago for *P. japonica* and *P. rubra*, respectively. Our study indicates that information from the intron sequences of nuclear genes can enhance our understanding of past genetic events in organisms.

Abbreviation list

PCR	Polymerase chain reaction
COI	cytochrome *c* oxidase I
MCMC	Markov chain Monte Carlo
MYR	million years
BP	before present

1. Introduction

Geographic history has a profound influence on the genetic structure of organisms, and the intensity of the influence is dependent on their life history (Avise, 2000; Cowen and Sponaugle, 2009). Populations of limited dispersal ability are more likely to be fragmented due to geographic changes and are thus more likely to record past geographic conditions in their genetic structure (Bohonak, 1999; Cowen and Sponaugle, 2009). In marine benthic metazoans, dispersal potential is largely associated with early life history, i.e., type of larval development (Cowen and Sponaugle, 2009). It has been shown that the modes of early life history have a significant influence even on the genomic evolution of metazoans (Romiguier et al., 2014).

Sea urchins belong to a group of marine animals whose genetic evolution as well as their life history has been extensively studied. Although most species of sea urchin spend the planktotrophic larval stage as pluteus, some species skip the planktotrophic stage and develop directly into juveniles (Strathmann, 1978). This process of direct development leads to restrictions in the gene flow (Hart, 2002). *Heliocidaris erythrogramma* is a sea urchin species that undergoes direct development. This species has been shown to have a relatively fragmented genetic population structure compared with the closely related species *H. tuberculata*, which develops through planktotrophic pluteus larvae (McMillan et al., 1992).

The Japanese sand dollar *Peronella japonica* is widely distributed in shallow water along the Japanese coastline. The fertilized eggs of this species develop into pluteus larvae with clearly elongated larval arms supported by the skeleton (Okazaki and Dan, 1954). However, the digestive system does not differentiate properly in the larval body; they are thus lecithotropic, and the larval stage lasts only a few days before metamorphosis (Okazaki and Dan, 1954). Therefore, this species has relatively limited dispersal ability, and its genetic structure is likely to be more prone to effects from changes in the marine environment.

* Corresponding author at: Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan.
E-mail address: hwada@biol.tsukuba.ac.jp (H. Wada).
[1] Present address: School of Marine Science and Technology, Tokai University.

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The marine environment around Japan experienced dynamic changes during the Cenozoic Era. The main islands of Japan and the Ryukyu Archipelago were subjected to complex vertical and horizontal movement, as well as sea level changes caused by climate transitions, and these diastrophisms led to temporary fluctuations in the area of exposed land (Kizaki and Oshiro, 1977, 1980; Ujiie, 1990; Ota, 1998). The East China and Japan Seas were established and subsequently collapsed in these geological movements, and subsequent bifurcations of warm ocean currents, such as the Kuroshio and Tsushima Currents, were established with the emergence of the two seas (e.g., Kizaki and Oshiro, 1977; Chinzei, 1986). These environmental changes would be expected to have affected the distributions of sea urchin populations.

In this study, we examined variations in mitochondrial and nuclear genes and elucidated phylogenetic relationships to determine whether geographic events along the Japanese coastline left behind traces in the sequence variations in *Peronella japonica* and its relatives. We also discuss the relationships of the paleogeographic and paleoenvironmental conditions during the Cenozoic Era with the established phylogeny.

2. Materials and methods

2.1. DNA extraction, PCR amplification, and sequencing

Tissues were obtained from 26 and 14 individuals of *Peronella japonica* and *P. rubra*, respectively, taken from a total of five localities along the coastline around Japan (Table 1). We also obtained DNA from two clypeasterid species, *Astriclypeus manni* and *Clypeaster japonicus* belonging to other families of Clypeasteroida.

Genomic DNA was extracted from the gonads or sperm of *P. japonica* and from the mouth of *P. rubra*, *A. manni* and *C. japonicus* using a DNeasy tissue kit (Qiagen) following the manufacturer's protocol.

We initially analyzed COI haplotypes from 17 specimens of *P. japonica*. Polymerase chain reaction (PCR) amplification of the partial cytochrome *c* oxidase I (COI) gene was performed using Ex Taq DNA polymerase (Takara Bio, Inc., Tokyo, Japan) and echinoderm COI universal primers (Hoarearu and Boissin, 2010) under the following conditions: 94 °C for 1 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 7 min. The PCR products were treated with ExoSAP-IT (Affymetrix) prior to the sequencing reactions. Sequencing reactions using a BigDye(r) Terminator v3.1 cycle sequencing kit (Applied Biosystems) and sequencing were performed by Eurofins Genomics (Tokyo) using an ABI 3730XL DNA analyzer (Applied Biosystems). The sequences were deposited in GenBank, the European Molecular Biology Laboratory (EMBL), and the DNA Data Bank of Japan (DDBJ) under the Accession Numbers provided in Table 1.

