

Phylogeography of *Geothelphusa* freshwater crabs: unexpected dual dispersal routes via land and sea

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Abstract

Aim

Dispersal is an important factor that determines the potential for colonization to pioneer sites. Most decapods employ seaward migration for reproduction with a planktonic larval phase. However, true freshwater crabs spend their entire life cycle in freshwater. Therefore, it is expected that genetic regionality can be easily detected. In this study, we focused on the genetic structures of true freshwater crabs, *Geothelphusa* crabs. Herein, we reveal the evolutionary history and dispersal patterns of freshwater crustaceans, for which there is limited knowledge to date.

Location

Japanese Islands

Taxon

Geothelphusa dehaani (Decapoda, Potamidae)

Methods

We collected and genetically analyzed 283 specimens at 138 localities from freshwater habitats across the Japanese Islands. Phylogenetic analyses were conducted on 1,796 bp of the combined dataset (mtDNA COI, 16S, and nDNA ITS1, histone H3) and 569 bp of the mtDNA COI dataset. The demographic history of *G. dehaani* was simulated using Approximate Bayesian Computation analysis. A salt tolerance experiment was conducted to confirm the survival rate in seawater.

Results

The resulting of phylogenetic relationships detected 10 clades that were highly likely to be monophyletic. These 10 clades genetically exhibited an explicit pattern of geographical differentiation. Also, we confirmed the salt tolerance ability of these Japanese freshwater crabs.

Main conclusions

The highlights of this study were the discovery of several cryptic species/lineages or undescribed species, and the completely different heterogeneous dual dispersal pathways detected within a single species; i.e., both land and ocean routes. As a result of phylogenetic analysis, it was concluded that Japanese crabs are basically genetically divided by straits. However, strong evidence for dispersion via ocean currents was also detected (i.e., a “sweepstake”), and it was also determined that *G. dehaani* could survive in seawater. This is the first observation of such a unique mode of expansion of a species’ distribution area.

1 Introduction

Dispersal is an important factor that determines the potential for colonization to pioneer sites or migration to new habitats, subsequent persistence and the level of genetic connectivity between populations (Banks et al., 2010; García-Verdugo et al., 2017; Van der Stocken et al., 2019; Waters et al., 2020). Population division is also a key process contributing to an organism's current distribution (Su et al., 1996, 1998; Sota & Vogler, 2001; Tominaga et al., 2013, 2016; Takenaka & Tojo, 2019). The success of colonization to pioneer sites depends on the environmental habitats of the new sites. Phylogeographic patterns influence geographic barriers and the dispersal ability of specific organisms (Avice, 2000; Orsini et al., 2013; Takenaka & Tojo, 2019; Promnun et al., 2021).

Decapoda is a crustacean group diversified in oceans; some members are also adapted to freshwater areas and as well as terrestrial areas. Most decapods employ seaward migration for reproduction, with a planktonic larval phase, even though crabs grow in the freshwater areas of rivers or brackish areas (Kabayashi, 2000; Wowor et al., 2009; Abdullah et al., 2017), i.e., diadromy or catadromy. The distribution patterns and ecology of crabs in riverine environments in Japan has been reported by Kobayashi (2000). The planktonic larval phase(s) predominantly determines potential dispersal ranges (McConaughy, 1992; Rocha et al., 2008; York et al., 2008; Yorisue et al., 2020). Therefore, the geographic genetic structure of most decapods is influenced by ocean currents (Cook et al., 2008; Niikura et al., 2015; Abdullah et al., 2017; Yorisue et al., 2020).

Decapods, including crabs, have evolved to move from the ocean into freshwater zones via brackish water and/or intertidal zones (Pearse, 1927). There are a few species of crab that live in freshwater or terrestrial environments. True freshwater crabs reproduce and spend their entire life cycle in freshwater or terrestrial environments, i.e., without a planktonic larval phase (Kobayashi, 2000; Daniels et al., 2002; Shih et al., 2006, 2011), and they never encounter the ocean (Miyake, 1983), resulting in the relatively poor dispersal abilities of freshwater crabs (Aotsuka et al., 1995; Ikeda et al., 1998). Although adults have the ability for terrestrial movement, freshwater crabs have a strong dependency on riverine habitats (Cook et al., 2008; Daniel et al., 2015). Therefore, it is expected that their genetic structures are affected by river structures and it should be easy to detect genetic regionality (Daniels et al., 2003; Koizumi et al., 2012; Copilaş-Ciocianu et al., 2017).

