RESEARCH NOTE

Molecular evidence of the evolutionary origin of a Bonin Islands endemic, *Stenomelania boninensis*

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Oceanic islands, which have never been connected to continental land masses, often contain many endemic nonmarine species (e.g. Darwin, 1859; Freed, Conast & Fleischer, 1987; Shaw, 1996). The Bonin Islands (Ogasawara Islands), located approximately 1,000 km from the Japanese mainland, were formed by volcanic activity and have never been connected to Eurasia (Asami, 1970). Several endemic species have been recorded from the Bonin Islands (e.g. Kobayashi, 1978; Chiba, 2002) and some of these have undergone adaptive radiations within the archipelago (Chiba, 1999). The Bonin Islands rose above sea level, presumably between the Pliocene and the early Pleistocene (Asami, 1970; Kaizuka, 1977; Imaizumi & Tamura, 1984), and were subsequently colonized by nonmarine organisms. The geographic isolation of the Bonin Islands in the western Pacific Ocean provides an ideal setting for allopatric speciation (Takayama *et al*., 2005). Historical colonization of these islands from the Japanese mainland, Nansei Islands or other proximal regions presumably has led to the evolution of endemic species. *Stenomelania boninensis* (Lea, 1850) (Gastropoda: Thiaridae) is endemic to the Bonin Islands, where it typically lives in freshwater rivers. It is rarely found in higher salinity habitats where other congeners commonly occur. We hypothesize that *S. boninensis* was derived from a congener that dispersed from a proximal region. To test this hypothesis, we evaluated phylogenetic relationships among this species and congeners distributed in geographically proximal regions using molecular data. We also estimated the divergence time of *S. boninensis* relative to its putative ancestor to help elucidate the evolution of *S. boninensis*.

We collected six *Stenomelania* species (*S. boninensis*, *S. costellaris* [Lea, 1850], *S. rufescens* [Martens, 1860], *S. uniformis* [Quoy & Gaimard, 1834], *S. crenulatus* [Deshayes, 1838], *S. juncea* [Lea, 1850]) and two other freshwater cerithioidean species (*Thiara scabra* [Müller, 1774], *Sermyla riqueti* [Grateloup, 1840]) from Amami-Ohshima, Okinawa, Tanegashima, Ishigakijima, Yonaguni Islands, the Bonin archipelago and Kyushu (all in Japan, Fig. 1). The *Stenomelania* species have limited distributions in Japan (Masuda & Uchiyama, 2004), though some are broadly distributed in Asia (Benthem Jutting, 1956; Kuroda, 1963; Pace, 1973; Haynes, 2001). Snails were collected and stored at −20°C for molecular analysis. DNA was isolated using the following procedure: snail tissues were homogenized in a solution of 300 ml 2× CTAB and 10 mg/ml proteinase K, incubated at 60°C for approximately 1 h, extracted once with phenol/chloroform (v:v, 1:1) and precipitated with two volumes of ethanol. The DNA pellets were briefly washed in 75% ethanol, air-dried for approximately 30 min and dissolved in 50 ml of water.

We analysed the mitochondrial DNA encoding cytochrome oxidase *c* subunit I (COI) gene. As a primer pair, we used CO1-bf (5′- GGGGCTCCTGATATAGCTTTTCC-3′, Miura *et al*., 2006) and COI-6 (5′-GGRTARTCNSWRTANCGN CGNGGYAT-′3, Shimayama *et al*., 1990). PCRs were run for 35 cycles under the following conditions: denaturing at 94°C for 60 s, annealing at 45°C for 60 s, and extension at 72°C for 90 s. The 35 cycles were preceded by an initial denaturing at 94°C for 1 min and followed by a final extension of 72°C for 7 min. The PCR products of the samples were purified and sequenced using an automated sequencer. Sequences were aligned by ClustalW (Thompson, Higgins & Gibson, 1994), implemented in the BioEdit (Hall, 1999) for further phylogenetic analyses.

