Phenotypic divergence in viviparid snails in a recently converted freshwater lagoon

OSAMU KAGAWA^{1,*}, TAKUMI SAITO², SHOTA UCHIDA¹ & SATOSHI CHIBA^{1,3}

¹ Graduate School of Life Sciences, Tohoku University, 2–2–2 Katahira, Aoba, Sendai, Miyagi 980–8576, Japan

² Department of Biology, Faculty of Science, Toho University, 2–2–1 Miyama, Funabashi, Chiba 274–8510, Japan

³ Center for Northeast Asian Studies, Tohoku University, 41 Kawauchi, Aoba, Sendai, Miyagi 980–8576, Japan

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Abstract: Freshwater mollusks often show high intraspecific variability in shell morphology, which may cause incongruences between molecular phylogeny and morphology-based species taxonomy. However, it remains unclear how morphological variations in freshwater snails are associated with different habitats such as rivers, ponds, and lakes. In the present study, we investigated the morphological variation in the shells of the viviparid freshwater snail *Sinotaia quadrata* in the Kahoku Lagoon in Japan, which was converted from a brackish to a freshwater lagoon 60 years ago. This newly established freshwater habitat is particularly well-suited for studies on how morphology is associated with environments. We found clear differences in shell morphologies between the *S. quadrata* populations sampled inside and outside of the lagoon. The *S. quadrata* sampled in the lagoon had thicker and more elongated shells with wider apertures than those sampled outside of the lagoon. No relationship was found between shell morphology and the distance between sampling sites. This suggests that the divergence in shell morphology occurred over a fairly short time period by rapid adaptation or phenotypic plasticity. Although it is unclear whether these differences are caused by phenotypic plasticity or adaptation, the present findings suggest that the shell morphologies of freshwater snails easily change under the influence of the environment. The high flexibility in shell morphology should be carefully considered when managing or studying freshwater mollusks.

Key words: freshwater mollusks, shell morphology, *Sinotaia quadrata*, Viviparidae

Introduction

How taxonomists identify and discriminate species affects the estimations of biodiversity and the development of ecosystem conservation programs (Mace 2004, Lenat & Resh 2006, Garnett & Christidis 2017). DNA-barcoding and environmental DNA techniques are increasingly used to determine species composition in communities; however, the taxonomy of most eukaryotes remains primarily based on morphological characteristics (Deiner et al. 2017). On the other hand, many species show incongruences between molecular phylogeny and morphology-based taxonomy (Patterson et al. 1993, Irestedt et al. 2004, Havermans et al. 2010, Covain et al. 2016, San et al. 2018). In particular, freshwater snails often show re-

markable incongruences between molecular phylogeny and morphology-based taxonomy (Köhler & Glaubrecht 2006, Pfenninger et al. 2006, Lee et al. 2007, Du et al. 2013, Hirano et al. 2015, Becker et al. 2016, Köhler 2016, Saito et al. 2018a). This sometimes results in problems when estimating species diversity for conservation programs for freshwater mollusks (Saito et al. 2018b). Potential causes of these incongruences include introgressive hybridization, ancient polymorphisms, the presence of cryptic species, rapid evolution, and highly plastic morphological traits (Glaubrecht & Köhler 2004, Lee et al. 2007, Miura et al. 2013, Köhler 2016, Hirano et al. 2019). These phenomena have been suggested by phylogenetic studies; however, many of these studies have not clarified the mechanisms underlying these incongruences. In particular, environmental pressures influence the selection of genotypes that underlie the expression of suitable phenotypic traits (Pres-ton & Roberts 2007), as well as the expression of phe-

*Corresponding author: Osamu Kagawa; E-mail, ok.osamukagawa@gmail.com

Fig. 1. *Sinotia quadrata*. 1A: a representative individual sampled outside of the Kahoku Lagoon. 1B: a representative individual sampled within the Kahoku Lagoon. The Position shown with a white point indicate where we measured shell thickness.

notypic plasticity, in which phenotypes change according to the environment (Hollander et al. 2006, Hoverman & Relyea 2009, Dunithan et al. 2012, Cazenave & Zanatta 2016). Nevertheless, the relationships between phenotype and environments of freshwater mollusks have not been fully considered in these phylogenetic studies.

