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Morphological and Genetic Variation Between the Japanese Populations of the Amphidromous Snail *Stenomelania crenulata* (Cerithioidea: Thiaridae)

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Freshwater gastropods often have limited dispersal capability and small geographic ranges, and face severe threats from habitat loss and degradation. However, in addition to the scarcity of knowledge on their life history traits, species taxonomy has not been adequately resolved and boundaries between intra- and interspecific variation remain unclear for many taxa. One such example of an indeterminate species boundary with implications for conservation issues is the relationship between the thiarid snails *Stenomelania crenulata* in Okinawa and southwards (ranked as CR+EN in the 2012 Japanese Red List) and *S. rufescens* in mainland Japan (VU). The results of our multidisciplinary investigation into variation in the shell morphology and mitochondrial (COI) and nuclear (ITS-1) gene sequences suggest that *S. rufescens* represents a geographic variant and a junior synonym of *S. crenulata*. The widespread geographic range of *S. crenulata*, spanning a few thousand kilometers north to south, is possible due to an amphidromous life cycle that involves a marine planktotrophic larval phase and upstream migration after settlement in estuaries. Nevertheless, there is recognizable morphological and genetic differentiation between distant populations, probably reflecting a relatively short pelagic duration and possibly also infrequent transoceanic dispersal; metamorphic competence is achieved in two weeks in full seawater and even more rapidly in brackish water. The Okinawan population, with only a few known localities, therefore deserves the high conservation priority; conservation efforts need to involve the proper maintenance of migration pathways including all marine, brackish and freshwater environments.

Key words: amphidromy, endangered species, freshwater ecology, Gastropoda, larval dispersal, PLD

INTRODUCTION

The world's freshwater gastropod fauna faces unprecedented threats from habitat loss and degradation (Lydeard et al., 2004; Strong et al., 2008). Freshwater species are particularly vulnerable to habitat change, as they tend to have limited dispersal capability and are often restricted to small hydrographic systems, including rivers, lakes, streams, and swamps (Régnier et al., 2009). In addition to low vagility, the most sensitive species are habitat specialists or comparatively long-lived species with a long maturation time or low fecundity (Strong et al., 2008). Anthropogenically introduced species accelerate the threat of extinction (Lydeard et al., 2004; Lysne et al., 2008). Invasive snails can negatively affect native taxa directly through competition for resources such as food and space or indirectly through changes in ecosystem function (Hall et al., 2003; Kerans et al., 2005). According to the 2006 IUCN Red List, 520 freshwater gastropod species are facing a major threat of extinction around the world (Strong et al., 2008). In Japan, 19 species are considered to be endangered (Ministry of the Environment, 2012). Of these, nearly one third (six) belong to the caenogastropod family Thiaridae.

Thiaridae comprises 135 described species from the tropics to warm temperate waters in Central and South America, on some Caribbean Islands, Africa, and in particular Southeast Asia to Australia and other western Pacific islands (Bandel et al., 1997; Strong et al., 2011). Thiarids inhabit both in lotic and lentic freshwater environments, with some taxa tolerating brackish conditions in stream mouths and estuaries (Glaubrecht et al., 2009). The current taxonomic account of the group recognizes 13 species in Japan (Masuda and Uchiyama, 2004; The Japanese Association of Benthology, 2012). The Ministry of the Environment classifies six of these in the Threatened I (CR+EN: Critically Endangered + Endangered) category, one in the Threatened II (VU: Vulnerable), and four in the Near Threatened (NT) in the latest, revised version of the Red List of Japan (2012). However, it remains unclear whether this categorization can be justified for all species and whether it is based on appropriate information on their natural history. For example, the "Near Threatened" category of the list contains

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Melanoides tuberculata (Muller, 1774) and *Tarebia granifera* (Lamarck, 1822), which may have been introduced into Japan from Southeast Asia. These species are considered to be invasive on many tropical and subtropical islands around the world (Pointier et al., 1993; Facon et al., 2005; Appleton et al., 2009; Sasaki et al., 2009). They reproduce mainly parthenogenetically and the young are released as metamorphosed juveniles, enabling them to successfully colonize ephemeral pools and artificial water bodies (Facon et al., 2006; Appleton et al., 2009).

Early ontogenetic traits also seem to play a fundamental role in determining the geographic distribution, population genetic structure, and hence the vulnerability of indisputably native freshwater snails. Certain thiarids are known to have rather wide-ranging distributions on scattered Pacific islands, which have supposedly been achieved with a life cycle trait called amphidromy (Strong et al., 2011). Amphidromy is a strategy involving migration of juveniles from marine to fresh water, where the growth from juvenile to adult, attainment of sexual maturity and spawning take place (McDowall, 2007). Freshwater snails of Neritidae and Neritiliidae (Neritimorpha) are better-documented amphidromous animals (e.g., Schneider and Lyons, 1993; Kano and Kase, 2003; Kano, 2011) along with gobiid fish and atyid and palaemonid shrimps (Lord et al., 2012; Castelin et al., 2013). The metamorphosed juveniles of nerites settle at river mouths and then migrate (often over 10 km) upstream where they remain for the rest of their life cycle (Kano, 2009). The energy cost of migration is compensated by lower predation pressure in the upper reaches and by the increased upstream availability of food for these animals, all of which graze on microalgae (Schneider and Lyons, 1993; McDowall, 2007). The longevity of their larvae in the ocean seems to exceed three or four months, and such a long larval period results in widespread geographic distribution and homogeneous genetic structure across the distribution range of the species (Crandall et al., 2010; Fukumori and Kano, 2014).

