Genetic divergence of the genus *Pomacea* (Gastropoda: Ampullariidae) distributed in Japan, and a simple molecular method to distinguish *P. canaliculata* and *P. insularum*

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Abstract

Snails of the genus *Pomacea* in several regions of Japan showed genetic divergence. Phylogenetic analysis based on the mitochondrial cytochrome *c* oxidase subunit I gene segregated them into two major clades, one corresponding to *P. insularum*, the other to *P. canaliculata*. We developed a simple molecular method to distinguish between the two species to investigate their distribution in Japan. We identified *P. canaliculata* at all 16 sampling locations except Iriomote Isle, and *P. insularum* at four locations (Iwata, Fukuyama, Ishigaki Isle, and Iriomote Isle), supporting a previous report that *P. canaliculata* is the dominant species in Japan. Morphological comparison of the two species collected from the same habitat (Ishigaki Isle) revealed that most *P. canaliculata* had clear dark spiral bands on their shells, and that *P. insularum* had no or only faint spiral bands. However, it is difficult to distinguish the two species using only shell morphology.

Key words: *Pomacea canaliculata*; *Pomacea insularum*; invasive species; mitochondrial DNA; distribution

INTRODUCTION

The genus *Pomacea* belongs to the freshwater gastropod family Ampullariidae. In the 1980s, *Pomacea* spp. were introduced into East and Southeast Asia from South America as food (Halwart, 1994), but they are now regarded as invasive alien species (Carlsson et al., 2004), and have become serious pests of rice (Halwart, 1994; Wada, 1999, 2004; Cowie, 2002). Snails referred to as *P. canaliculata* (Lamarck) were introduced into Japan in 1981, where they are now an important constraint to the spread of low-cost direct seeding of rice (Wada, 2004).

The taxonomy of the genus *Pomacea* is based on shell, egg mass, and soft tissue morphology (Thiengo et al., 1993; Cowie et al., 2006); however, no clear criteria to distinguish among species have been established because of intraspecific variation and probable environmental influences on the variation (e.g. Estebenet and Martin, 2003). These un-

clear morphological criteria have confused taxonomy and identification within the genus.

The snails in Japan were considered to be *P. insularum* until the early 1980s. Habe (1986), however, concluded that they were *P. canaliculata* according to his observations of shell morphology: the shell of *P. canaliculata* is usually olive colored with clear spiral bands, whereas that of *P. insularum* is usually yellow-brown colored with pale spiral bands, and often grows larger.

Recently, Rawlings et al. (2007) reported that the nucleotide sequences of the mitochondrial *12S*, *16S*, and *COI* (cytochrome *c* oxidase subunit I) regions are more reliable for classification of *Pomacea* species. On this basis, we tried to re-identify the *Pomacea* species distributed in several regions of Japan.

MATERIALS AND METHODS

Snail collection. Snails with shells higher than

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2 cm were used for this study because it was easier to isolate the foot muscle of larger snails in DNA extraction. We collected snails at 18 sites in 10 prefectures in central and southern Japan from April to October 2007 (Table 1, Fig. 1). The snails were either used immediately or kept alive in plastic containers until use.

DNA extraction, PCR amplification, and sequencing. Total genomic DNA was extracted with

Table 1. Collection sites and sample ID of *Pomacea* species in this study

Sample ID ^a	Locality	Habitat
H1	Kitaura, Ibaraki	water pathway
H2	Iwata, Shizuoka	water pathway
H3	Nara, Nara	paddy field
H4	Kakogawa, Hyogo	paddy field
H5	Inami, Hyogo	paddy field
H6	Yurihama, Tottori	water pathway
H7	Fukuyama, Hiroshima	water pathway
S1	Matsuyama, Ehime	paddy field
K1	Kamimine, Saga	water pathway
K2	Kikuchi, Kumamoto	water pathway
R1	Iheya Isle, Okinawa	paddy field
R2	Nago, Okinawa	paddy field
R3	Kin, Okinawa	paddy field
R4	Ishigaki Isle, Okinawa	water pathway
R5	Ishigaki Isle, Okinawa	pond
R6	Ishigaki Isle, Okinawa	paddy field
R7	Iriomote Isle, Okinawa	pond
R8	Iriomote Isle, Okinawa	paddy field

^a First letter of each ID indicates the collection district (H: Honshu Isle, S: Shikoku Isle, K: Kyushu Isle, R: Ryukyu archipelago).

