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PHYLOGENY OF THE LAND SNAILS *BRADYBAENA* AND *PHAEOHELIX* (PULMONATA: BRADYBAENIDAE) IN JAPAN

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ABSTRACT

The Japanese Archipelago harbours high diversity of endemic bradybaenid land snails. However, there have been few systematic studies of these snails. The resolution of the taxonomy and phylogenetic relationships of these bradybaenid land snail taxa is important both for describing species diversity and for promoting the conservation of these land snails. We investigated the molecular phylogeny of *Bradybaena* and *Phaeohelix* using the CO1 and internal transcribed spacer genes, to clarify whether morphological traits and the current species taxonomy of these genera reflect their phylogenetic relationships. Our results show that the Japanese species in these genera are genetically divided into three clades, and the geographical distribution pattern of the lineages tends to reflect phylogenetic relationships. Although the nominal species taxonomy of these genera was not consistent with their molecular phylogenetic relationships, their shell and genital morphology reflected phylogenetic relationships to some extent. Inferred phylogeny and observed genital morphology showed that *Phaeohelix submandarina*, *P. miyakejimana* and *Bradybaena circulus oceanica* from Hachijo-kojima Island belong to *P. phaeogramma*. In addition, the distinction between *Bradybaena* and *Phaeohelix* was not supported by molecular phylogeny, showing instead that *Phaeohelix* should be synonymized with *Bradybaena*. This study suggests that a further taxonomic revision of Japanese Bradybaenidae is needed and, to address this issue, genital anatomy is useful in addition to molecular phylogenetic analyses.

INTRODUCTION

Recent progress in molecular phylogenetic studies has often revealed incongruence of existing taxonomy with molecular species delimitation and phylogenetic relationships. Addressing these issues is important for describing species diversity patterns and promoting the conservation of this diversity (Pimm *et al*., 1995; Whittaker *et al*., 2005; Sahney, Benton & Ferry, 2010; Davies & Buckley 2011; Deans, Yoder & Balhoff, 2012). Such incongruence is common among land snails, because their low mobility results in a high level of local adaptation and genetic divergence among populations (Chiba, 1999; Davison *et al*., 2005; Pfenninger *et al*., 2005; Schilthuizen *et al*., 2006). Further studies are needed to clarify how existing taxonomy and phenotypic differences among land snail taxa reflect their phylogenetic relationships.

East Asia is known as a hotspot for terrestrial molluscan diversity. Particularly high species diversity exists for Bradybaenidae within the Japanese Archipelago (Azuma, 1982; Minato, 1988). In Japan, 13 genera and 183 species of Bradybaenidae have been recorded (Minato, 1988). However, systematic studies of Japanese Bradybaenidae have been limited to a few genera (Hirano *et al*., 2014). Here we focus on two bradybaenid genera, *Bradybaena* and *Phaeohelix*, which comprise a number of morphologically similar species and subspecies in East Asia.

Three species of *Bradybaena* have been recorded in the Japanese Archipelago (Azuma, 1982; Minato, 1988). *Bradybaena similaris* (Férussac, 1821) is a foreign species that currently inhabits the whole archipelago, and its native locality is assumed to be South East Asia (Kuroda & Habe, 1949). *Bradybaena pellucida* Kuroda & Habe in Habe, 1953 is considered to be a Japanese endemic (type locality: Sata-misaki, Kyushu; Habe, 1953), but has recently spread throughout Japan as an alien species (Seki, Inoue & Asami, 2002; Nishi, 2013). These two species are distinguished by their mantle colour and the microsculpture pattern on the penial wall (Seki, Wiwegweaw & Asami, 2008), but they often produce hybrids. *Bradybaena circulus* inhabits the Ryukyu Islands and several oceanic islands and comprises three subspecies (Fig. 1). *Bradybaena circulus circulus* (Pfeiffer, 1846) is endemic to Okinawa Island and adjacent islands (Habe, 1956). *Bradybaena circulus oceanica* (Habe, 1962) is distributed in several oceanic islands, the Hachijo Islands and the Daito Islands (Habe, 1962; Azuma, 1982; Azuma & Azuma, 1994). *Bradybaena circulus hiroshihorii* (Kuroda, 1973) is distributed in the Danjo and Uji Islands (Kuroda, 1973; Tomiyama, 1984).