To clarify the relationship between morphology and environment, we investigated morphological variations in *Sinotia quadrata* (Fig. 1). *S. quadrata* is broadly distributed across East Asia, and occurs in various environmental habitats (e.g., rivers, water channels, rice fields, wetlands, and lakes) (Masuda & Uchiyama 2004, Hirano et al. 2015). Based on fossil records, Japanese populations of *S. quadrata* were most likely introduced from the Asian continent around 4,000 years ago (Kurozumi 2013). Furthermore, a recent molecular phylogenetic study suggested that *S. quadrata* from the Asian continent and Japan could not be distinguished by two mitochondrial molecular markers (Hirano et al. 2015). In addition, this phylogeny also showed that *S. quadrata* found inside and outside of Lake Biwa in Shiga Prefecture, Japan, had quite different shell morphologies, but shared the same haplotype. However, the relationship between phenotype and environment has not been investigated quantitatively. Therefore, we focused on Kahoku Lagoon to test the morphological variation in *S. quadrata* between *S. quadrata* found inside and outside of the lagoon. Kahoku Lagoon was formed over 1,000 years ago and was originally brackish (Kahokugata Lake Institute 2013). However, this lagoon was converted to a freshwater lagoon as a result of a reclamation project that began in 1963 (Kawahara & Takahashi 2001), and subsequently, because of the desalination of the lagoon, brackish organisms disappeared from Kahoku Lagoon, and were replaced by freshwater organisms (Kahokugata Lake Institute 2013). *S. quadrata* currently occur inside of the

current Kahoku Lagoon and in water channels connected to the lagoon (Nomura & Takahashi 2006). As such, the populations of *S. quadrata* within Kahoku Lagoon settled in this lagoon less than 60 years ago. This means that the desalinated Kahoku Lagoon can be considered a new habitat for *S. quadrata*, and therefore this lagoon provides an excellent opportunity to elucidate the relationship between phenotype and environment. Using this unique model system, we aimed to evaluate how the morphology of *S. quadrata* differs between *S. quadrata* that occur inside and outside of Kahoku Lagoon.

Materials and Methods

Study sites and sampling

The sampling of *S. quadrata* was conducted around Kahoku Lagoon, Kanazawa City, Japan (36°39'05.4"N, 136°40'08.2"E). The survey sites included six locations in Kahoku Lagoon ($n=54$ *S. quadrata*) and 13 locations in the surrounding area ($n=127$ *S. quadrata*) (Fig. 2 and Table 1). As the concentration of calcium ions in the water affects the shell morphology of freshwater mollusks (Marin et al. 1996, Rundle et al. 2004), the calcium ion concentrations were measured using an ion meter at each collection site (LAQUAtwin-Ca-11, accuracy $\pm 20\%$, Horiba, Japan, note that a calcium ion data of sampling site 5 was missing value). Collected *S. quadrata* were brought back to the laboratory within one week, boiled at 100°C for 5 minutes, and stored in 99.5% ethanol.

Quantification of shell morphology

Elliptic Fourier analysis was used to describe and quantify shell morphology (Williams et al. 2012, Doyle et al. 2018, Jackson & Claybourn 2018). Images of *S. quadrata*

shells were acquired using a digital camera (K-S2, Ricoh, Japan), from which elliptic Fourier analysis was performed using SHAPE (Iwata & Ukai 2006). Since shell size or shell thickness are not taken into account in elliptic Fourier analysis, shell width as shell size was measured from the image using Image J (Schneider et al. 2012), and shell thickness was measured using a point micrometer (CPM15,

accuracy ± 0.001 mm, Mitsutoyo, Japan) (Fig. 1B).

Statistical analysis

In order to investigate whether the outline of shells differ between *S. quadrata* sampled from inside and outside the lagoon, we constructed generalized linear mixed models (GLMMs) with Gaussian distributions. In the GLMMs, principal component scores (PC1 to PC5) obtained by elliptic Fourier analysis were included as dependent variables, and a model was constructed with each of the principal component scores. The information on whether the individual *S. quadrata* was collected from within the lagoon or from outside the lagoon was included as the fixed effect. The inside/outside of the lagoon status was converted to 0/1 in the analyses. To account for differences in sampled locations, the sampling site was included as a random effect. The significance of the models was examined by comparing the full model and the null model using a likelihood ratio test with chi-square distributions.

In order to determine whether shell thickness and size of *S. quadrata* differ inside and outside the lake, we conducted analysis using the same method (GLMMs with Gaussian distributions). The shell thickness and size (shell width) were designated as a dependent variable in each model, and the habitat (inside and outside of the lagoon) was included as the fixed effect, with sampling site included as a random effect. In addition, since shell thickness is affected by shell size, in the model that included thickness as a dependent variable, shell size was added as a fixed effect.