True freshwater crabs, which have adapted to inland water and also wet terrestrial environments, are distributed globally and there are over 1,300 freshwater species out of 6,700 known species of brachyuran crabs (Yea et al., 2007). These freshwater crabs currently constitute eight families (Pseudothelphusidae, Trichodactylidae, Potamonautidae, Deckeniidae, Platythelphusidae, Potamidae, Gecarcinucidae, and

Parathelphusidae: Martin & Davis, 2001; Yea et al., 2007). A freshwater crab taxon, i.e., *Geothelphusa* Stimpson, 1858 (Potamidae), which inhabits mountain streams and/or upper rivers is endemic to Taiwan and the Japanese Islands. In particular, many species are distributed in Taiwan and the Ryukyu Islands (Shokita, 1996; Shokita et al., 2002; Shih et al., 2006, 2007). *Geothelphusa dehaani* is a species endemic to the Japanese Islands, which is widely distributed in Honshu, Shikoku, Kyushu, and surrounding islands (e.g., Yakushima Island, Tanegashima Island, Nakanoshima Island, the Koshikijima Islands, the Oki Islands, Fukuejima Island, Sado Island, and the Kinkazan Island) (Suzuki and Naruse, 2012). On the Osumi Peninsula (Kyushu Island), *Geothelphusa exigua* Suzuki and Tsuda, 1994 is distributed, and *Geothelphusa marmorata* (Suzuki and Okano, 2000) is distributed on Yakushima Island, *Geothelphusa koshikiensis* (Suzuki and Kawai, 2011) is distributed on the Koshikijima Islands, and *Geothelphusa mishima* (Suzuki and Kawai, 2011) is distributed on the Mishima Islands (Fig. 1). In the Ryukyu Islands, different *Geothelphusa* species are distributed on each island.

The habitats of these *Geothelphusa* species are restricted to small streams and ponds with clean freshwater. These habitat characteristics suggest that the vagility or dispersal capability of these crabs is low and led to the genetic differentiation among populations within the species (Nakajima & Masuda, 1985; Ikeda et al., 1998). Regarding *G. dehaani*, populations of Yakushima and Nakanoshima and the Koshikijima Islands have a blue body color, although the body color of the Kyushu Island populations of this species are red (Okano et al., 2000). Chokki (1980) reported that with respect to biogeographical coloration of *G. dehaani*, this species can be differentiated into three color types. Similarly, Suzuki (1992) reported on the regional characteristics of the body color of *G. dehaani* in the North-eastern region of Honshu Island. However, since then all three such body color types have been detected in various regions within Honshu, Shikoku and Kyushu (Chokki, 1976, 1980; Suzuki & Tsuda, 1991; Suzuki, 1992; Furuya and Yamaoka, 2017).

To date, there has been no study of *Geothelphusa* crabs using molecular markers covering their distributional ranges. In this study, we focused on genetic structures at the regional population level and the evolutionary process of true freshwater crabs. These crabs are particularly suitable organisms for discussion regarding how the process of formation of freshwater decapod populations has occurred. Herein, we reveal the evolutionary history of *Geothelphusa* crabs and their genetic regionality, and subsequently compare the genetic structures and dispersal patterns of freshwater crustaceans as observed in some previous studies.

2 Materials & Methods

2.1 Sampling of materials

Molecular phylogenetic analyses were conducted using 283 specimens including 268 targeted ingroup specimens. Specimens were collected from 138 localities at freshwater habitats across the Japanese Islands between 2015 and 2021. This area reasonably represents almost all of the distribution ranges of *Geothelphusa dehaani* (Toyota, 2019). Almost all the specimens were fixed in 99.5% ethanol in the field, although several specimens were fixed in the laboratory. Detailed information on the specimens used in this study is summarized in Table 1 and Figure 1. Specimens were identified based on the morphological characteristics of the male 1st pleopods (Toyota & Seki, 2019), which are considered to exhibit key identifying characteristics.