Phylogenetic trees were constructed using PAUP* (Swofford, 2001) and MEGA4 software (Tamura *et al*., 2007). Topologies were generated using neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) algorithms. The best evolutionary model for the NJ and ML analyses (HKY + I + G) was selected by the Modeltest program (Posada & Crandall, 1998). The MP analysis was conducted using heuristic searches with 100 random additional sequence replications and a CNI search algorithm. The ML analysis was performed with an initial tree obtained by stepwise addition of sequences and the TBR branch swapping algorithm. For all trees, support values for internal nodes were generated by bootstrap resampling with 1,000 replicates. In each analysis, sequences of two confamilial species, *T. scabra* and *S. riqueti*, were used as outgroups. We examined the consistency of the evolutionary rate among *Stenomelania* species in order to determine whether application of a molecular clock was appropriate. For this purpose, we used a tree-wide likelihood ratio rate test (Felsenstein, 1981). Likelihood scores with and without molecular clock were calculated using the same parameter as the ML analysis described above. The outgroup was pruned from the trees prior to this test. To identify individual taxa that may have evolved heterogeneously, we used the branch-length test (Takezaki, Rzhetsky & Nei, 1995). Branch lengths were calculated based on HKY + G model (using only transversion substitutions). One haplotype was selected from each species and tested for deviation from neutral evolution. Standard errors were estimated by bootstrapping procedures (1,000 replicates). This test was conducted using LINTREE (http://www.bio.psu.edu/People/Faculty/Nei/Lab/lintre/lintrdos.zip). After the elimination of taxa that showed a heterogeneous evolutionary rate we constructed a linearized tree (Takezaki *et al*., 1995) based on the NJ topology using MEGA4. Since no fossil data are available for a molecular clock calibration in *Stenomelania*, we estimated the divergence times using relatively slow (1.8% per million years, Wilke, 2003) and fast (2.9% per million years, Haase, Marshall & Hogg, 2007) calibrations derived from the CO1 gene in another caenogastropod family (Hydrobiidae). Sequences

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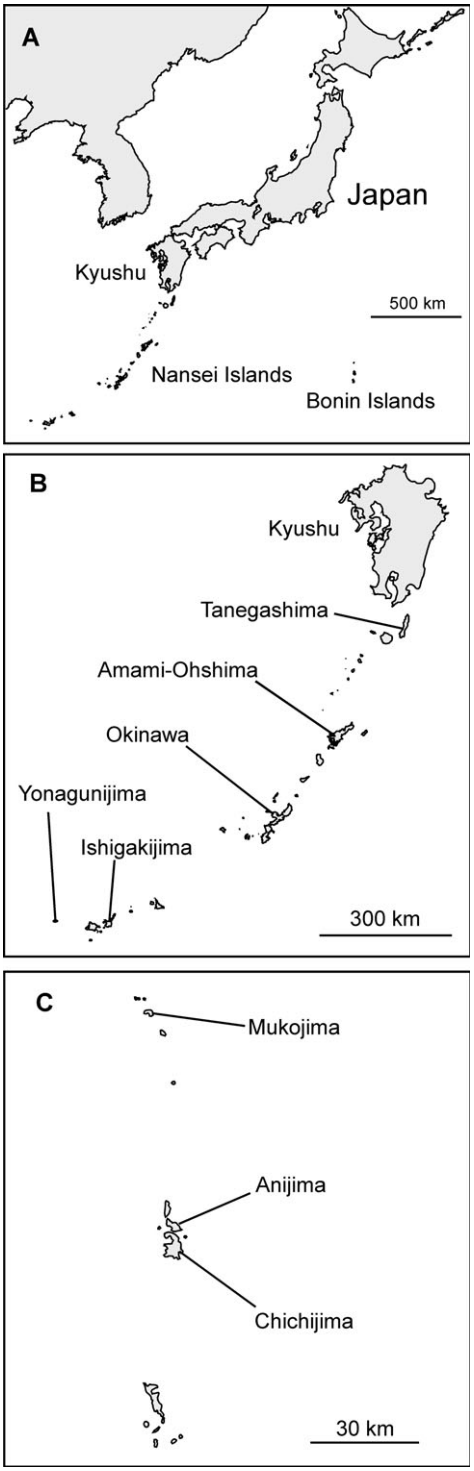