Although relatively little is known about the amphidromous life cycle in Thiaridae, evidence from different lines of investigation suggests the presence of a marine larval phase in the genus *Stenomelania*. Veliger larvae of *S. crenulata* (Deshayes, 1838), found swimming in the freshwater reach of a river in India, were successfully maintained for two weeks weeks, but only in brackish water (Seshaiya, 1940). Upstream migration from the lower brackish reach to upper freshwater environment has been documented in *S. rufescens* (Martens, 1860) in Japan; laboratory experiments have verified higher seawater tolerance in juveniles than adults in this species (Okazaki and Wada, 2007). The protoconch shape of *S. punctata* (Lamarck, 1822) from the Philippines is reported to be characteristic of planktotrophic cerithioideans, further corroborating the presence of the prolonged larval phase (Bandel et al., 1997). Freshwater animals with this life cycle are able to colonize new habitats across the sea and therefore potentially less susceptible to the risk of extinction than those spend their entire life in small hydrographic systems (Sodhi et al., 2010; Lee and Jetz, 2011). Thus, more detailed information on the ecological and ontogenetic characteristics is needed for each species to ensure effective conservation (Cote et al., 2010; McLane et al., 2011).

Even more critical for the protection of endangered animals is a proper systematic definition of the target species. Conservation efforts, if limited by the lack of meaningful classifications, are not maximally effective, or worse, can be actively harmful to the long-term preservation of biodiversity (Perez and Minton, 2008). The taxonomy of freshwater gastropods has been largely based on morphology, primarily using shared shell characters (Perez and Minton, 2008), although phenotypic plasticity has been frequently reported in their shells (e.g., Holomuzki and Biggs, 2006) and the boundary between intra- and interspecific variation is, in many cases, unclear (Strong et al., 2008). A number of early studies have resulted in the recognition of a few conchologically variable and widespread species, or conversely in the unwarranted enormous inflation of nominal taxa, including species, subspecies, and “morphs” (Lysne et al., 2008; Strong et al., 2008). Thiarid species, among others, have extensive intraspecific variation arising from ontogenetic, ecophenotypic, geographical, and/or individual genetic differences (Strong et al., 2011). One such example of an indeterminate species boundary involving conservation issues is the relationship between *S. crenulata* in Okinawa and *S. rufescens* in the mainland Japan.

Stenomelania rufescens was first described by Martens (1860) based on shells collected by von Siebold from an unknown locality in mainland Japan, possibly around Nagasaki, Kyushu Island (Habe, 1976). Snails identified as belonging to this species inhabit the lower reaches of streams and creeks in Honshu, Shikoku and Kyushu (Kuroda, 1929), as well as on Amami Island (Kimura and Kimura, 2008), which is located 300 km south of Kyushu. The shell attains a length of 50 mm and is characterized by the inconspicuous surface sculpture of the whorls (Masuda and Uchiyama, 2004; Fukuda and Kimura, 2012). *Stenomelania crenulata*, on the other hand, shows a more conspicuous shell sculpture (Kubo, 2012). The latter species was described by Deshayes (1838), presumably based on material collected from the Philippines (Reeve, 1859), and is considered to distribute from India (Seshaiya, 1940) to Okinawa Island, Japan (Masuda and Uchiyama, 2004; see also Masuda and Fukuda, 2006). Both *S. rufescens* and *S. crenulata* are included in the Japanese Red List, with the latter included in higher category (CR+EN) than the former (VU) (Ministry of the Environment, 2012).

However, the taxonomic status of the two taxa as independent species, and hence the suggested levels of vulnerability, has come into question from a genetic point of view. Miura et al. (2008) obtained exactly the same sequence for the cytochrome oxidase *c* subunit I (COI) gene from the specimens of the mainland Japan and Okinawa and pointed out the possibility of either introgression between the two species or conspecificity. Meanwhile, no morphological, ecological or other relevant criterion has been provided for a revised taxonomy of the two species or subspecies or populations. DNA sequence information should always be compared with other corroborating evidence as an indicator of species delimitation (DeSalle, 2006).

In the present study, we investigate the relationship between *S. rufescens* and *S. crenulata* by comparing shell morphology and molecular sequence data from both mitochondrial and nuclear DNA. Furthermore, we aim to provide

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more information on the presence or absence of the amphidromous life cycle and the ability of oceanic dispersal as the veliger larva by illustrating the geographic distribution of conchological and genetic variations. Our overall goal is to provide a solid basis for future conservation strategies of these taxa, as well as other thiarid freshwater snails, by combining knowledge from various biological disciplines.

MATERIALS AND METHODS

Materials

The study specimens were collected from fresh or brackish water in ditches, estuaries and lower stream reaches in the southwestern part of Japan (Table 1) and preserved in 95–99% ethanol. In this section and in the Results, the snails from the mainland Japan (Honshu, Shikoku and Kyushu) and Okinawa are tentatively called *S. rufescens* and *S. crenulata*, respectively, adopting the previous species identification (Masuda and Uchiyama, 2004; Masuda and Fukuda, 2006; Fukuda and Kimura, 2012; Kubo, 2012).

A total of 66 specimens including 22 individuals of *S. rufescens* and 44 of *S. crenulata* were measured for the biometric parameters of the shell (Table 1; Fig. 1A). Of these, only 58 specimens (17 and 41 from the respective taxa) were examined also for the shell sculpture (Fig. 1B) as the remaining eight shells had badly eroded surfaces. Molecular analyses were conducted for 20 specimens, ten from each taxon, representing as many localities and conchological variations as possible (Fig. 2; see below). Shell specimens from Kaohsiung, Taiwan and the Philippines, loaned from the National Museum of Nature and Science, Tokyo (NSMT; Fig. 3), were also examined for the comparison of the conchological characters with the Japanese specimens.

Table 1. Localities, sampling dates and numbers of specimens used for morphological and molecular analyses.