The genus *Phaeohelix*, which is endemic to Japan, was described as a subgenus of *Bradybaena* (Kuroda & Habe, 1949), but is currently considered a separate genus because of the presence of an accessory dart sac (Minato, 1978). *Phaeohelix*

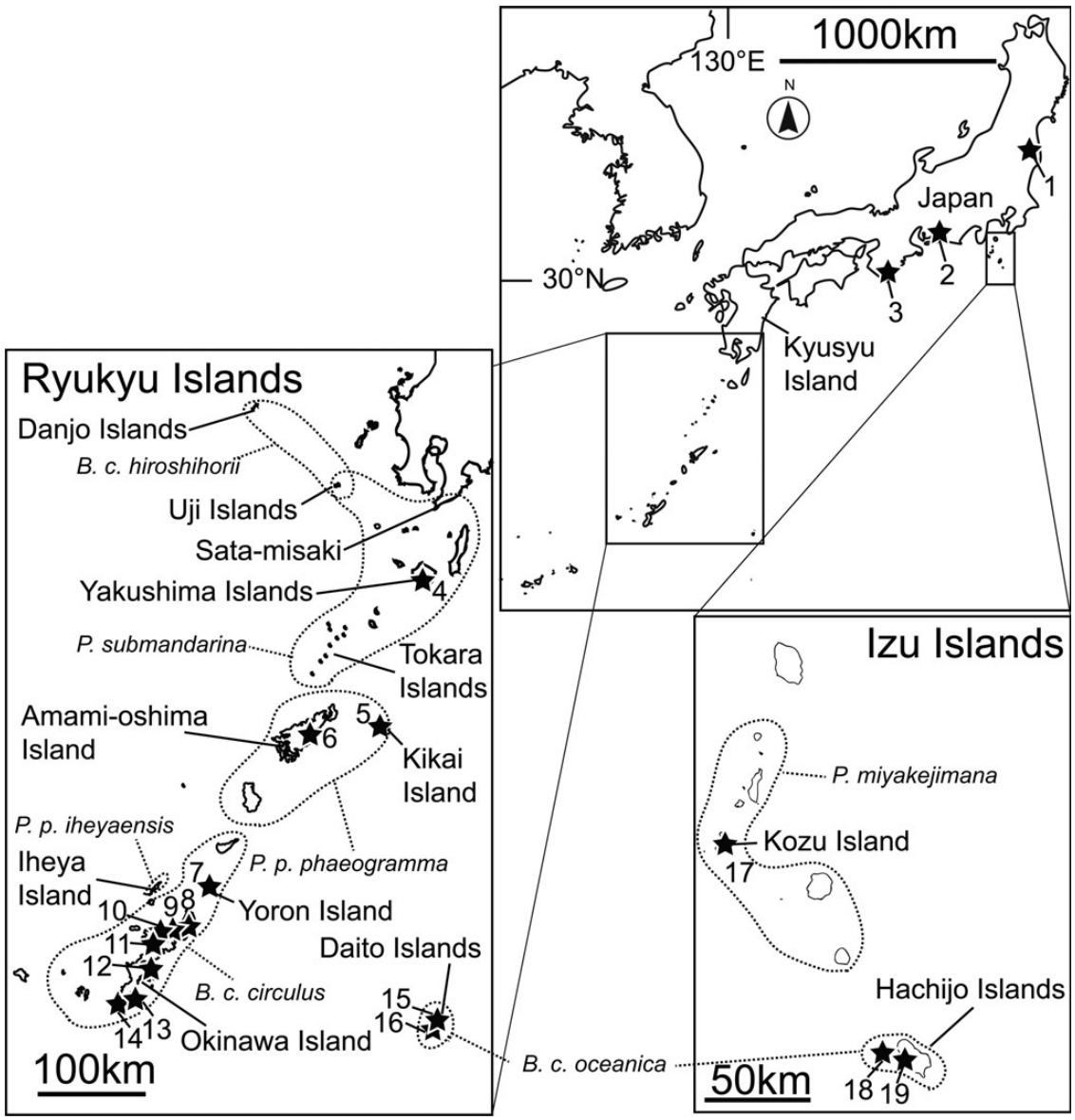

Figure 1. Map showing the sampling sites and distribution areas of *Bradybaena* and *Phaeohelix*. Sites 1–3 are in mainland Japan, 4–16 are in the Ryukyu Islands and 17–19 are in the Izu Islands. The numbers corresponds to the site numbers in Table 1. The dashed line indicates the distribution area of each species.