Different calcium ion concentrations may cause differences in shell morphology, and therefore the difference in

Fig. 2. Map of Kahoku Lagoon and the surrounding area. Points indicate the sampling sites included in this study. Sites numbered 1–6 are within Kahoku Lagoon, and sites numbered 7–19 are outside of Kahoku Lagoon.

Table 1. Descriptions of sampling sites, average shell sizes, and numbers of individual *Sinotia quadrata* sampled.

Site number	Place	Environments	Ca ²⁺ (mg/L)	Mean of the shell size $\pm$ s. e. (mm)	n
1	inside of the Lagoon	rock, sand, mad, emerging plants	27	18.3 $\pm$ 0.23	12
2	inside of the Lagoon	mad, emerging plants	48	22.5 $\pm$ 1.31	2
3	inside of the Lagoon	mad, rock, emerging plants	25	18.3 $\pm$ 0.24	18
4	inside of the Lagoon	rock, mad	23	16.6 $\pm$ 0.33	8
5	inside of the Lagoon	sand, mad, emerging plants	26	15.4	1
6	inside of the Lagoon	rock, sand, mad, emerging plants	NA	19.9 $\pm$ 0.30	13
7	rice field, water channel	mad	27	16.9 $\pm$ 0.50	15
8	water channel	mad, wall of concrete	52	17.3 $\pm$ 0.24	10
9	water channel	mad, wall of concrete	53	20.4 $\pm$ 0.43	14
10	water channel	mad	29	18.8 $\pm$ 1.16	3
11	river	rock, mad	27	20.4 $\pm$ 0.72	4
12	water channel, rice field	mad	28	20.2 $\pm$ 0.94	11
13	water channel, rice field	mad	41	18.9 $\pm$ 0.36	14
14	water channel, rice field	mad	26	15.4 $\pm$ 0.35	3
15	water channel, rice field	mad	16	16.8 $\pm$ 0.47	5
16	water channel, rice field	sand, mad	35	21.1 $\pm$ 0.32	19
17	water channel	mad	12	19.9 $\pm$ 0.40	16
18	water channel, rice field	mad	27	17.5 $\pm$ 1.00	6
19	water channel	mad, wall of concrete	40	17.0 $\pm$ 0.40	7

calcium ion concentrations between sampling sites inside and outside of the lagoon was examined using the Mann–Whitney U test.

The difference in shell morphology may be determined by the geographical distance between sampling sites, and not by differences in environmental pressures. Therefore, we conducted a Mantel test that examined the correlation between the matrices, in order to investigate whether the morphological differences between the sampling sites correlated with the geographical distances between the sampling locations. The values for morphological traits were included as principal component scores (PC1 to PC5) and morphological measurements (shell width and thickness), respectively.

All analyses were performed in R-3.4.1. (R Development Core Team 2005). GLMMs were performed using package Lme4 (Bates et al. 2019). Mann–Whitney U tests were performed using package exactRankTests (Hothorn & Hornik 2019). Mantel tests were performed using package vegan (Oksanen et al. 2019).

Results

Results of the principal component analysis of the elliptic Fourier analysis showed that at least 83% of the variation in *S. quadrata* shell morphology was explained by the first five principal components (PC1: 54.9%; PC2: 10.5%; PC3: 9.26%; PC4: 4.83%; PC5: 4.03%). The outline from each principal component was reconstructed using the results of the elliptic Fourier analysis (Fig. 3). GLMMs, which tested whether PC scores were different inside and outside the lagoon, showed that the values for PC2 and PC3 differed significantly between sampling sites located inside and outside of the lagoon, respectively (PC2: $P=0.002$; PC3: $P=0.047$, Table 2, Fig. 4). These principal component scores were lower for the shells of *S. quadrata* sampled inside than those sampled outside of the lagoon (Table 2, Fig. 4). However, the other principal components were not significantly different between sampling sites located inside and outside of the lagoon (PC1: $P=0.857$; PC4: $P=0.423$; PC5: $P=0.616$) (Table 2).