Figure 1. Sampling areas. **A.** East Asia. **B.** Kyushu, Nansei Islands. **C.** Bonin Islands. *Stenomelania boninensis* was collected from Chichijima (27°03′N, 142°12′E), Anijima (27°07′N, 142°12′E) and Mukojima (27°40′N, 142°08′E) (Bonin Islands). *Stenomelania costellaris*, *S. uniformis* and *Thiara scabra* were collected in Amami-Ohshima (28°10′N, 129°20′E). *Stenomelania rufescens* was collected in Kyushu (32°35′N, 131°42′E) and Tanegashima (30°27′N, 130°57′E). *Stenomelania crenulatus* was collected in Okinawa (26°39′N, 127°53′E). *Stenomelania juncea* was collected in Yonagunijima (24°27′N, 122°59′E). *Sermyla riqueti* was collected in Ishigakijima (24°24′N, 124°08′E).

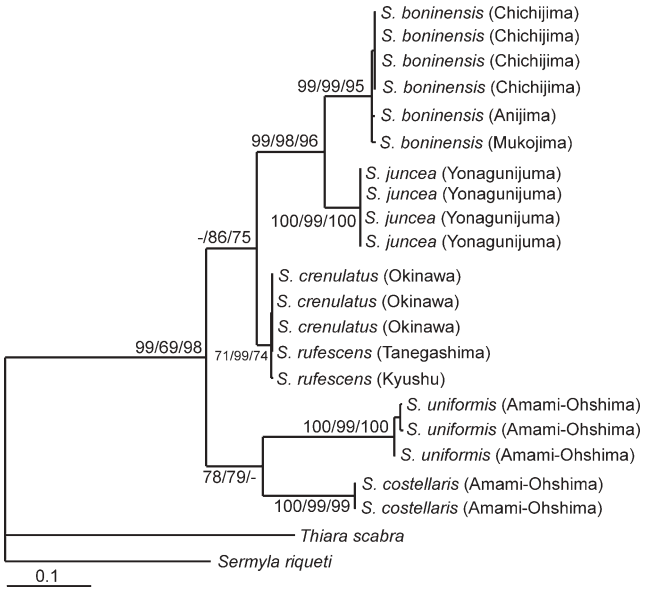

Figure 2. Maximum-likelihood tree based on the mitochondrial COI gene. Numbers near nodes indicate bootstrap support (values $<50\%$ not shown) for the different analyses (neighbor-joining/maximum parsimony/maximum likelihood).

analysed in this study were deposited in GenBank (accession no. EU273758–EU273769 and EU416201).