includes three species (Azuma, 1982; Minato, 1988). *Phaeohelix submandarina* (Pilsbry, 1890) is distributed from the Tokara Islands to the southernmost part of Kyushu (Kuroda, 1955; Fig. 1). *Phaeohelix miyakejimana* (Pilsbry & Hirase, 1903) inhabits the Izu Islands, with the exception of the Izu-oshima and Hachijo (Habe, 1977; Minato, 1978). *Phaeohelix phaeogramma* has two subspecies and inhabits Amami-oshima, the Tokunoshima and Kikai Islands (*P. p. phaeogramma* (Ancey, 1888)) and Iheya Island (*P. p. iheyaensis* (Pilsbry & Hirase, 1905)) (Habe, 1956; Fig. 1).

In this study, we investigate molecular phylogenetic relationships among the *Bradybaena* and *Phaeohelix* species in Japan. We examine how shell and anatomical characteristics reflect phylogenetic relationships and how evolutionary history has caused geographic patterns of morphological variations. In addition, we revise the taxonomy position of these species in the light of molecular phylogeny, and shell and anatomical characteristics.

MATERIAL AND METHODS

Samples

Sampling was performed on mainland Japan, the Izu Islands and the Ryukyu Archipelago. We collected 27 individuals in total, consisting four species/subspecies of *Bradybaena* and three species/subspecies of *Phaeohelix* (Fig. 1, Table 1). In the phylogenetic analysis we used *Acusta despecta sieboldtiana* as an outgroup, because *Acusta* is the closest sister group for *Bradybaena* and *Phaeohelix* (Hirano *et al.*, 2014). A fragment of the foot muscle from each individual was stored in 99.5% ethanol for DNA extraction, and the remaining soft tissue was stored in 70% ethanol after dissection and observation of the genital morphology.

Genetic analyses

Total DNA was extracted according to a method modified from Sokolov (2000). Muscle tissue was homogenized in 800 μl of

Table 1. Sampling information of *Bradybaena* and *Phaeohelix* specimens used in the present study.

Taxon	Site no.	Locality	CO1	ITS	Specimen ID
Bradybaena circulus circulus	12	Hamahigajima, Uruma, Okinawa	AB902854	AB902880	KC1760
	14	Hyakuna, Nanjo, Okinawa	AB902855	AB902881	KC1955
	8	Mt. Terukubi, Kunigami, Okinawa	AB902856	AB902882	KC1996
	7	Yoron Is., Kagoshima	AB902857	AB902883	KC2669
	9	Mt. Nekumachiji, Ogimi, Okinawa	AB902858	AB902884	KC3612
			AB902859	AB902885	KC9071
	11	Mt. Katsuu, Motobu, Okinawa	AB902860	AB902886	KC9130
B. c. oceanica	15	Kitadaito Is., Okinawa	AB902861	AB902887	KC3285
	16	Minamidaito Is., Okinawa	AB902862	AB902888	KC5124
			AB902863	AB902889	KC5126
	18	Hachijo-kojima Is, Tokyo	AB902864	AB902890	KC8154
			AB902865	AB902891	HC0390
B. pellucida	2	Inasa, Hamamatsu, Shizuoka	AB902866	AB902892	HCkhk
			AB902867	AB902893	HC1354
			AB902868	AB902894	HC1355
B. similaris	3	Nachikatsuura, Wakayama	AB902869	AB902895	KC4592
	1	Aoba, Sendai, Miyagi	AB852697	AB852967	KC8276
	19	Hachijo Is., Tokyo	AB902870	AB902896	HC0391
	10	Yohena, Nago, Okinawa	AB902871	AB902897	HC0888
	13	Itoman, Okinawa	AB902872	AB902898	HCitmn
Phaeohelix miyakejimana	17	Kozu Is., Tokyo	AB902873	AB902899	HC1388
			AB902874	AB902900	HC1389
			AB902875	AB902901	HC1390
P. phaeogramma phaeogramma	5	Kikai Is., Kagoshima	AB902876	AB902902	KC4276
			AB902877	AB902903	KC4277
	6	Amami-oshima Is., Kagoshima	AB902878	AB902904	HC1396
P. submandarina	4	Yakushima Is., Kagoshima	AB902879	AB902905	KC2714
Acusta despecta sieboldtiana			AB852623	AB852890	KC7906