Fig. 1. Geographical locations of *Pomacea* spp. collection sites. Letters in the figure indicate sample ID (see Table 1).

a Nucleon Phytopure DNA extraction kit (GE Healthcare UK Ltd., UK) using phenol-chloroform extraction. Soft tissue was removed from the shell, and approximately 150 mg of foot muscle was dissected out. The muscle was crushed in a Multi-Beads Shocker (Yasui Kikai, Japan) at approximately $710\times g$ for 1 min in 600 μL of supplied Reagent I and 200 μL of Reagent II. After centrifugation at $2,000\times g$ for 5 min, the supernatant (approximately 500 μL) was mixed with 250 μL of chloroform and 250 μL of TE-saturated phenol. After centrifugation at approximately $3,400\times g$ for 1 min, the aqueous (upper) phase was transferred to a new tube, and mixed with 100 μL of chloroform and 100 μL of Nucleon Phytopure DNA extraction resin suspension. After centrifugation at approximately $4,300\times g$ for 2 min, the supernatant was transferred to a new tube, mixed with 300 μL of isopropanol, and centrifuged at approximately $4,300\times g$ for 10 min. The DNA pellet was rinsed with 80% ethanol. Finally, the pellet was suspended in 50 μL of $0.1\times \text{TE}$ (1 mM Tris-HCl [pH 8.0] + 0.1 mM EDTA).

PCR was conducted with Takara ExTaq DNA polymerase (Takara, Shiga, Japan), the forward primer LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'), and the reverse primer HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al., 1994; Thaewnonngiw et al., 2004; Rawlings et al., 2007). The amplified region was the mitochondrial *COI* gene segment (658 bp), which was part of the sequence analyzed by Rawlings et al. (2007). The PCR conditions were 94°C for 2 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and a final extension of 72°C for 5 min. PCR products were purified with a Montage PCR centrifugal filter (Millipore Corp., Bedford, MA, USA) and suspended in 30 μL of $0.1\times \text{TE}$ (1 mM Tris, 0.1 mM EDTA, pH 8.0).

The PCR products of 75 snails were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100 sequencer (Applied Biosystems, USA).

Phylogenetic analysis. Phylogenetic analysis was conducted with CLUSTAL X (Thompson et al., 1997). After alignment of the *COI* sequences, a phylogenetic tree with bootstrap values (1,000 replications) was constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987).

Simple molecular method to distinguish between *P. canaliculata* and *P. insularum*. To reduce costs, we created a simple molecular method to distinguish *P. insularum* from *P. canaliculata* (see Results) using species-specific PCR primers. Two forward primers, PcanCOI (3'-TGG GGT ATG ATC AGG CC-5') and PinsCOI (3'-ATC TGC TGC TGT TGA AAG C-5'), were constructed as a multiplex primer from the nucleotide sequences of the *COI* gene of *P. canaliculata* and *P. insularum*, respectively. The reverse primer HCO2198 was common to both species. Thermal cycling conditions were 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. The sizes of the PCR products were analyzed by agarose gel electrophoresis.

Morphological observations. We compared morphological characteristics between the two species. Discrimination between *P. canaliculata* and *P. insularum* is difficult (Cowie et al., 2006; Rawlings et al., 2007) owing to the large variation within each species (Estebenet and Martin, 2003; Cowie et al., 2006). We compared shell morphology according to the criteria described by Habe (1986). To eliminate environmental effects on shell morphology, we compared snails collected from Ishigaki Isle (see Results and Table 2). Snails that spawned eggs were identified as female and were used for this comparison. We observed shell color

and the form of the spiral bands by eye.

RESULTS

Phylogenetic analysis of the genus *Pomacea* distributing in Japan

PCR with primers LCO1490 and HCO2198 amplified a 708 bp fragment from all tested snails. The snails were divided into three well-supported clades with three to five unique mitochondrial DNA sequences (Fig. 2). These clades included the same or similar sequences as the sequences of *P. canaliculata* and *P. insularum* reported by Rawlings et al. (2007) (Fig. 2). *P. canaliculata* Group A consisted of snails from H1, H3, H4, S1, K1, K2, R2, and *P. canaliculata* from Argentina and the USA. *P. canaliculata* Group B consisted of H2, H3, H6, S1, H7, K2, R1, R4, and R5. Although none of the sequences was reported, the group seemed to be *P. canaliculata* because this group had higher homology with *P. canaliculata* than any other species reported by Rawlings et al. (2007). The other clade consisted of snails from H2, H7, R4, R5, R7, R8, and *P. insularum* from Argentina, Brazil, and the USA. Seventeen of 658 bp were commonly replaced between *P. canaliculata* Group A and *P. canaliculata* Group B (Fig. 3). Fifty-two and 49 of 658 bp in the sequence of *P. insularum* commonly differed from those of *P. canaliculata*

Fig. 2. Phylogenetic relationships among *Pomacea* snails in Japan, and their relation to reported *Pomacea* sequences (in underlined gothic letters, reported by Rawlings et al., 2007). Snails collected from each site are shown as sample ID (see Table 1). Numbers in parentheses following sample ID indicate number of samples belonging to each clade. NJ method was used to construct the phylogenetic tree from mitochondrial COI sequence (658 bp). Numbers listed above branches indicate bootstrap value (1,000 replications).