After measuring the shells, we found that the average shell size of *S. quadrata* sampled from sites inside the lagoon was 18.54 ± 0.22 mm (mean $\pm$ s.e.) and the average shell size of *S. quadrata* sampled outside the lake was 18.97 ± 0.21 mm (mean $\pm$ s.e.) mm. The shell size was not significantly different between *S. quadrata* sampled from sites located inside and outside the lagoon ($P=0.979$, Table 2). Shell thickness showed a significant positive correlation with shell size ($P=0.001$, Fig. 5, Table 2). Additionally, the shells tended to be thicker in *S. quadrata* sampled within the lagoon than *S. quadrata* sampled outside the lagoon, although the difference was not statistically significant ($P=0.052$, Fig. 5, Table 2). Calcium ion concentrations did not differ between sites located inside and outside of the lagoon ($P=0.320$) (Table 1), and, therefore, calcium ion concentration was not related to the morphological variations in *S. quadrata* shells.

There was no correlation between *S. quadrata* shell morphology and geographical distance between sampling sites (Table 3).

Fig. 3. Contour illustrations reconstructed by elliptic Fourier analysis of *Sinotia quadrata* shells. The outlines represent the mean of the shape, plus and minus two standard deviations (S.D.) for each principal component. The first row is an overlap of the three.

Table 2. The results of generalized linear mixed models (GLMMs) used in this study. In order to investigate whether the shell morphologies differ between *S. quadrata* sampled from inside and outside the lagoon, we constructed GLMMs with Gaussian distributions.

Shell morphology	Intercept		Place				Shell size			
	Estimate	S. E.	Estimate	S. E.	χ^2	P value	Estimate	S. E.	χ^2	P value
PC1	−0.0017	0.0099	0.0022	0.012	0.033	0.86	—	—	—	—
PC2	−0.013	0.0038	0.016	0.0045	9.6	0.0020	—	—	—	—
PC3	−0.0051	0.0031	0.0074	0.0037	3.9	0.047	—	—	—	—
PC4	0.00087	0.0019	−0.0017	0.0023	0.63	0.86	—	—	—	—
PC5	0.00082	0.0020	−0.0011	0.0024	0.25	0.42	—	—	—	—
Shell size	18.57	0.80	−0.027	0.95	0.00070	0.98	—	—	—	—
Shell thickness	−0.14	0.090	−0.073	0.038	3.8	0.052	0.031	0.0046	41	0.0013

Fig. 4. Plots of the top three principal components obtained by elliptic Fourier analysis of *Sinotia quadrata* shells. Distributions on the upper and right sides of the plots show the density of the points. Fig. 4A indicates the relationship between PC1 and PC2. Fig. 4B indicates the relationship between PC2 and PC3. Black square symbols indicate *S. quadrata* collected from inside of Kahoku Lagoon. Grey circle symbols indicate *S. quadrata* collected from locations outside of Kahoku Lagoon, including rice fields, rivers, and water channels.

Fig. 5. Plot indicating the relationship between shell size and shell thickness. The line is the regression line between shell size and shell thickness, and the grey range along the line indicates the 95% confidence interval. Distributions on the upper and right sides of the plots show the density of the points. Black square symbols indicate *S. quadrata* collected from inside of Kahoku Lagoon. Grey circle symbols indicate *S. quadrata* collected from locations outside of Kahoku Lagoon, including rice fields, rivers, and water channels.

Discussion

The results of the elliptic Fourier analysis showed that PC2 and PC3 of *S. quadrata* were significantly different between sites located inside and outside of the lagoon (Table 2). Lower values for PC2 indicate a more elongated, cylindrical shape, while higher values for PC2 reflect a

Table 3. The results of Mantel tests used to analyze whether the morphological differences in *Sinotia quadrata* shells between the sampling sites correlated with the geographical distances between the sampling locations.

Shell morphology	R	P value
PC1	0.046	0.073
PC2	-0.059	0.985
PC3	0.028	0.176
PC4	-0.023	0.734
PC5	0.032	0.173
Shell size	0.394	0.122
Shell thickness	0.039	0.134

more conical shape (Figs. 3, 4). PC3 represents differences in the bulge of the outer edge of the shell's aperture; higher values indicate wider apertures (Figs. 3, 4). Therefore, because these PC scores reflect the expansion rate of shell aperture size with growth, the *S. quadrata* inside the lagoon have elongated cylindrical shells, while the *S. quadrata* outside the lagoon have conical shells. Shell size did not differ between the sites located inside and outside of the lagoon (Table 2), suggesting that the lagoon environment has no effect on *S. quadrata* shell size. On the other hand, regarding shell thickness, the *S. quadrata* sampled inside of the lagoon tended to have thicker shells than those sampled outside the lagoon ($P=0.052$) (Fig. 5) (Table 2). In addition, shell thickness correlated positively with shell size (Fig. 5, Table 2). Furthermore, our Mantel tests showed that there was no significant correlation between shell morphology and geographic distance (Table 3), and this suggests that morphological differences do not reflect spatial autocorrelation. In summary, our results suggest that

the *S. quadrata* shells are the same size both inside and outside of the lagoon, but the shells of *S. quadrata* sampled inside the lagoon are thicker than those of *S. quadrata* sampled outside the lagoon.