The specimens are kept in the collections of the authors: HC, Hirano collection; KC, Kameda collection.

lysis buffer and incubated at 60°C for 1 h. Saturated KCl (80 μl) was added to the lysate, and the solution was incubated for 5 min on ice and then centrifuged for 10 min. The supernatant (700 μl) was transferred to a new tube, extracted once with phenol/chloroform solution, and precipitated with an equal volume of 2-propanol. DNA pellets were rinsed with 70% ethanol, vacuum-dried, and dissolved in 50 μl Tris-EDTA buffer.

To estimate the phylogenetic relationships among the collected snails, we sequenced fragments of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene and nuclear ribosomal internal transcribed spacer (ITS) regions 1 and 2. The primers used in PCR were LCO1490 (5′-GGTCAACAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATC-3′) (Folmer *et al*., 1994) for CO1, and 18d (5′-CACACCGCCCGTCGCTACTACCGATTG-3′) (Hillis & Dixon, 1991) and Its-4 (5′-TCCTCCGCTTATTGATATGC-3′) (Innis *et al*., 1990) for ITS. The PCR amplification was conducted under the following reaction conditions: an initial denaturation for 2 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 40°C (CO1) or 50°C (ITS), and 1.5 min at 72°C with a final extension for 2 min at 72°C. The PCR products were purified using Exo-SAP-IT (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and sequenced using the PCR primers and two internal primers, cl5.8r (5′-GCGTTCAAGATGTCGATGTTCAATG-3′) (Kameda, Kawakita & Kato, 2007) and lsITS2f (5′-GGTGGATCACTCGGCTCGTGCG-3′) (Kameda, Kawakita & Kato, unpubl.). Sequencing was performed using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and electrophoresed using an ABI 3130xl sequencer (Applied Biosystems). The resulting CO1 and ITS sequences have been deposited in the DDBJ/EMBL/GenBank databases (Table 1).

Phylogenetic analysis

The CO1 sequence alignment was straightforward and required no gaps. The ITS sequences were aligned using MUSCLE v. 3.8 (Edgar, 2004) to eliminate the uncertainty in the alignments in this region. GBLOCKS v. 0.91b (Castresana, 2000) was used to select regions in the aligned sequences that were confidently aligned for analysis (Table 2, Supplementary material, Appendix 1). Phylogenetic trees were obtained using maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods, and MP analyses were performed with MEGA5 (Tamura *et al*., 2011). An MP tree was obtained using the subtree-pruning-regrafting algorithm with search level 1 in which initial trees were obtained with the random addition of sequences (10 replicates). Prior to the ML and Bayesian analyses, we used the Kakusan4-4.0.2011.05.28 program (Tanabe, 2011) to select the appropriate models for sequence evolution (Table 2). Based on the selected models, ML analysis was performed with 500 iterations of the likelihood ratchet (Nixon, 1999; Vos, 2003) using TreeFinder (Jobb, 2008) and Phylogears2 v. 2.2.2012.02.13 (Tanabe, 2012). Nodal support for the MP and ML analyses was assessed using bootstrap analyses with 1,000 replications. The Bayesian analysis was performed using MrBayes v. 3.1.2 (Ronquist & Huelsenbeck,

Table 2. Information on sequence alignments and models of sequence evolution for maximum likelihood and Bayesian analysis.

Alignment	Length of alignment	Excluded sites	Model of sequence evolution
CO1 position1	178	–	J2_Gamma
CO1 position2	178	–	J3_Gamma
CO1 position3	177	–	GTR_Gamma
ITS	1,257	32–35, 90, 146, 301–303, 318–323, 362, 445–450, 511, 516–517, 522, 550–551, 558, 572–573, 811–812, 863–865, 986, 1,041–1,046, 1,088–1,089, 1,186–1,191, 1,216, 1,231–1,232, 1,241–1,242	TVM_Gamma