Amplification of the fourth intron of *Alx1* was performed with a Prime Star GXL (Takara Bio, Inc.) using primers designed in the fourth and fifth exons of *P. japonica Alx1* (Forward: 5′-GTTTCAAAAACAGAA GGGCAAAAT-3′, Reverse: 5′-AGTCAAACTCCCTCCTCAGGTT-3′). The intron position was inferred by comparison with the genome sequence of *Strongylocentrotus purpuratus Alx1* (Sea Urchin Genome Sequencing Consortium, 2006). The same set of primers was used for amplification of the fourth intron of *P. rubra Alx1*. The amplified DNA was sequenced as described for COI. Several additional primers were designed for sequencing the full length of the *Alx1* intron. In order to analyze haplotype distribution, additional specimens were analyzed for *Alx1* intron.

2.2. Phylogenetic analyses and estimation of divergence time

The alignments for two nuclear genes were determined based on the maximum nucleotide similarity using a MEGA6 (Tamura et al., 2013) and MAFFT v. 6.864 (Katoh and Toh, 2008). Pairwise base differences were calculated using PAUP* 4.0 beta 10 software (Swofford, 2003).

To determine the phylogenetic relationships among COI haplotypes, we performed phylogenetic analyses using the maximum likelihood (ML) and Bayesian inference (BI) methods. Model selection for ML and BI analyses was performed using the Akaike information criterion (AIC) in jModeltest v. 0.1.1 (Posada, 2008). For ML and BI analyses, we also performed a partitioning scheme following recent studies (e.g., (Brandley et al., 2005; Wiens et al., 2010)). The schemes used for the data were non-partition and three-partition strategies by codon position. These strategies were assessed using ML implemented in Treefinder, v. October 2008 (Jobb, 2008). The three-partition strategy was selected as optimal for the COI gene. In this strategy, SYM + I, F81 + G, and GTR + I + G were selected as the best models for the first, second, and third positions, respectively.

ML analysis was performed using Treefinder under the models selected in the above process. The confidence of the branches in the ML was determined using bootstrapping (Felsenstein, 1985) with 1000 replicates in Treefinder. Tree topologies with bootstrap proportions of ≥70% were regarded as sufficiently resolved nodes (Huelsenbeck and Hills, 1993; Shaffer et al., 1997).

BI using the Markov chain Monte Carlo (MCMC) technique was also performed using MrBayes 3.2 software (Ronquist et al., 2012). We initiated four independent analyses with a random starting tree that ran for 10 million generations. We used the program Tracer 1.5 (Rambaut and Drummond, 2007) to determine when the log likelihood of sampled trees reached stationary distribution. Because apparent stationarity of the MCMC runs was reached at no later than one million generations, we conservatively discarded the first 2.5 million generations from each run as burn-in, and sampled one of every 100 generations from the remaining 8 million generations to calculate the posterior probability for each branch in the Bayesian tree. Bayesian posterior probabilities (BPPs) ≥0.95 were considered significant support (Larget and Simon, 1999) (Huelsenbeck et al., 2001).

BEAST 1.8.0 (Drummond et al., 2012) was used with a relaxed clock model and with a lognormal distribution and Yule process to obtain Bayesian estimates of the timing of diversification events. The Hasegawa, Kishino, and Yano (HKY) model (Hasegawa et al., 1985) was selected as the best model for both noncoding sequences using jModeltest. The program ran for 10 million generations, with sampling occurring every 1000 generations for each analysis, assuming the calibration point between *P. japonica* and *P. rubra* as 45.6 Myr BP (see above). A burn-in of 20% was applied to obtain the node age estimates using TreeAnnotator 1.8.0 (Drummond et al., 2012).

3. Results and discussion

3.1. Mitochondrial DNA sequences did not resolve the genetic structure of P. japonica

We examined the partial COI sequences from a total of 17 individuals of *P. japonica* from five localities around Japan and obtained 10 haplotypes (Table 1, Fig. 1A, E). *P. japonica* distributes from the main island of Japan (Honshu) to Kyushu and Okinawa. We analyzed these sequences in three species of clypeasterid with that of *Strongylocentrotus purpuratus* as outgroups. In total, we analyzed COI sequences from four species of Clypeasteroida (*P. japonica*, *P. rubra*, *Clypeaster japonicas*, *Astriclypeus manni*) and one Camarodonta (*Strongylocentrotus purpuratus*; Jacobs et al., 1988). The sequence differences within species were relatively low (0–1.9%, 0–13 bp), whereas the sequence differences among the four echinoid species ranged from 12.4 to 20.9% (84–141 bp).

Fig. 2 shows the ML derived from 645 bp of mitochondrial COI gene. The BI tree (not shown) was almost identical to the ML tree. Monophyly was supported for the genus *Peronella* as well as *P. japonica* and *P. rubra* by high bootstrap values. However, the intraspecific variations within *P. japonica* were low. The populations of this species on the Japanese coastline seem to be slightly fragmented either due to recent divergences or because they are panmixed assemblages. Therefore, we