Locality	Coordinates	Date	Morphology*	DNA
"*Stenomelania rufescens*"				
Kochi, Shikoku Is.	33°28′N, 133°29′E	Jun. 14, 2003	6 (3)	3
Nobeoka, Kyushu Is.	32°35′N, 131°41′E	May 6, 2004	3 (2)	2
Nobeoka, Kyushu Is.	32°35′N, 131°42′E	–	–	1†
Shibushi, Kyushu Is.	31°26′N, 131°03′E	Jun. 13, 2006	1 (1)	1
Ibusuki, Kyushu Is.	31°17′N, 130°37′E	Apr. 7, 2004	12 (11)	4
Tanegashima Is.	30°27′N, 130°57′E	–	–	1†
Stenomelania crenulata				
Motobu, Okinawa Is.	26°39′N, 127°54′E	Oct. 3, 2009	21 (20)	4
Motobu, Okinawa Is.	26°39′N, 127°54′E	–	–	3†
Nago, Okinawa Is.	26°33′N, 128°02′E	Oct. 4, 2009	23 (21)	6

*Numbers denote specimens measured for biometric parameters shown in Fig. 1A, followed by specimens (in parentheses) observed also for surface sculpture as in Fig. 1B. †Miura et al. (2008).

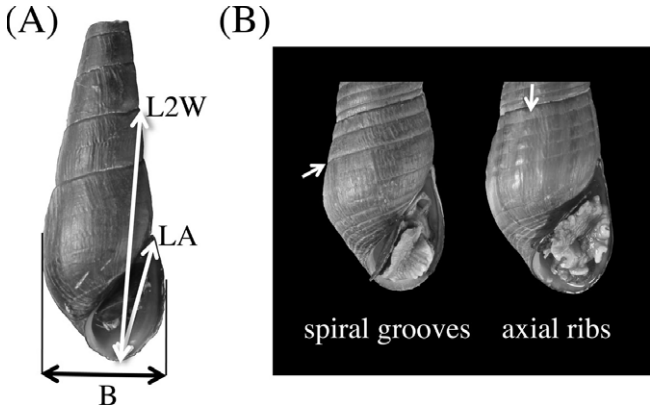


Fig. 1. **(A)** Morphometric characters used in present study. Abbreviations: B, shell width; L2W, height of first and second body whorls; LA, maximum dimension of aperture. **(B)** Shell surface sculpture. Arrows point to spiral groove (left) or axial rib (right).

Morphological analyses

The following biometric parameters of the shell were measured to 0.1-mm precision using an electronic caliper: L2W, height of the first and second body whorls; B, shell width; LA, maximum dimension of the aperture (Fig. 1A). L2W, rather than shell height, represents the overall shell size, as the apex was eroded away in most specimens. These parameters were consistently used in a previous study on thiarid systematics (Glaubrecht et al., 2009). In addition, the presence or absence of spiral grooves and/or axial ribs was determined in the upper-half surface of a shell whorl with a width of approximately 10 mm (Fig. 1B). This standardization allowed us to compare specimens at different ontogenetic stages; spiral grooves tend to be more conspicuous in earlier whorls than in later whorls in the same specimen (Fig. 2). All specimens had a few, sometimes faint, spiral grooves in the base of the shell and these grooves were not considered in the analysis of shell differentiation. Statistical tests were conducted using the software package R version 2.15.1.

Molecular analyses

Total DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN Inc., Valencia, CA, USA). For further purification, 20 μl of GeneReleaser (BioVentures Inc., Murfreesboro, TN, USA) was added to 1 μl of the total DNA following the described protocol. Polymerase chain reactions (PCR) were used to amplify 1250-bp and approximately 520-bp fragments (excluding the primer sequences) of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and nuclear internal transcribed spacer 1 (ITS-1) region, respectively. The primers used for the amplification were LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′; Folmer et al., 1994) and COI-6 (5′-GGRTARTCNSWRTANCGNCGNGGYAT-3′; Shimayama et al., 1990) for the COI gene, and ITS-f pulmo (5′-GTCGTAACAAGGTTTCCGTAGGTGAAC-3′) and 5.8 ks (5′-GGCTGCGCTCTTCATCGACTCACGA-3′; new primers) for the ITS-1 region. PCR reactions were carried out in a final volume of 10 μl [0.5 μl genomic DNA template (c. 100 ng), 7.15 μl ddH_2O, 1.0 μl Takara ExTaq buffer, 0.8 μl dNTPs (2.5 μM each), 0.25 μl of each primer (20 μM), and 0.05 μl Takara ExTaq enzyme]. After an initial denaturation for 2 min at 94.5°C, the reaction solution was run for 30 cycles with the following parameters: denaturation for 30 s at 94.5°C, annealing for 40 s at gene-specific temperatures, followed by an extension for 60 s at 72°C. Annealing temperatures of 42°C were used for COI amplification and 52°C for ITS-1 amplification. Successful PCR products were cleaned using ExoSAP-IT (USB, Cleveland, OH, USA) following the described protocol. Both strands were directly cycle-sequenced using the amplification primers with the BigDye Terminator Cycle Sequencing Kit, version 3.1 on an ABI 3130 sequencer (Applied Biosystems, Foster City, CA) at the Atmosphere and Ocean Research Institute, The University of Tokyo.

The COI sequences were aligned by eye in MEGA 5 (Tamura et al., 2011) as there was no indel or amino acid substitution among study specimens. Three sequences reported by Miura et al. (2008) with the DDBJ/EMBL/Genbank accession numbers EU273765, EU273767 and EU416201 were also included in the COI dataset to represent three more localities for *S. rufescens* and *S. crenulata* (Table 1). A haplotype network was constructed using the program