2.2 DNA extraction, amplification, sequencing and alignment

Total genomic DNA was extracted from a tissue sample from ethanol-preserved specimens and DNA was purified using a DNeasy Blood & Tissue Kit (Qiagen, Hilden), according to the manufacturer's instructions. Total DNA was used to amplify fragments of mitochondrial DNA (mtDNA) cytochrome c oxidase subunit I (COI), 16S rRNA, nuclear DNA (nDNA) internal transcribed spacer 1 (ITS1) and histone H3. The primer sets used for polymerase chain reaction (PCR) and annealing temperatures are listed in Table 2. rTaq (TOYOBO, Osaka) was used for COI, ExTaq (Takara, Shiga) for 16S, ITS1, and histone H3. PCR products were purified using ExoSAP-IT (GE Healthcare, Buckinghamshire). Purified DNA fragments were used for cycle sequencing reaction with a BigDye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems, California) and sequenced on an automated DNA Sequencer (ABI3130xl DNA analyzer; Applied Biosystems, California).

All of the sequence data have been submitted to the DNA data bank of Japan (DDBJ database) and accession numbers are provided in Table 1. Sequence alignment and editing were conducted using MEGA ver. 6.06 (Tamura et al., 2013) and CLC working bench (CLC bio, Aarhus). All sequence data were aligned automatically by Clustal W (Thompson et al., 1994) instrumented in MEGA ver. 6.06 (Tamura et al., 2013).

2.3 Phylogenetic analysis

Phylogenetic analyses were conducted on 1,796 bp of the combined dataset (mt DNA COI, 16S, and nDNA ITS1, histone H3) and 569 bp of the mtDNA COI dataset, using the maximum likelihood method (ML; Felsenstein, 1981) and Bayesian inference (BI; Huelsenbeck et al., 2001). ML analysis was performed with RAxML (Stamatakis, 2014) using the GTR + G + I model and 1,000 bootstrap replicates. Bayesian inference was conducted using MrBayes5D ver 3.1.2 (Tanabe, 2008) and the HKY85 + G + I model for the combined dataset and the HKY85 + G model for the mtDNA COI dataset, which is the best fit model selected based on the Schwartz's Bayesian Information

Criterion (BIC; Schwartz, 1978) in Kakusan 4 (Tanabe, 2007). Two Markov Monte Carlo chains were run for 10 million generations with sampling every 1000 generations. The first 10% of the sampling trees were discarded as burn-in. Bayesian log trace files were visualized by Tracer ver. 1.6 (Rambaut & Drummond, 2007) and we confirmed the effective sampling size (ESS) was more than 200. Both ML and Bayesian phylogenetic trees were visualized by FigTree ver.1.3.1 (Rambaut, 2009).

2.4 Demographic history

Demographic history of *Geothelphusa dehaani* was simulated using a combined dataset. Based on the results of phylogenetic analysis, Clades 1–5 and Clade 7 were clustered in six groups for demographic analysis. Because only single specimens were detected in this study, Clade 6 was excluded from the analysis. To test the phylogenetic relationship and the possibility of oceanic dispersal by the Kuroshio Current in this species, approximate Bayesian computation (ABC) analysis implemented in DIYABC ver. 2.1.0 (Cornuet et al., 2014) were used to compare five scenarios. As shown in Figure 2, the genealogical position of Clade 4 was tested in the analysis. All possible summary statistics were included and 6,000,000 simulations were run for the scenario. The best scenario was selected from the results of direct estimation and the logistic regression estimate of the posterior probabilities for scenarios. All the DIYABC results (summary statistics) are shown in Figure 2.

2.5 Salt Tolerance Evaluation

A salt tolerance experiment was conducted to confirm the survival rate in seawater. We collected *G. dehaani* including adults and juveniles from the Izu Peninsula (St. 88) and Nagano (St. 19) (Table 1), and one more site (Sanada, Ueda, Nagano: 36.4963, 138.3622) not used for genetic analysis. In a laboratory, *G. dehaani* were reared in artificial seawater (Tetra Marine Salt Pro; USA) for 15 days, and their survival was confirmed every day.

As artificial water, 34 g of “Tetra Marine Salt Pro” were added to 1,000 mL tap water (salinity of ca. 3.4%), which is a general seawater concentration, with salinity of 6.8% (Tetra Marine Salt Pro is 68 g in 1,000 mL tap water), which is twice that concentration, were used for the salt tolerance experiment. The rearing container was a Plastic case (NAKAYA KAGAKU SANGYO CO.; Japan; 173×118×118 mm). No food was given during the experiment to avoid its influence; it is unlikely that food will be eaten if a crab disperses by ocean currents (there is no problem in terms of survival even if no food is given for 15 days).