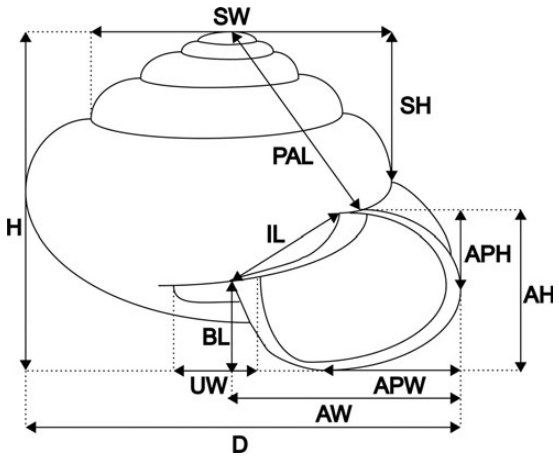

Figure 2. Shell measurements used in the morphological analysis. Abbreviations: H, hell height; D, shell diameter; AH, aperture height; AW, aperture width; BL, height of basal lip; IL, length of inner lip; AL, aperture length; APH, height from uppermost part of aperture to periphery; ABW, width from lowermost part of aperture to periphery; SH, spire height; SW, spire width of spire; UW, umbilicus width; PAL, length from uppermost part of aperture to protoconch (PAL).

2003). The analysis was performed using two simultaneous runs, which consisted of running four simultaneous chains for 5 million generations and sampling the trees every 1,000 generations. We discarded the first 501 trees as burn-in, and the remaining samples were used to estimate the tree topology, branch length and substitution parameters.

Morphological analysis

To investigate the interspecific variation in genital traits, we examined genital anatomy using a stereomicroscope. We used only fully mature shells that had a strongly thickened outer lip. The male genital system in this group consists of the following parts: penis, epiphallus, vas deference, dart, and dart-related organs such as the dart sac, accessory dart sac and mucus gland. For each snail, the presence/absence of the accessory dart sac was recorded; the other traits were not recorded because they were always present. Structures were not measured because lengths of genital organs can be highly variable even within species, perhaps due to sexual selection and/or interspecific interaction (Kameda, Kawakita & Kato, 2009). Furthermore, relative size of the genital characters has not previously been used for species identification in *Bradybaena* and *Phaeohelix*. Instead, we opened the penial wall longitudinally and examined the internal microsculpture of the penial chamber, which is considered to be important for species recognition (Gomez, 2001; Seki, Wiwegweaw & Asami, 2008).

For the shell morphological analysis, the following shell characteristics were measured from digital images (Fig. 2): shell height (H), shell diameter (D), aperture height (AH), aperture width (AW), height of basal lip (BL), length of inner lip (IL), height from the uppermost part of the aperture to the periphery (APH), width from the lowermost part of the aperture to the periphery (ABW), height of spire (SH), width of spire (SW), umbilicus width (UW) and length from the uppermost part of the aperture to the protoconch (PAL). We used only adult shells that have strongly thickened outer lips; shells with an incomplete aperture or apex were excluded from the analysis. In total, 58 individuals were available for morphometric analysis (Supplementary Material, Appendix 2). Based on these measurements, we conducted a canonical discriminant analysis (CDA) and compared CDA scores for each clade. The CDAs were conducted with XLSTAT (Addinsoft). In the canonical discriminant analyses the three characteristics that had larger absolute values of standardized scoring coefficients were treated as the major variables.

RESULTS

Phylogenetic relationships

MP analysis of the combined data resulted in a single most parsimonious tree [1,022 steps, consistency index excluding uninformative characteristics (CI) = 0.575045, retention index = 0.837079]. The results of ML and Bayesian analyses were largely consistent with the relatively well-supported clades. Only posterior probabilities $\geq$0.95 were considered statistically significant, while bootstrap support values larger than 70% were considered highly supported. Hence, support values below this significance level are not discussed below.

The inferred phylogeny demonstrated that the species examined were separated into three major clades (Fig. 3). *Bradybaena circulus circulus* and *B. c. oceanica* of the Daito Islands belonged to clade A. Clade B comprised *B. c. oceanica* of Hachijo-kojima Island and the genus *Phaeohelix*, and *Phaeohelix* was paraphyletic to *B. c. oceanica*. *Bradybaena similaris* and *B. pellucida* formed clade C. The molecular phylogenetic analysis revealed that *B. c. oceanica* was not monophyletic but consisted of two different lineages. In addition, *B. c. oceanica* of Kitadaito Island and of Minamidaito Island were not monophyletic.