The phylogenetic relationships within *Stenomelania* based on the COI gene (873 bp) are shown in Figure 2. Haplotypes of each of four *Stenomelania* species (*S. boninensis*, *S. costellaris*, *S. uniformis*, *S. juncea*) formed clades that were supported by high bootstrap values (95–100%) for all tree algorithms (NJ, MP and ML, Fig. 2), while *S. rufescens* and *S. crenulatus* were included in a single clade. The observed pattern may indicate either introgression between *S. rufescens* and *S. crenulatus* or conspecificity. (This issue should be further studied using both mitochondrial and nuclear genes.) *Stenomelania boninensis* was depicted as sister to *S. juncea*, with high bootstrap values (96–99%, Fig. 2). This result strongly suggests that the endemic *S. boninensis* and *S. juncea* share a most recent common ancestor. Although we did not analyse every Asian *Stenomelania* species, we nonetheless consider it appropriate to conclude that *S. boninensis* is most closely related to *S. juncea* since the congeners that were not included in our study are not distributed in close proximity to the Bonin Islands. The uniformity of evolutionary rate among *Stenomelania* species was not rejected by the likelihood ratio test (difference in $-\ln = 3.56$, $P = 0.99$). However, the branch-length test indicated that the substitution rates of *S. rufescens* and *S. crenulatus* are significantly different from those of the other *Stenomelania* species ($z = 2.40$, $P < 0.05$ for *S. rufescens* and $z = 2.64$, $P < 0.05$ for *S. crenulatus*). After elimination of these sequences, the branch-length test did not reject the null hypothesis of uniformity of evolutionary rate (data not shown). Therefore, we constructed the linearized tree without *S. rufescens* and *S. crenulatus* sequences (Fig. 3). This tree indicates that two major groups of *Stenomelania* diverged about 15–8 million years ago (Ma) (Event A in Fig. 3). The subsequent divergence between *S. juncea* and *S. boninensis* occurred about 5–3 Ma (B in Fig. 3). Colonization of the Bonin Islands by terrestrial organisms probably began between the Pliocene and the early Pleistocene (Asami, 1970; Kaizuka, 1977;

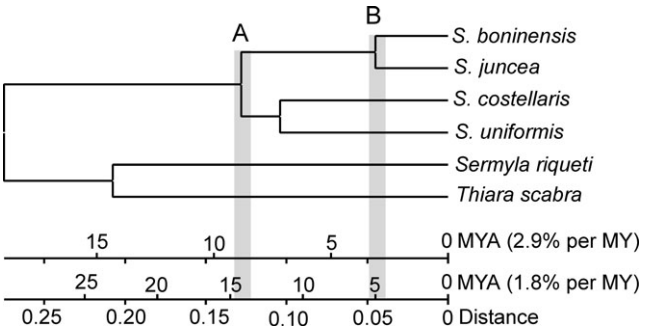

Figure 3. A linearized tree for the *Stenomelania* COI gene. The branch lengths were re-estimated under the assumption of rate constancy. Letters indicate divergence events discussed in the text. The upper bar refers to estimated divergence times based on an evolutionary rate of 2.9% per million years (Haase *et al*., 2007) while the lower bar is based on a rate of 1.8% per million years (Wilke, 2003).

Imaizumi & Tamura, 1984) and is consistent with the estimated time of divergence between *S. juncea* and *S. boninensis*. However, our estimated divergence times are tentative since these were not based on either the fossil record or local geological events. Note that colonization in the opposite direction, from the Bonin Islands to Yonagunijima, is unlikely since *S. juncea* is widely distributed in Asia (Benthem Jutting, 1956; Kuroda, 1963; Pace, 1973; Haynes, 2001; Masuda & Uchiyama, 2004). Colonization histories similar to that proposed have been described for other Bonin Islands species. For example, a fresh water goby, *Rhinogobius* sp., probably colonized the Bonin Island about 3.2–2.9 Ma (Mukai *et al*., 2005). *Stenomelania boninensis* exhibits morphological variation among the islands of the Bonin archipelago (e.g. Kuroda, 1929). However, our results indicate that there is little genetic variation among haplotypes found in each island (genetic distance = 0.007–0.008). The dispersal history of *S. boninensis* among the Bonin Islands is obscured by the low bootstrap values of the relevant portion of our phylogenetic hypothesis (<65%). Although genetic distances among the observed haplotypes were small, there were no haplotypes shared among the islands (Fig. 2), suggesting limited gene flow. We speculate that the insular *S. boninensis* populations rapidly attained their unique morphological features following recent dispersal events. Additional studies, such as detailed ecological and genetic comparisons of *S. boninensis* populations within the archipelago, are needed to further clarify the mechanisms underlying their morphological variations.

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