In the experiment, freshwater crabs were reared in three experiments: seawater, twice the concentration of seawater, and freshwater (tap water). After 15 days, the freshwater crabs reared in freshwater were then placed in seawater, and the freshwater

crabs reared in seawater were placed in freshwater, respectively. The freshwater crabs reared in twice the concentration of seawater did not survive for 15 days, so the experiment was terminated.

3 Results

3.1 Phylogenetic analyses

The results of phylogenetic relationships observed for *Geothelphusa dehaani* specimens, based on the mtDNA COI region (569 bp) for 283 specimens, and based on the combined datasets of the mtDNA COI (560 bp) and 16S rRNA (532 bp) regions, and the nDNA ITS (440 bp) and histone H3 (264 bp) regions using 58 specimens, are shown in Figures 3 and 2, respectively. The phylogenetic cladogram based on each data set detected 10 clades, and almost all of the clades were consistent between the COI dataset and the combined total dataset (Table 1). The distribution of the collection sites of the specimens in each clade is shown in Figure 1. These 10 clades were highly likely to be monophyletic (Figs. 2, 3). Genetic relationships between respective clades based on the mtDNA COI region dataset were not observed (Fig. 3), while genetic relationships based on the combined dataset were more clearly observed (Fig. 2). However, the phylogenetic cladogram could not reliably reveal the relationships among clades 3 to 6.

Based on the mtDNA COI region, Clade 1 is mainly distributed in Honshu, with some specimens from Shikoku and Sado Island. Clade 2 is distributed in Western Honshu, and Suo-Oshima Island, the Oki Islands, and a single specimen from Kyushu was included. Clade 3 is distributed on the Tokara Islands and Yakushima Island (i.e., the Northern Ryukyu Islands). Clade 4 is distributed on the Izu Peninsula, the Boso Peninsula, and the Miura Peninsula. Clade 5 is mainly distributed in Kyushu, with one specimen from Shikoku. Clade 6 is distributed on Tanegashima Island. Clade 7 is distributed in Northwestern Kyushu (i.e., the Shimabara Peninsula, and the Amakusa Islands). Clade 8 is distributed in Shikoku and Kyushu. Clade 9 is mainly distributed in Shikoku, and some in Honshu. Clade 10 is distributed in Kyushu. The genetic distances (p -distances) between respective clades are shown in Table 3. Clades 8 and 9 had a greater genetic distance from each other than the genetic distances observed between other clades. The genetic diversity (i.e., both the haplotype diversity and nucleotide diversity) of each clade is shown in Table 4. In addition, recent expansion of the effective population sizes of Clade 1 was clearly indicated (see Tajima's D and Fu's F_s ; Table 4).

Regarding the inconsistent results of phylogenetic analyses based on the dataset of only the mtDNA COI region and the combined dataset, specimens from Sado Island (St. 12) were found to have contradicting results (Table 3; Figs. 2, 3). A specimen from

Tanegashima Island (St.100; Clade 6) was analyzed by means of both the COI based dataset and the combined dataset, and was also found to be greatly genetically differentiated from the other clades. A specimen from Kyushu (St. 95; Clade 10) was also found to be greatly genetically differentiated from the other clades based on only the mtDNA COI region.

3.2 Demographic history

ABC analysis was conducted in order to reveal the most probable phylogenetic relationships between clades which had less reliability based on phylogenetic analysis results, and to reveal the genealogical position of Clade 4 that mainly consists of specimens from isolated peninsulas. In a comparison of the direct estimation results of calculated posterior probabilities between scenarios, Scenario 2 was the most supported and the second most supported scenario was Scenario 5 [posterior probabilities and confidence intervals of Scenarios 1–5: 0.17 (0.00–0.51); 0.29 (0.00–0.68); 0.17 (0.00–0.50); 0.13 (0.00–0.42); 0.24 (0.00–0.62), respectively]. From the results of the logistic regression estimate for scenarios, Scenario 5 was the most supported [posterior probabilities and confidence intervals of Scenarios 1–5: 0.00 (0.00–0.00); 0.01 (0.01–0.02); 0.04 (0.03–0.04); 0.01 (0.01–0.01); 0.94 (0.93–0.94), respectively].