Morphological variations

To investigate variations in shell morphology among the three clades identified in the molecular analysis, CDA was performed based on seven shell measurements (Wilks's lambda; value = 0.0861854, $F = 8.8231$, $P < 0.001$; Fig. 4, Supplementary material, Appendix 3). The first and second canonical components explained 76.46 and 23.54% of the total variance, respectively, which accounted for a total of 100% (Appendix 3). A higher score on CAN1 indicates a flatter shell and smaller aperture (positive D, negative H and AW), whereas a higher score on CAN2

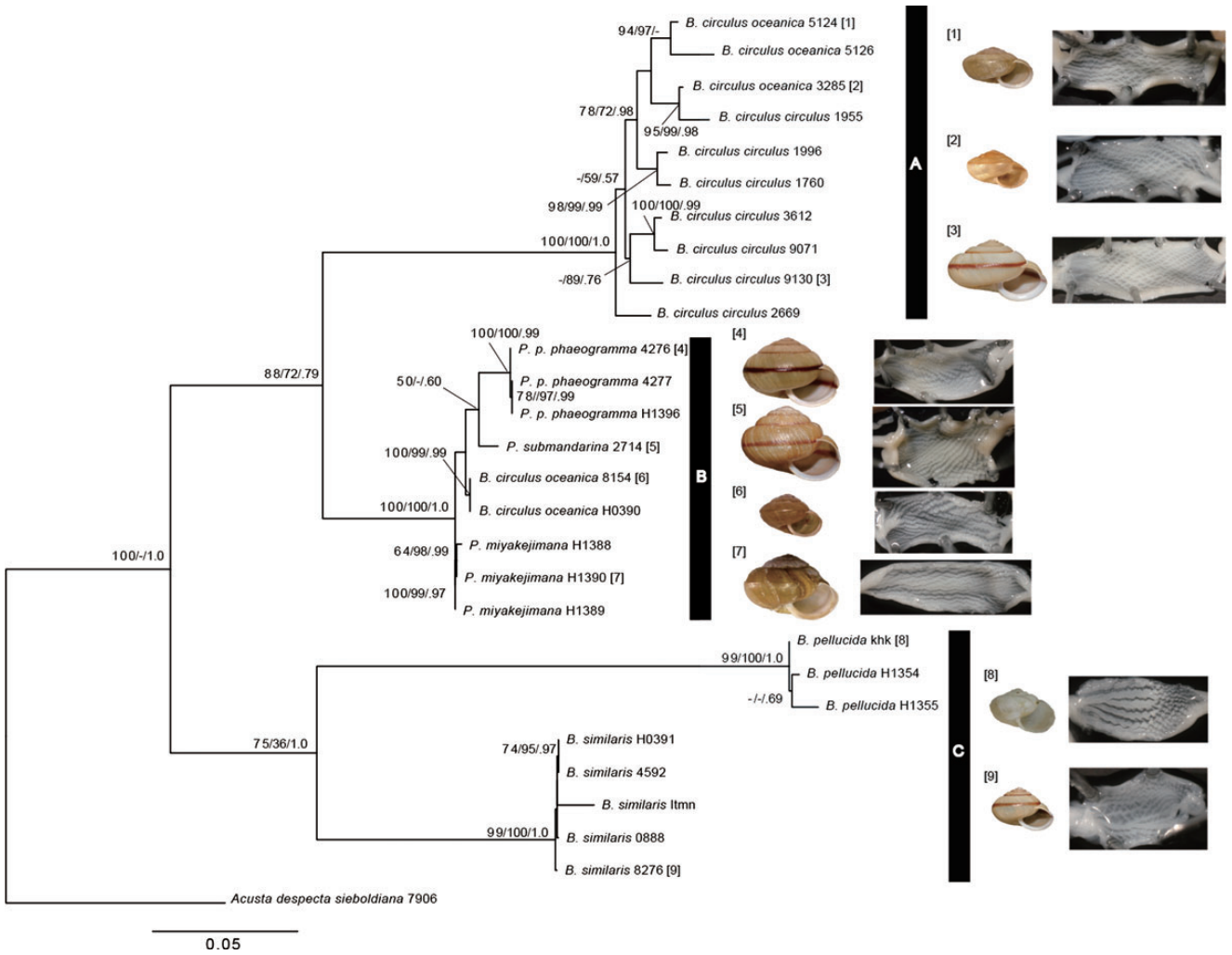

Figure 3. Maximum likelihood tree of specimens of *Bradybaena* and *Phaeohelix* based on combined sequences from the internal transcribed spacer and CO1 genes (−ln likelihood = −9260.9853). Each OTU label represents a species/subspecies name followed by the specimen ID. Numbers in brackets next to taxon labels correspond with each shell and penial wall image. Bars to right of tree indicate each clade. Numbers on branches indicate maximum likelihood and maximum parsimony bootstrap values followed by Bayesian posterior probabilities. Scale bar = 0.05 substitutions per site.

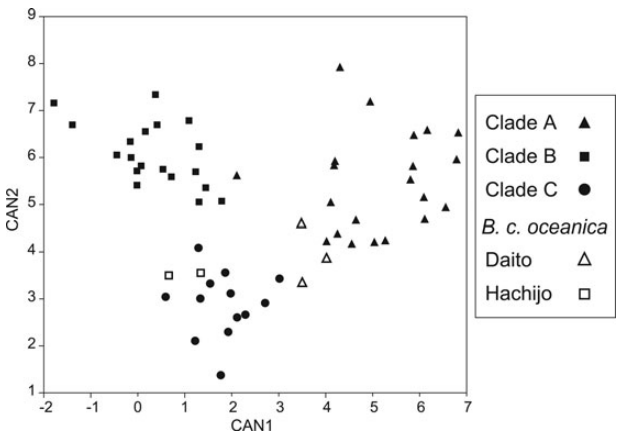

Figure 4. Plots of results from canonical discriminant analysis based on measurements of the shells of *Bradybaena* and *Phaeohelix* specimens. The symbols to the right of the figure indicate each clade (see Fig. 3).

indicates a smaller body whorl and aperture (positive SW, negative BL and APH). The shells of clade A are flatter than those of clade B; the shells of clade C are characterized by a larger body whorl and aperture (Fig. 4). The CDA results also showed that the shell shape of *B. c. oceanica* from clades A and B resembles that of clade C, although the plot ranges do not always overlap.