Fig. 3. Representative nucleotide sequences of mitochondrial COI segment (658 bp) from *Pomacea* species in Japan. Shaded and boxed sequences indicate common and species-specific primers, respectively. The specific name and group (see Fig. 2) are shown on the left side of each sequence.

Group A and *P. canaliculata* Group B, respectively (Fig. 3).

Discrimination between *P. canaliculata* and *P. insularum* by the simple molecular method

Comparison of the nucleotide sequences between *P. canaliculata* and *P. insularum* suggested that nucleotide differences in the COI region were valid differentiators (Fig. 3). Multiplex PCR with primers PcanCOI, PinsCOI, and HCO2198 amplified a longer segment (666 bp) from snails in the *P. canaliculata* clade, and a shorter segment (390 bp) from snails in the *P. insularum* clade (Fig. 4). The two fragments were not simultaneously detected in any snail.

Using this simple molecular method, we deter-

Fig. 4. Difference in the length of amplified segments between *P. canaliculata* (solid arrow) and *P. insularum* (open arrow). The fragments amplified by the simple molecular method (see text) were 666 bp and 390 bp in length in *P. canaliculata*, and *P. insularum*, respectively.

Fig. 5. Typical shells of *P. canaliculata* (upper) and *P. insularum* (lower) from Ishigaki Isle. Scale bar: 5 cm.

Table 2. Numbers of snails identified as *P. canaliculata* and *P. insularum* based on a simple molecular method

Sample ID	Number of snails (accession nos)	
	<i>P. canaliculata</i>	<i>P. insularum</i>
H1	16 (AB433757)	0
H2	9 (AB433758)	2 (AB433776)
H3	22 (AB433759-60)	0
H4	8 (AB433761)	0
H5	8	0
H6	16 (AB433762-63)	0
H7	7 (AB433764)	4 (AB433777)
S1	15 (AB433765-66)	0
K1	17 (AB433767)	0
K2	21 (AB433768-71)	0
R1	6 (AB433772)	0
R2	10 (AB433773)	0
R3	9	0
R4	3 (AB433774)	5 (AB433778)
R5	4 (AB433775)	4 (AB433779)
R6	1	6
R7	0	10 (AB433780)
R8	0	4 (AB433781)

mined which *Pomacea* species inhabits each collection site. We found *P. canaliculata* at all sampling sites except Iriomote Isle (R7, R8) (Table 2). On the other hand, we found *P. insularum* in only four locations: Iwata (H2), Fukuyama (H7), Ishigaki Isle (R4, R5, and R6), and Iriomote Isle (R7, R8).

Morphological comparisons

Typical shells of both species collected on Ishigaki Isle are shown in Fig. 5. No differences in shell color were apparent. In addition, differences in the form of spiral bands were not clear, although most *P. canaliculata* (7/9) had clear dark spiral bands on their shells, and all ten *P. insularum* seemed to have no or only faint spiral bands.

DISCUSSION

Our results show that both *P. insularum* and *P. canaliculata* are distributed in Japan. Phylogenetic analysis divided the snails into three major clades, two in *P. canaliculata* and one in *P. insularum* (Fig. 2). This indicates that *P. insularum* was also introduced into Japan, probably in the 1980s, and has survived in several regions. Although *P. insularum* is also present in Japan, *P. canaliculata* still seems to be the major species in most parts. We found *P. insularum* in only four locations (Table 2) but it could be found in other regions of Japan.

The form of the spiral band on the shell differed between *P. canaliculata* and *P. insularum* to some extent (Fig. 5); however, this cannot be a definitive character to distinguish the two species because it is often ambiguous. Thus, at present, the simple molecular method (Fig. 4) is a rapid effective method for the classification of *P. canaliculata* and *P. insularum*.

Recently, Hays et al. (2008) investigated genetic diversity in the genus *Pomacea* introduced into

Southeast and East Asia. According to their work, 4 species of *Pomacea* were introduced: *P. canaliculata*, *P. insularum*, *P. scalaris*, and *P. diffusa*. They reported that *P. canaliculata* is distributed in Malaysia, China, Indonesia, Laos, Myanmar, Philippines, Thailand, Vietnam, South Korea, Taiwan and Japan, while *P. insularum* is distributed in Malaysia, Cambodia, Singapore, Thailand, Vietnam, South Korea and Taiwan. Our study revealed a new distribution range of *P. insularum* in East Asia. In addition, Hays et al. (2008) suggested that *P. canaliculata* and *P. insularum* introduced into Asia have multiple origins. Our results that *P. canaliculata* consisted of two groups (Fig. 2) may mean that *P. canaliculata* was introduced into Japan from multiple origins.

The possibility of hybridization between *P. canaliculata* and *P. insularum* is of interest. We found *P. canaliculata* with pale spiral bands, a characteristic of *P. insularum*. In addition, we found both species at some sites (Table 2), suggesting the possibility of hybridization. Further approaches, such as experimental crossing and comparison of nuclear DNA sequences, are needed to answer this question.

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