3.3 Salt tolerance evaluation

Since the results of the phylogenetic analysis indicated the probability of ocean current- based dispersion (i.e., the Kuroshio current), we evaluated the salt tolerance ability of *G. dehaani*. As a result, no significant difference in survival was detected between freshwater and seawater species for at least 15 days (Table 5; Fig. 4). However, Clade 4 of *G. dehaani* could not survive when the concentration of the seawater saltiness was doubled ($n = 5$). We also investigated whether or not they could survive when they arrived in new freshwater environments after dispersal via ocean currents. When they were reared in freshwater after being reared in seawater for 15 days, there was no detrimental effect on their survival. There was no difference in salt tolerance between Clades 1 and 4.

4 Discussion

The diversity of the *Geothelphusa* genus is very wide, especially in Southeast Asia and the Ryukyu Islands, so it is thought that the origin of the freshwater crab *Geothelphusa* was in the more southern regions, with subsequent migration northward through the Ryukyu Islands (Shih & Ng, 2011; Shih et al., 2004, 2011). Regarding Honshu, Shikoku, Kyushu, and the surrounding islands, *Geothelphusa exigua* is distributed in the southern part of Kyushu, i.e., the Osumi Peninsula (Suzuki & Tsuda, 1994), *Geothelphusa marmorata* is distributed on Yakushima Island (Suzuki & Okano,

2000), and *Geothelphusa mishima* is distributed on the southwestern islands of Kyushu (i.e. Kuchinoerabu Island, Kuroshima Island; Suzuki & Kawai, 2011), *Geothelphusa koshikiensis* is distributed on the Koshiki Islands (Suzuki & Kawai, 2011). As such, there is a high degree of species diversity in the south western parts of the Japanese Islands. Also, in this study, it was revealed that the four genetic clades (i.e., Clades 5, 7–8 and 10) within the species of *G. dehaani* are distributed in Kyushu, and since the genetic diversity of southwestern Japan is high, it is considered that *G. dehaani* also originated from the southwestern direction and migrated northward.

4.1 Genetic differentiation by island

It is known that many crustaceans disperse over a wide area via ocean currents due to their planktonic life cycle during their larval stages in brackish waters and/or the ocean. Therefore, they have significant potential to disperse via ocean currents (McConaughy, 1992; Rocha et al., 2008; York et al., 2008; Abdullah et al., 2017; Yorisue et al., 2020). It is well documented that the distributional ranges of geographical lineages of marine organisms are greatly influenced by ocean currents (Rocha et al., 2008; York et al., 2008). The larvae of decapods are generally dispersed over wide areas by oceanic surface currents (Cook et al., 2008; Page et al., 2008). However, as for the freshwater crab, *G. dehaani*, it spends its entire life in freshwater, so it has no stages in which it can migrate via the ocean. Therefore, *G. dehaani* was expected to be an easy species in which to detect genetic differentiation within each geographical region. As a result, *G. dehaani* was shown clearly to be composed of nine major genetically differentiated clades, based on the sets of mtDNA COI, 16S rRNA, and nDNA histone H3 regions. Genetic differentiation of each clade was primarily detected between islands. This means that each strait functions as a large genetic barrier for this freshwater crab, as was initially expected. Other related species of the same *Geothelphusa* genus were distributed around Kyushu and the Ryukyu Islands, which are also being primarily divided by their islands (Nakajima & Masuda, 1985; Suzuki & Okano, 2000; Suzuki & Kawai, 2011). The distribution patterns observed by which different species inhabit each island (i.e., tend to be speciated at the island level) could be a general pattern for freshwater crabs.

Freshwater species that adapted to mountain streams, to which freshwater crabs are adapted, have been reported to be more likely to exhibit genetic regionality (Tominaga et al., 2013, 2016; Takenaka & Tojo, 2019; Takenaka et al., 2019; Tojo et al., 2021). Similarly, freshwater crabs in other regions (e.g., Taiwan, China, Africa) have been reported to be genetically differentiated on a regional basis (Daniels et al., 2006; Shih et al., 2006). However, as a result of our genetic analysis of *G. dehaani*, no genetic regionality was detected within each island (however, in the large islands such as Honshu, Shikoku and Kyushu, several intra-islandic genetic lineages were detected).