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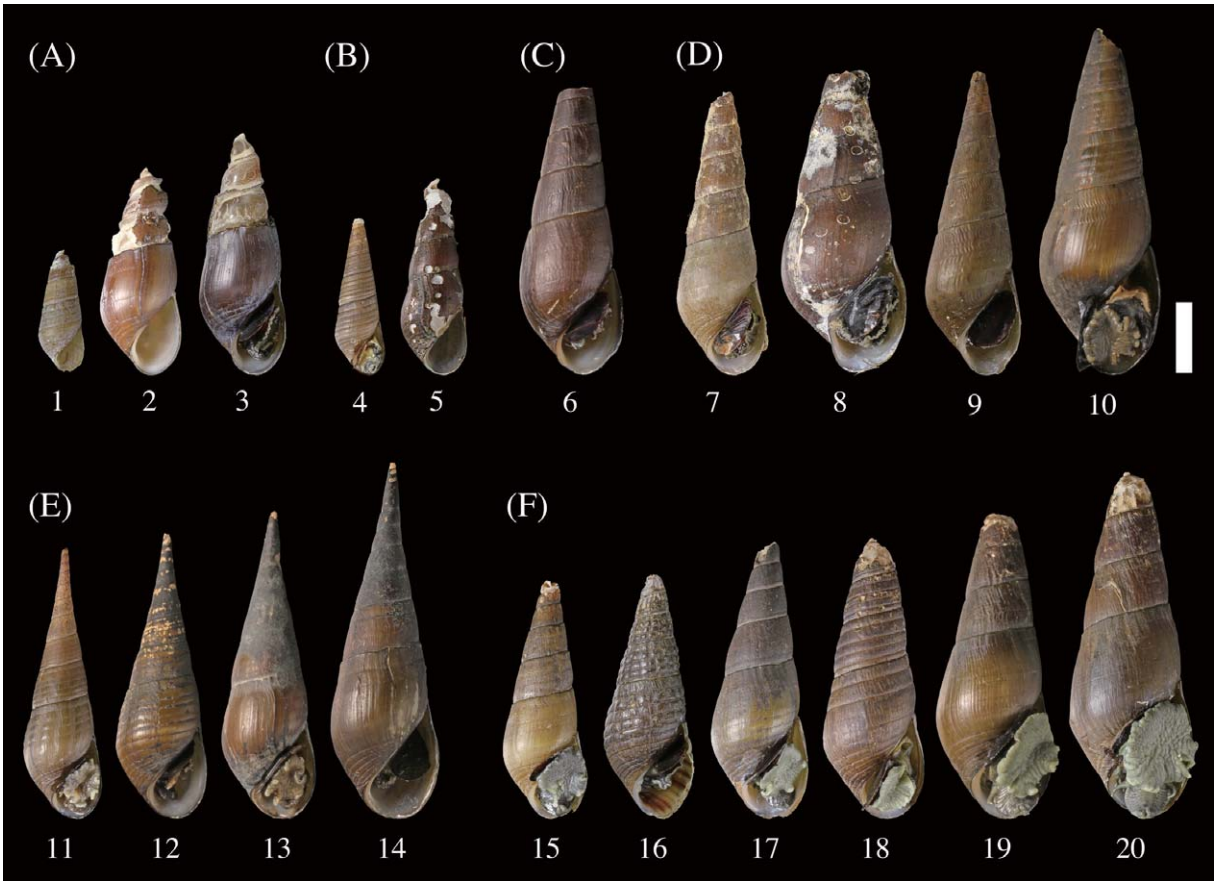


Fig. 2. "*Stenomelania rufescens*" from mainland Japan **(A–D)** and *S. crenulata* from Okinawa **(E, F)** used for molecular analysis; numbers shown below specimens correspond to those in Fig. 6. Sampling localities: **(A)** Kochi, **(B)** Nobeoka, **(C)** Shibushi, **(D)** Ibusuki, **(E)** Motobu and **(F)** Nago (see Fig. 7). Scale bar = 10 mm.

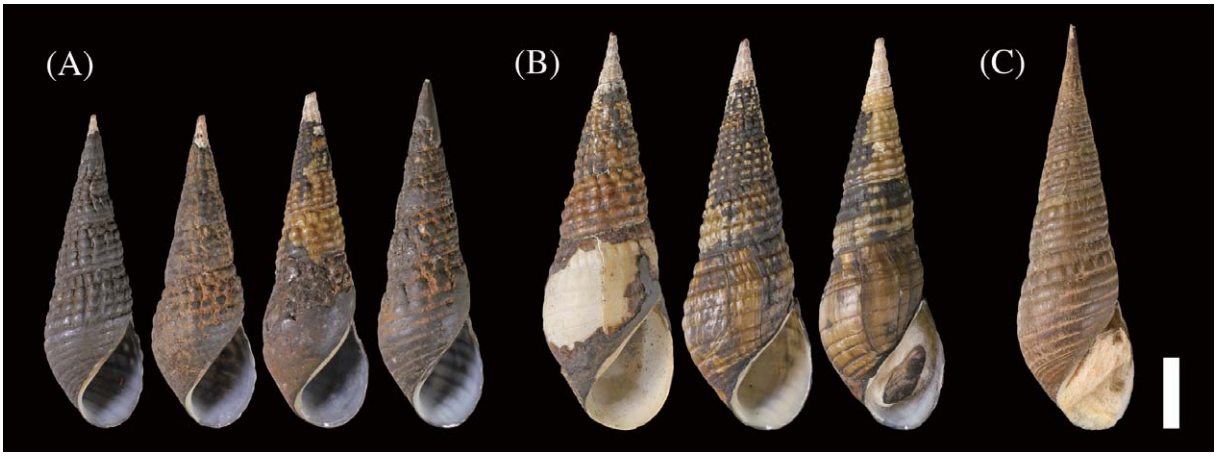


Fig. 3. *Stenomelania crenulata* from Taiwan **(A, B)** and Philippines **(C)**. **(A)** "Kaohsiung," NSMT-Mo78510. **(B)** "Qianzhen, Kaohsiung," NSMT-Mo78511. **(C)** "Philippines," NSMT-Mo78508. Scale bar = 10 mm.