The genital morphology, particularly the internal microsculpture of the penial wall, was clearly different among clades (Fig. 3). In clade A, all individuals had a grid-like microsculpture that was entirely on the penial wall. In clade B, all individuals had pleated diagonal wrinkles on the penial wall. Some of these wrinkles bore partial zigzag crenulations. Within clades A and B, penial internal sculpture was mostly identical among species. In contrast, the microsculpture patterns were different among the species in clade C, as reported in a previous study (Seki, Wiwegweaw & Asami, 2008). The penial inner sculpture of the species in clade C was consistent with the findings of a previous study (Seki, Wiwegweaw & Asami, 2008) and different from those in the other two clades. In addition, the presence/absence of an accessory dart sac reflected the molecular

phylogeny. All individuals in clade B had an accessory dart sac, whereas clades A and C had no accessory dart sac.

DISCUSSION

Phylogenetic analyses demonstrated that the studied bradybaenid species in the Ryukyu and Izu Islands are grouped into two clades. Clade A is distributed in the Daito Islands and the central part of the Ryukyu Islands (Okinawa and adjacent islands), while clade B consists of populations from the Izu Islands and the northern part of the Ryukyu Islands (Kikai and Yakushima Islands; Figs 1 and 3). The phylogeographic patterns suggest that divergence between clades A and B occurred within the Ryukyu Islands, and that each population subsequently migrated from the continental islands (Ryukyu Islands) to the oceanic islands (Daito Islands and Izu Islands for clades A and B, respectively).

Divergence between clades A and B may reflect the geographical history of the Ryukyu Islands. Species with relatively poor dispersal ability such as amphibians show divergence within the Ryukyu Islands; thus large genetic differences have been identified between the species on the Amami Islands and those on the Okinawa Islands (Kameda, Kawakita & Kato, 2007; Kuramoto *et al*., 2011), reflecting the history of separation of these islands. The phylogeographical patterns observed in this study are consistent with these patterns, suggesting that the same episode of geographical isolation caused the divergence between clades A and B.