Since adult *G. dehaani* has some capability for terrestrial movement, it was considered possible that gene flow could occur over a wide area within larger scale islands.

Regarding the inconsistency in the genetic clade of the Sado Island population, the Sado specimen was positioned in Clade 1 based on genetic analysis of the mtDNA COI region; however, the Sado specimen was positioned in Clade 2 based on genetic analysis of the combined datasets. If this inconsistency in the genetic positioning of the Sado Island population is correct, the Sado Island population may be derived from an introgression between ancestor populations of Clade 1 observed in Honshu and of Clade 2 observed in western Japan and the Oki Islands. However, only a single sample from Sado Island could be analyzed. Regarding the cladogram based on the mtDNA COI region, it was composed of short branches except for haplotype H36 detected from Sado Island, and only H36 was observed to be genetically differentiated from the other haplotypes with long branches. The cladogram based on the combined dataset indicates genetic differentiation between the genotype G14 detected from Sado Island and other genotypes. Therefore, since there is the possibility of an error due to the single sample, it is necessary to further carefully investigate the phylogenetic relationship of the Sado Island population. Clades 6 and 10 also were based on single samples, so further investigation is also needed, but there is no doubt that they are genetically differentiated from the other clades.

4.2 Unexpected distribution expansion: dispersal via two different routes

We have shown *G. dehaani* to be genetically differentiated between islands, having migrated northward terrestrially, but one exceptional clade was detected. That is, Clade 4, detected on the Izu Peninsula, the Miura Peninsula, and the Boso Peninsula. These specimens were all closely related to the clade detected in Kyushu, Shikoku and the Northern Ryukyu Islands (Clades 3, 5–7). As for the results of the ABC analysis, Clade 4 was evaluated to be monophyletic with Clades 5 and 6, and these clades were also evaluated as monophyletic with Clade 3. This means that the Clade 4 shows a disjunct distribution that is closely related to the clades that are widely geographically separated across Honshu and Shikoku. Also, the Izu, Miura and Boso Peninsulas are connected by the Honshu landmass; however, Clade 4 was only detected on these three peninsula areas (Clade 4 was not detected in the inland areas of Honshu), and as such, these populations are isolated from each other. From this distribution pattern, it is considered that an ancestor lineage of Clade 4 migrated from Kyushu or the Northern Ryukyu Islands via ocean currents (Fig. 5). However, since *G. dehaani* lives in the upper regions of rivers for its entire life unlike many crustaceans (Nakajima & Masuda, 1985), this result was unexpected. Shokita (1996) and Yea et al. (2007) also reported that *G. dehaani* cannot survive in seawater. Even so, it was unclear whether or not they could survive for a number of days in the ocean. In order to investigate the potential for

ocean current dispersion, we experimentally evaluated the seawater tolerance of *G. dehaani*. This result was surprising and contrary to our initial expectations. In our seawater tolerance experiments, *G. dehaani* survived for at least 15 days in seawater environments. In fact, previous study showed that two freshwater crab families (Potamidae and Gecarcinucidae) can survive in sea water (Esser & Cumberlidge, 2011).

In addition, we determined that *G. dehaani* survived when returned to freshwater after being reared in seawater for 15 days. That is, it was shown that from a physiological and ecological viewpoint that oceanic dispersal of *G. dehaani* is a possibility. Seawater tolerance for as long as 15 days provides the potential for migration via ocean currents that could carry this crab a considerable distance. It was also shown that it could be possible for *G. dehaani* to subsequently survive in freshwater areas after migrating to the Izu Peninsula via ocean currents. Although some cases of seawater crabs migrating via ocean currents have been reported, cases of pure freshwater crabs doing so are extremely rare (Shih et al., 2004; Daniels, S. R. 2011; Jesse et al., 2009; Esser & Cumberlidge, 2011). Considering the geographical genetic structure of *G. dehaani* in this study, the most likely interpretation is that that ocean current dispersion has occurred.

In addition, Clade 2 detected from Suo-Oshima Island, western regions of Honshu and Sado Island was also suggested to migrate via ocean currents. Clade 2 may have migrated via an ocean current on the Sea of Japan side, compared to Clade 4 which would have migrated via Pacific Ocean currents (Fig. 5). There are strong ocean currents (i.e., the "Kuroshio" currents) southwest to northwest on the Pacific Ocean side and the Sea of Japan side, and these conclusions are consistent with these current flow paths (Fig. 5).