TCS 1.21 (Clement et al., 2000) with a connection limit of 95%. The calculation of pairwise F_{ST} values and their significance as well as the exact test were performed in Arlequin 3.11 (Excoffier et al., 2010). The ITS sequences were aligned with ProAlign ver. 0.5 (Löytynoja and Milinkovitch, 2003), and regions with posterior probabilities below 90% were regarded as alignment-ambiguous sites and were excluded in the succeeding analyses. For purposes of comparison, a homologous ITS sequence was determined for *Stenomelania juncea* (Lea, 1850) from Yonaguni Island, Okinawa (24°27′N, 123°00′E) with the methods described above.

Geographic distribution

The sampling sites of the specimens for the above analyses were plotted on a distribution map of *S. rufescens* and *S. crenulata*.

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Sampling localities were also plotted for other conspecific specimens deposited in our laboratory. Moreover, information on their distribution in Japan was gathered from museum specimens, previous literature records, including illustrated books and government reports, as well as from various websites (for details see Supplementary File S1 online). The records of occurrence in the literature and on websites, that may have referred to erroneously identified specimens, were classified into two categories: one with a photograph of *S. rufescens* or *S. crenulata* and the other without any material for our verification.

This map does not necessarily reflect the present status of the species distribution and occurrences; some of the localities are historical and are represented only by records from the 19th and 20th centuries. For example, Brot (1874) and Hartman (1897) described *Melania loebbeckii* and *M. yokohamensis* based on conspecific specimens from Yeddo (Tokyo) and Yokohama in Tokyo Bay Area, while all localities in the eastern part of Honshu Island have subsequently been lost (e.g., Kimura, 2009). We aimed to show the evolutionary consequence of past and present dispersal and/or vicariance events on their geographic distribution rather than the ecological impacts of recent human activities, which will be dealt with elsewhere.

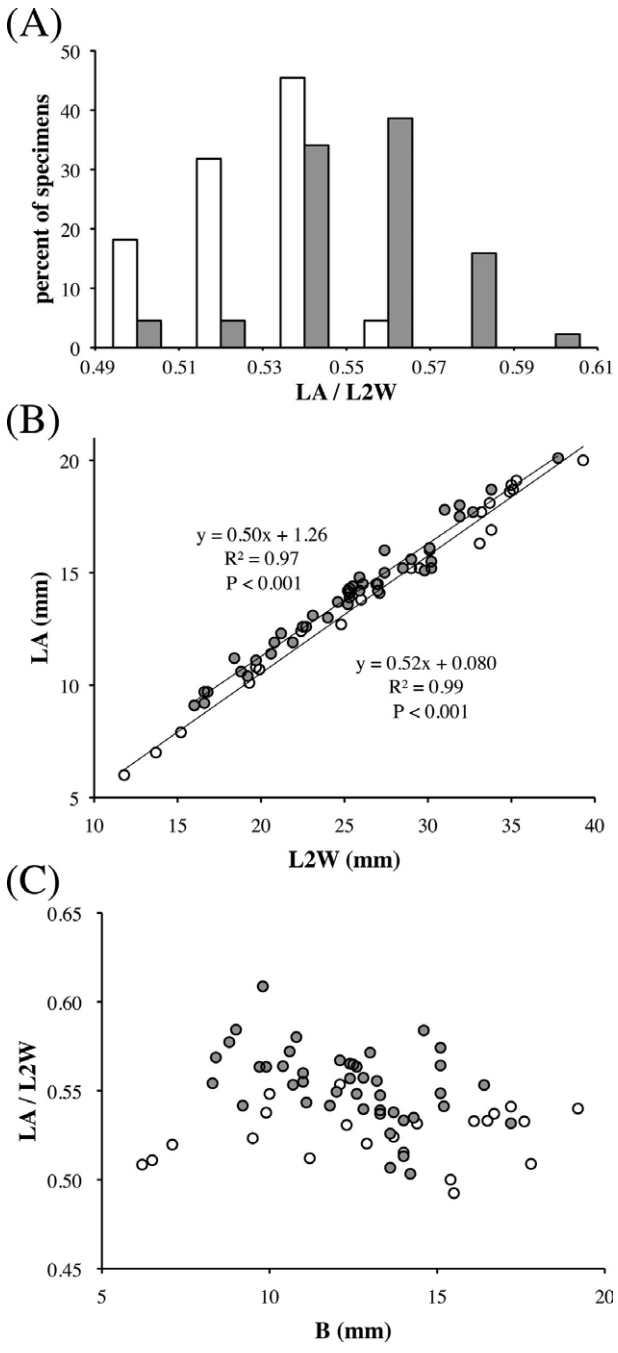


Fig. 4. Relationship of maximum dimension of aperture (LA) and height of first and second body whorls (L2W) in *S. crenulata* (gray) and "*S. rufescens*" (white). **(A)** Frequency distribution of LA/L2W. **(B)** Correlation between LA and L2W. **(C)** Relationship between LA/L2W and shell width (B); aperture of *S. crenulata* is more longitudinally elongated in juveniles than in fully-grown individuals; invariable apertural shape of "*S. rufescens*" results in straight slope of spire.

RESULTS

Morphological analysis

The ratio of the aperture size to the entire shell (here approximated by LA/L2W; Fig. 1A) differed significantly between *S. rufescens* and *S. crenulata*, with a proportionally smaller aperture in the latter (Mann-Whitney's *U* test, $P < 0.000001$). The histogram of this ratio reveals different peaks for the two taxa and also a considerable overlap in the interval of 0.53–0.55 (Fig. 4A). A scatter diagram for LA/L2W shows a strong correlation in each taxon ($R^2 \geq 0.97$, $P < 0.001$; Fig. 4B); when this ratio was plotted against the shell width (B) an inverse correlation is observed only in *S. crenulata* (Pearson's $r = -0.34$, $P = 0.025$; *S. rufescens*: Pearson's $r = 0.092$, $P = 0.69$; Fig. 4C). The shell aperture of *S. crenulata* is therefore more longitudinally elongated in juveniles than in fully-grown individuals. This ontogenetic change explains the proportionally thinner early whorls and wider penultimate and last whorls, which result in the concave slope of the spire (e.g., Fig. 2: specimens 11–14). On the other hand, the invariable shape of the aperture in the ontogeny of *S. rufescens* results in the straight slope of the