The different environmental pressures inside and outside of the lagoon potentially led to the observed morphological differences in *S. quadrata* shells. Predators represent one such environmental pressure. Different shell morphologies result in several defense mechanisms against predators. For example, in freshwater snails in the genus *Physa*, globular shells interfere with predation from fish and elongated shells from crayfish (Dewitt et al. 2000). Furthermore, the narrowness of the shell's aperture and its thickness affect shell-breaking predators (Appleton & Palmer 1988, Trussell 2006, Konuma & Chiba 2007). In recent years, there was a huge outbreak of crayfish in Kahoku Lagoon (Kahokugata Lake Institute 2013). Furthermore, cyprinid fishes also occur in Kahoku Lagoon (Kahokugata Lake Institute 2013), and these fish feed on freshwater mollusks (Imamura & Hashitani 1957, Correia et al. 2005, Moriyama 2007). Thus, crayfish and cyprinid fishes, which can be predators of freshwater mollusks, may have caused differences in shell morphology between the sampling sites located inside and outside of Kahoku Lagoon.

Furthermore, water physical environments are another factor that may underlie the morphological differences in *S. quadrata* shells. Some morphological features of the shell function may reduce the stress caused by wave and/or water current effects (Way et al. 1993, Kihira 1996, Urabe 1998, Le Pennec et al. 2017). There are waves inside the lagoon (Shirai & Kawahara 2005), and there are many environments in which water flows in one direction outside the lagoon. Due to the large differences in these physical properties, morphological differences in *S. quadrata* shells may occur.

Additionally, as freshwater mollusks produce shells using calcium ions in the water, the concentration of these ions in the environment affects shell thickness (Marin et al. 1996, Rundle et al. 2004). However, this effect can be ignored in the present study because there were no differences in the calcium ion concentrations inside and outside of the lagoon. Alternatively, fast-growing snails develop thin, globular shells that accommodate more body mass than their thicker, more elongated counterparts (Kemp & Bertness 1984). If the *S. quadrata* in the lagoon grow slowly, they are likely to have thick, elongated shells. This mechanism may be able to explain the morphological differences observed in this study.

Our results show that *S. quadrata* sampled within the lagoon had elongated cylindrical shells. The *S. quadrata* population within the lagoon has been established in the last 60 years because of the history of the lagoon. The origin of the lagoon population is not clear; however, it is highly possible that the lagoon population was introduced from the surrounding area, because freshwater habi-

tats within and outside the lagoon are connected by water channels (Fig. 2). In addition, even if the *S. quadrata* population within the lagoon and the *S. quadrata* population outside the lagoon have different origins, it is likely that the two populations have a certain gene flow due to these water channels. The dispersal ability of freshwater mollusks including Viviparidae in small and connected habitats (Ribi 1986, Ribi & Arter 1986, Statzner et al. 2008, Kappes & Haase 2012) may also facilitate this. Furthermore, our Mantel tests showed that there was no significant correlation between shell morphology and geographic distance (Table 3), and this suggests that morphological differences do not reflect the origin and/or primitive history of immigration of each population. Considering these factors, the morphological differences between *S. quadrata* sampled within the lagoon and outside the lagoon are likely to have developed after the desalination of the lagoon. That is to say, the observed changes may be a consequence of either phenotypic plasticity or rapid morphological evolution. To distinguish between these two mechanisms underlying morphological change, further studies using high-resolution genetic markers are required. Nevertheless, the present findings suggest that morphological variation occurred in *S. quadrata* as a result of their habitat differences between flowing water and the lagoon. A similar qualitative relationship between shell morphology and habitat has also been observed in *S. quadrata* sampled inside and outside of Lake Biwa (Hirano et al. 2015). This relationship may be general in *S. quadrata*, although further in-depth studies are needed. Thus, the consistent relationship between morphological variation and habitat suggests that the environment of the lake affects the morphology of viviparid mollusks. Furthermore, the presumed flexibility in shell morphology of freshwater mollusks may emphasize the importance of considering habitat environment in species assessment, identification, and studies that examine shell morphology.

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