In this way, these dispersion events across dispersion barriers for such species by random and/or abnormal events, such as typhoons, hurricanes, floods, changes in the flow routes of ocean currents, and wind direction, are known as "Sweepstakes" events, as suggested by Simpson (1940). For example, the distribution of mammals, amphibians and reptiles that could not cross the sea in Madagascar were explained as "sweepstakes" events (Stankiewicz et al., 2006; Katinas et al., 2013). For *G. dehaani*, almost all clades and species are separated by sea regions, whereby only a few had any potential to be dispersed by ocean currents. Therefore, the existence of parapatric distributions with the clade dispersed on the mainland of Honshu is interesting.

Japanese organisms originating from the southern and western regions migrated to the Japanese Islands via land bridges and expanded their distribution areas across land in a northward direction (Motokawa & Kajihara, 2017; Tojo et al., 2017). Also, it has been reported that species adapted to environments along coasts and organisms that spend a part or all of their lives in the sea rode ocean currents and thereby expanded their distribution areas (Niikura et al., 2015; Abdullah et al., 2017; Ueno et al., 2020;

Nakahama et al., 2022). The dispersion routes and methods may be dependent on the environments that they are adapted and/or ecological features. Under such circumstances, it was suggested that *G. dehaani* dispersed via land and ocean toward northern regions via dual routes that differed greatly depending on the system. To date, cases of such dual route species dispersal across both land and ocean have not been reported. We have identified a group that has expanded its distribution area using these dual heterogeneous routes. The evolutionary history of *G. dehaani* estimated in this study is considered to be as follows. The ancestral lineage of *G. dehaani* firstly distributed in a southwestern region and then migrated northward via land bridges. After this expansion of its distribution area within Honshu, the ancestral lineage of Clade 4 dispersed from southern Kyushu and the Northern Ryukyu Islands to the Izu Peninsula, the Miura Peninsula and the Boso Peninsula via ocean currents. As a result, multiple clades of different origins were established within Honshu.

Similarly, within the taxa that are thought to have migrated via the Pacific Ocean, some clades of freshwater shrimps of the *Palaemon* genus distributed in the southern Kyushu region and were also found to have distributed to the Izu Peninsula, the Kii Peninsula and the Muroto Peninsula (Cho et al., 2019). However, some questions remain: “Why was only Clade 4 observed to have migrated via ocean currents?”, and “Why was Clade 4 detected on only three peninsulas?”. What these three peninsulas have in common is that they were once independent islands from Honshu (Shiba, 2016; Takahashi, 2019; Kikuchi, 1997). From a population genetics point of view, it is difficult to change the frequency of the gene pool of an already established population by way of a small number of outside individuals being dispersed to such already established populations. Therefore, in the case of former islands which were isolated from Honshu, freshwater crabs were previously absent, so freshwater crabs successfully dispersed to such islands and could settle into the islands and establish populations. Of course, it cannot be denied that the crabs that make up Clade 1 may have dispersed across the strait to Izu and the other two peninsulas (currently the Izu, Miura and Boso Peninsula), which were once independent islands. However, it would have been difficult to disperse due to the strong ocean current, the “Kuroshio”. In addition, such dispersion from Clade 1 is unlikely from the genetic structures of these peninsula populations. From these viewpoints, the possibility that the crabs in Clade 1 were dispersed after being connected to the land as a peninsula should also be rejected. Therefore, it is highly possible that freshwater crabs settled in their optimal niches on as yet unpopulated islands, i.e., the proto Izu Peninsula Island. After that, the population of the Izu Peninsula probably made “stepping stone” migrations, dispersing on to the former islands of the Miura Peninsula and then the Boso Peninsula, which are located further northeast.

4.3 Finding several cryptic species

Genetic analyses have often revealed the existence of cryptic species (Pereira-da-Conceicao et al., 2012; Vuataz et al., 2013; Rutschmann et al., 2014, 2017; Bisconti et al., 2016, 2018; Saito et al., 2018; Yano et al., 2020; Takenaka et al., 2021). In this study, it was revealed that the scale of genetic differentiation observed between the populations of Honshu, Shikoku, and Kyushu, which were morphologically identified as *G. dehaani*, at an interspecific level or greater. In particular, Clades 8 and 9 detected in Shikoku and Kyushu have a larger genetic distance (p -distance) than that between other clades. Although the monophyly of Clades 1–7 was highly supported, the monophyly of Clades 1–8 was not so strongly supported. When including Clade 9, Clades 1–9 was not evaluated as being monophyletic. It is necessary to investigate morphological characteristics in more detail, but at least two cryptic species were found on Shikoku and Kyushu in this study.