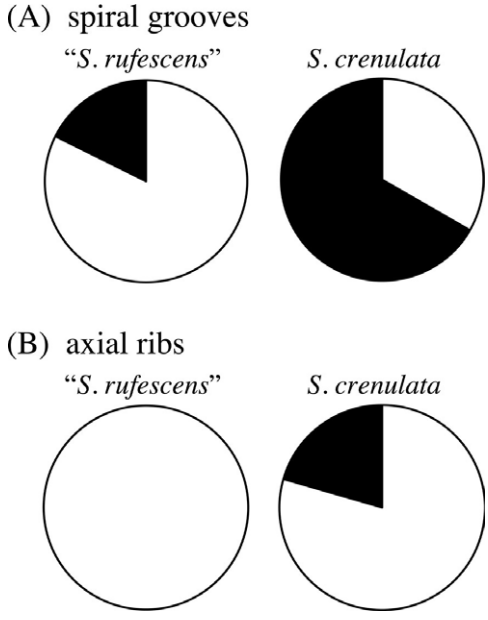


Fig. 5. Comparison of shell surface sculptures between *Stenomelania crenulata* and "*S. rufescens*"; presence (black) or absence (white) of spiral grooves (**A**) and axial ribs (**B**).

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spire (e.g., specimen 9). The two taxa could not be differentiated by statistic analyses of other shell parameters (Mann-Whitney's *U* test, $P > 0.05$).

Stenomelania rufescens and *S. crenulata* also show different trends in the development of shell sculpture (Fig. 5). The former exhibits the spiral grooves and axial ribs less frequently than the latter (Fisher's exact probability test, $P < 0.01$); the axial ribs are always lacking in *S. rufescens*.

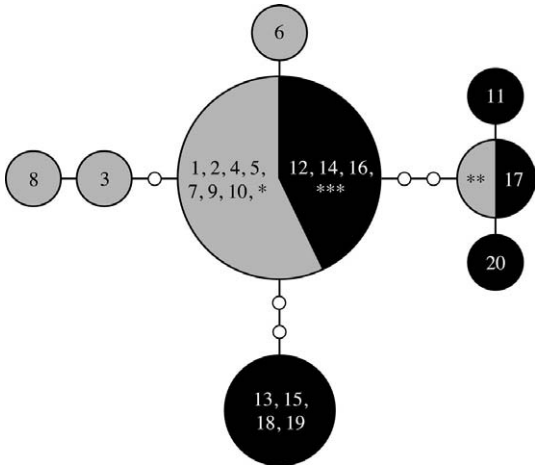


Fig. 6. COI haplotype network for *Stenomelania crenulata* (gray) and "*S. rufescens*" (black). Circle sizes reflect frequencies of haplotypes. Numbers in circles correspond to those in Fig. 2. Small open circles represent hypothetical haplotypes, which were not detected in study specimens. Asterisks indicate sequences from Miura et al. (2008): *EU416201 from Motobu, **EU273767 from Tanegashima and ***EU273765 from Nobeoka (see Table 1; Fig. 7).

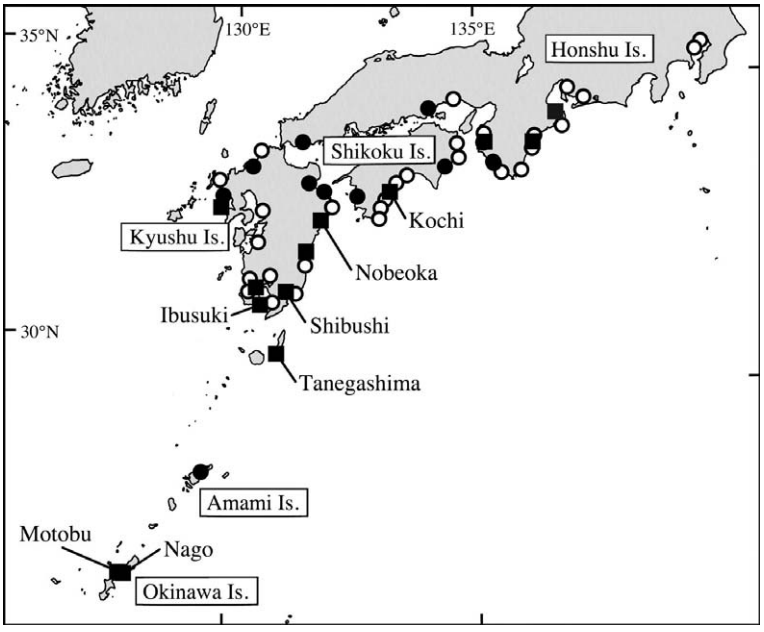


Fig. 7. Geographic distribution of *Stenomelania crenulata* and "*S. rufescens*" in Japan. Solid squares denote collection sites of study specimens; sites for sequenced snails are shown with locality names. Solid and open circles denote localities in previous literatures and websites with and without a photograph of a specimen for identification, respectively (see Supplementary File S1 online).

Molecular analysis

The nucleotide sequences generated from *S. rufescens* and *S. crenulata* in the present study have been deposited in the DDBJ/EMBL/Genbank under accession numbers AB920316–AB920326. The amplified sequences of the mitochondrial COI gene (1150 bp; AB920319–AB920326) were longer than those reported in Miura et al. (2008) and only the homologous 785 bp were used in the succeeding analyses. There were 12 variable and eight parsimony-informative sites, all of which were at the third codon positions; all but one substitution were transitions. The most common haplotype, found in 14 out of 25 individuals, was connected to seven other haplotypes with one to four steps (Fig. 6). The first and third most common haplotypes, which were separated by three steps, were represented by both *S. rufescens* and *S. crenulata*. The two taxa were significantly different in the pairwise F_{ST} (0.11; $P = 0.018$), while such significance was not detected by the exact test of population differentiation ($P = 0.097$). No correlation was found between COI haplotype and two morphological characteristics, i.e., presence or absence of the spiral grooves and axial ribs (exact test, $P > 0.05$).