Dispersal from the Ryukyu Islands to the Daito and Izu Islands is likely to have occurred as a result of transportation by the oceanic current that flows from West to East along the Japanese Islands. The individuals of *B. c. oceanica* from Kitadaito and Minamidaito Islands were not monophyletic, suggesting that colonization from Okinawa Island to the Daito Islands has occurred at least twice, despite the long distance from Okinawa Island (*c.* 400 km; Fig. 1). These data suggest that populations within geographically close oceanic islands are not necessary derived from a single ancestor that reached the island, but could be derived from multiple colonization events from the same remote source (Parent & Crespi, 2006; Cowie & Holland, 2008). The possibility of dispersal between the two oceanic islands could not be assessed because of small sample size, so it remains unclear whether the entire populations of the Minamidaito and Kitadaito Islands have an independent origin. Further study is necessary to reveal the history of colonization into and within the Daito Islands.

With the exception of the southernmost part of Kyushu, clade B does not inhabit the mainland of Japan (Habe, 1953), suggesting that populations in the Izu Islands (*P. miyakejimana*) resulted from long-distance dispersal over 2,000 km from the northern Ryukyu Islands. Such long-distance dispersal to the Izu Islands is likely fairly common even in species with low mobility, because examples of a similar distribution of the same land snail genus or species in the northern Ryukyu and Izu Islands have been reported in *Yakuena* and *Trishoplita* (Habe, 1977). However, it is not clear whether *P. miyakejimana* of the north Izu Islands and *B. c. oceanica* of the south Izu Islands originated from multiple colonization from the Ryukyu Islands or a single migration and subsequent diversification within the Izu Islands, because support values among the island populations were low. Further phylogeographic studies are necessary to reveal the detailed dispersal history of this group.

Morphometric analysis demonstrated that each clade has a different trend in shell morphology (Fig. 4; Supplementary material, Appendix 3). However, *B. c. oceanica* in clades A and B has unusually small shells and resembles clade C. The convergence of shell shape between two *B. circlus oceanica* lineages could imply adaptation to the environments of oceanic islands. However, other oceanic island populations, such as *P. miyakejiman* from Kozu Island, have a large shell that is unlike *B. c. oceanica* in Hachijyo-kojima Island, despite the fact that these populations are phylogenetically close. Thus, environmental differences between continental and oceanic islands may not in fact be a major cause of the divergence in shell morphology for these land snails. Although causes of the geographical patterns of shell morphologies detected in this study are unclear, shifts in shell morphology have likely occurred as a result of a local adaptation to the environment of each island.

In contrast with shell shape, genital morphology is relatively conservative and distinctive among the clades of *Bradybaena* and *Phaeohelix* (Fig. 3). Among the studied species, four types can be recognized based on the microsculpture on the internal penial wall and the presence/absence of an accessory dart sac. In the original description, the accessory dart sac was not indicated for *B. c. oceanica* from the Izu Islands (Habe, 1962). However, based on our observation, an accessory dart sac does in fact exist in *B. c. oceanica* from the Izu Islands. Therefore, the presence of an accessory dart sac characterizes clade B. The morphology of the penis and microsculpture on the internal penial wall can function in mechanical isolation during cross-species mating (Seki, Wiwegweaw & Asami, 2008) and, in some cases, these traits show a clear difference among different biological species (Seki, Wiwegweaw & Asami, 2008). The function of the accessory dart sac is unknown, but it most likely reflects sexual selection. Although the degree of reproductive isolation between the populations in clades A and B is unclear, the above findings suggest that the two clades likely represent separate biological species. In contrast, nominal species within clades A and B should be considered as conspecific, because they cannot be discriminated based on genital anatomy, including the internal sculpture of the penial chamber. Accordingly, clades A and B can be identified as *B. circulus* and *P. phaeogramma*, respectively.

This study also raises issues of the validity of the genus *Phaeohelix*. Based on phylogenetic and morphological evidence, *Phaeohelix* is not a separate genus but should be synonymized with *Bradybaena*. This study suggests that a further taxonomic revision of Japanese Bradybaenidae is needed and shows that, to address this issue, genital anatomy is useful in addition to molecular phylogenetic analyses.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *Journal of Molluscan Studies*.

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