Approximately 520-bp fragments amplified from the ITS-1 region (AB920316–AB920318) were trimmed by excluding low quality regions near the primers. The aligned data matrix had 432 sites, 421 of which remained after masking alignment ambiguous sites. The final dataset contained only one variable site (at the position 21 near the 18S rRNA gene), which was heterozygous within two individuals, and therefore no further analysis was conducted to test for population differentiation. A pairwise sequence comparison with the closest known species *S. juncea* (see Miura et al., 2008) revealed an uncorrected distance of 1.9%: eight homozygous sites were substituted between them (accession number: AB921550).

Geographic distribution

Stenomelania rufescens has been recorded from the coastal areas of Honshu, Shikoku and Kyushu Islands as well as from Tanegashima and Amami Islands (Fig. 7; Supplementary File S1 online). Most localities in Honshu and Shikoku occur along the Pacific sides; there are a few records from rivers flowing into the Seto Inland Sea, while none on the Sea of Japan coast. A number of localities have been found in Kyushu, along both eastern and western shorelines facing the Pacific Ocean and East China Sea, respectively, while there has been no report from the continental side of the latter sea, i.e., from Korea or China (e.g., Kuroda, 1929; Min Duk-Hi, 2004). The present distribution seems to be restricted to the Kii Peninsula and southward in the mainland Japan (e.g., Kimura, 2009).

Previous records of *S. crenulata* have been confined to Okinawa and Amami Islands in Japan (Fukuda and Kimura, 2012; Kubo, 2012), while neither a published figure nor a voucher specimen was available to verify the occurrence on the latter island. Two shells of *Stenomelania* collected from Amami and shown in Kimura and Kimura

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(2008: figs 29, 30) closely match those of *S. rufescens* from the main islands of Japan in lacking the spiral grooves and axial ribs and in having the straight slope of the spire.

These localities of *S. rufescens* and *S. crenulata* appear to be continuously distributed from the southern coast of Honshu to Okinawa Island with the influence of the warm Kuroshio Current and its branch, Tsushima Current. The small volcanic and coralline islands between Tanegashima and Okinawa Islands (except Amami) are not inhabited by any *Stenomelania* species, probably due to the lack of a suitable habitat. Yaeyama Islands between Okinawa Island and Taiwan also lack *S. rufescens* or *S. crenulata*, while congeneric species are abundant (e.g., Masuda and Uchiyama, 2004).

The museum specimens from Taiwan and an unknown locality in the Philippines (Fig. 3) most closely resemble some of the Okinawan individuals, with their conspicuous surface sculpture (Fig. 2: specimens 12 and 16). While their spiral grooves and axial ribs are even more clearly carved than those of the Okinawan shells, the slope of their spires is straight, as in *S. rufescens* from the mainland Japan. Overall, the specimens from Taiwan and the Philippines are conchologically similar enough to be considered conspecific with the Okinawan individuals, and this justifies the use of the name *S. crenulata* (described presumably from the Philippines) for the Japanese specimens. The geographic distribution of the study species ranges from Tokyo to at least the Philippines via Okinawa and Taiwan, a distance of approximately 3000 km in a straight line.

DISCUSSION

Our multidisciplinary investigation on the variation and distribution of *Stenomelania crenulata* and *S. rufescens* suggests that *S. rufescens* described from mainland Japan represents a geographic variant and a junior synonym of *S. crenulata*. The morphometry of the shell shows that the only significant discrepancy between the Okinawan and mainland populations exists in the ratio of the aperture size to the entire shell but with a considerable overlap, and no statistical difference was detected in other quantitative conchological characters (Fig. 4). The observation of the spiral grooves and axial ribs reveals that the manifestation of these sculptures is higher in the specimens from Okinawa and southwards than those from the mainland Japan, although both conditions are found in one population (Figs. 2 and 5). The individuals of the two geographic regions share the first and third most common haplotypes of the COI gene (Fig. 6). The exact test for the COI dataset did not detect population differentiation between the two regions, although the pairwise F_{ST} value was significant (see below). The nuclear ITS sequences were invariable among the study specimens after removal of a heterozygous site, contrasting with 1.9% distance to their putative closest relative, *S. juncea* (eight homozygous substitutions among 421 sites). These morphological and genetic differences between *S. crenulata* and "*S. rufescens*" appear to fall within the range of intraspecific variation, irrespective of the apparently discontinuous estuarine habitats in the islands, which are hundreds of kilometers apart.

This widespread geographic range of *S. crenulata* is possible through interisland dispersal by the amphidromous life cycle and marine planktotrophic larval phase. Their occurrences are restricted to coastal areas (Fig. 7) and no specimen has been found from an upstream part of a major river or a closed inland water body. Previous investigations on the early development and ecology of *S. crenulata* showed that this species requires saline water only in the larval period (Seshaiya, 1940; but see below) and that metamorphosed juveniles migrate from the lower brackish reaches to upper freshwater environments (Okazaki and Wada, 2007). Molecular studies on amphidromous animals often show a lack of genetic structuring across an extensive geographic range that spans over thousands of kilometers, indicating continuous gene flow across distant populations (Crandall et al., 2010; Cook et al., 2012; Taillebois et al., 2013). Indeed, the geographic distribution of *S. crenulata*, particularly in temperate Japan (Fig. 7), shows a striking similarity to the distribution pattern exhibited by many species of shallow marine benthos with a planktotrophic larval phase (e.g., Yorifuji et al., 2012). The Kuroshio and Tsushima currents carry pelagic larvae from the tropical and/or subtropical islands to the southern and western coasts of the mainland Japan, while insufficient tolerance to low-temperature stress seemingly prevents the colonization and establishment of these species along the northern coasts and along the Sea of Japan (e.g., Spalding et al., 2007).

However, the transoceanic dispersal of *S. crenulata* seems to take place only in rather rare, sporadic events, at least between remote islands, and may occur only on an evolutionary time scale. As mentioned above, there are slight but recognizable morphological and genetic differences between Okinawan and the mainland populations. The occurrence of the same COI haplotypes in the two regions is thus likely the result of incomplete lineage sorting but not ongoing gene flow. Conspecific specimens from Taiwan and the Philippines also show minor conchological differences from the Japanese material (Fig. 3). Such differentiation of local populations most probably results from a shorter pelagic larval duration (PLD) in *S. crenulata* than in many tropical and subtropical amphidromous animals. For example, two amphidromous neritid snails maintain intraspecific population heterogeneity among five South Pacific archipelagos (i.e., Vanuatu, Fiji, Samoa, Society Islands and Marquesas) presumably with PLDs of a few or several months (Crandall et al., 2010; Fukumori and Kano, 2014). The lack of significant genetic differentiation has also been documented for the gobiid fish *Sicyopterus lagocephalus* (PLD: 130–260 days) among western Pacific islands (Lord et al., 2012) and the palaemonid prawn *Macrobrachium lar* (90 days) between the northwest Indian and northwest Pacific oceans (Castelin et al., 2013). These have undergone a recent range expansion through the region, colonizing each archipelago over a short amount of time. In contrast, the veliger larvae of *S. crenulata* achieve metamorphic competence in two weeks from hatching in full seawater; PLD is further reduced in brackish water, possibly suggesting preference to the latter environment (Hidaka, unpublished data). Most of their larvae may therefore remain in the estuary of the natal river and perhaps only few are carried out to the open ocean and remain in the pelagic environment for only a few weeks—hence the restricted gene flow among distant populations. Moreover, thiarid species including *S. crenulata*

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are generally less abundant than neritid snails and some other amphidromous animals in estuaries and streams in Japan (e.g., Masuda and Uchiyama, 2004). The limited number of female individuals would result in less fecundity, fewer larvae and fewer opportunities of oceanic dispersal than in more abundant animals, hence differentiation of the local populations.

Stenomelania crenulata was originally described by Deshayes (1838), presumably based on material collected from the Philippines (Reeve, 1859). The specimen figured in Reeve (1859: pl. 5) shows the sculpture and outline of the shell that are similar enough to those of the present specimens from Okinawa, Taiwan and the Philippines. We therefore consider *S. crenulata* as the senior synonym of a few names based on Japanese specimens, including *Melania rufescens* Martens, 1860 from Japan (probably Nagasaki; Habe, 1976), *M. loebbeckii* Brot, 1874 from Yeddo (Tokyo) and Nagasaki, *M. dunkeri* Heimburg, 1884 from Higo (Kumamoto) and *M. yokohamensis* Hartman, 1897 from Yokohama. Some authors regard *Bulimus torulosus* Bruguière, 1789 from Madagascar as a senior synonym of *S. crenulata* and use the former species name (*S. torulosa*) for snails from western Pacific islands (Benthem-Jutting, 1956; Starmühlner, 1984; Ueng, 2011). However, the original description of *B. torulosus* was not accompanied by a figure of the type material (Bruguière, 1789). The succeeding authors apparently have not examined the type (missing in the collection of Muséum National d'Histoire Naturelle, Paris; V. Héros, personal communication; see also Tillier and Mordan, 1983) or topotypic specimens (but see Brot, 1874 for his opinion on the type locality of this species). Given the limited dispersal capability (see above), the occurrence of the same thiarid species is implausible in the Indian and Pacific islands, which are separated by 11,000 km, without anthropogenic introduction. Indeed, few amphidromous neritid species have such a wide-ranging distribution covering both Madagascar and Japan, even with the generally much higher levels of dispersal in that group (Fukumori and Kano, 2014). We thus tentatively conclude *S. crenulata* is the proper name for the study species. It is desirable that future studies include specimens from as many Asian countries as possible, for both molecular and morphological analyses, to establish the southern or western limits of the distribution area and to evaluate connectivity among populations in the entire species range. The taxonomic identity of the veliger larvae reported from India as *S. crenulata* (Seshaiya, 1940) cannot be ascertained, although their morphological and ecological characteristics are comparable with those of Okinawan specimens of the species (Hidaka, unpublished data).

The results of the present study demonstrate the importance of the local populations for management and conservation of *S. crenulata*. The 500-km apart Okinawan and mainland populations have been recognized as separate species and included in the Threatened I (CR+EN) and Threatened II (VU) categories of the Japanese Red List, respectively (Ministry of the Environment, 2012). While the independent species status of the former is not supported by our results, its historical independence as an intraspecific population warrants the high conservation status and efforts. The occurrence in Okinawa Island is, to our knowledge, restricted to two localities (see also Kubo, 2012). There is also a 900-km geographic gap and morphological discrepancies, hence plausible genetic differentiation, between the Okinawan population and the nearest known population to the south in Taiwan. Curiously, no population has been found in Yaeyama Islands where other thiarid snails occur in comparable freshwater environments. It is unclear whether the absence in Yaeyama and rarity in Okinawa Island are attributable to anthropogenic impact, but the local extinction of population due to habitat loss and degradation has indeed been documented for the present species in rivers feeding Tokyo Bay and many other areas in the mainland Japan (Kimura, 2009; Fukuda and Kimura, 2012).

In conclusion, the conservation of the amphidromous snail *S. crenulata* should involve the proper maintenance of migration pathways including all marine, brackish and freshwater environments (see Thuesen et al., 2011; Cook et al., 2012), particularly for the isolated Okinawan population. More effective management and conservation measures can be obtained from future ecological and behavioral studies on suitable environments for adult and juvenile snails as well as for larval metamorphosis and settlement.

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