Besides the Ryukyu Islands, freshwater crabs also inhabit various other islands within the Japanese Islands, and these may contain even more cryptic species. For example, regarding *Geothelphusa sakamotoana* that was treated as one of the outgroups in this study, the H81 haplotype detected on Takara Island of the Tokara Islands is largely genetically differentiated from the haplotypes (H93–99) of *G. sakamotoana* detected on Okinawa-jima and Amami-Oshima Islands, although their phylogenetic relationship is not yet certain. It was also revealed that H93–97 detected in Okinawa Island and H98–99 detected in Amami-Oshima Island are largely genetically differentiated. Also, Clade 6 detected on Tanegashima Island and Clade 10 detected on Kyushu (only from site No.95) were genetically differentiated from the other clades, therefore they come from isolated genetic lineages limited to each geographical area.

Differences in the body color of *G. dehaani* have often been reported in many regions (Chokki, 1976, 1980; Nakajima & Masuda, 1985; Suzuki & Tsuda, 1991), but each of these previous studies focused on a restricted regional scale, so the details of the relationships between body color and phylogeny are unknown. Although a red body color and blue body color specimens of *G. dehaani* were collected within the same stream on Shikoku in this study, no detected genetic differences were found between each body color type (Fig. 1D, E: st. 116). This suggests that there is basically no relationship between body color and genetic differences in *G. dehaani* (H82 and H83: Table 1; Fig. 3). Previously, Aotsuka et al. (1995) reported two body colors (i.e., dark brown and blue) of *G. dehaani* that exhibited some degree of genetic differentiation using isozyme variation analysis. The population analyzed in a previous study (Aotsuka et al., 1995) constituted Clade 1 in the present genetic analyses results. Meanwhile, genetically differentiated crabs consisting of Clade 4 inhabited an area close to this site (Aotsuka et al., 1995), and the body color of the freshwater crabs in Clade 4 was pale bluish (this color treated to be as “blue” color in previous studies). Almost all samples

collected from the Izu Peninsula (Clade 4: Fig. 1F) and the Northern Ryukyu Islands (Clades 3 and 6) had blue bodies. Therefore, the contradiction between the previous study and our results may be explained by the possibility of the existence of hybrids in Clades 1 and 4. Aotsuka et al. (1995) conducted isozyme analysis, and found the existence of more polymorphisms than the genetic loci in our analyses, so it is possible that the results of the previous study may reflect more recent events. Therefore, it is highly possible that Clade 4 made a secondary contact, then hybridized with Clade 1 after migrating to the Izu Peninsula via ocean currents. To establish if this is the case, it is necessary to analyze more sensitive molecular markers (SNPs, SSR, etc.). It is not known whether the blue body color occurred following migration via ocean currents or as a result of the “founder effect”. It will be an interesting to investigate any correlations between body color differences and phylogenetic relationships.

In conclusion, it is known that the species distributed in the Ryukyu Islands are primarily differentiated by straits, and it was also found that species or genetic clades distributed in Honshu, Shikoku, and Kyushu are also fundamentally differentiated by straits. Therefore, it can be generally said that freshwater crab dispersal and/or connectivity between populations is restricted by straits and that speciation occurs between isolated populations.

However, strong evidence for dispersion via ocean currents was detected (i.e., a “sweepstake”), and it was also determined that *G. dehaani* could survive in seawater. At first glance, the results were contradictory, but since *G. dehaani* inhabits the upper reaches of river systems, it is thought that dispersion events via ocean currents are rare. That is, ocean current-based dispersal is unlikely to occur in habitats of large river systems. However, in small-scale river systems such as on small-scale islands and peninsulas, it is often the case that upstream-inhabiting freshwater crabs are swept away to sea areas during floods. As a result, it is thought that ocean current-based dispersal has occurred to such islands and/or peninsula habitats. In addition, even if freshwater crabs succeed in dispersal via ocean currents, it is unlikely that they can successfully colonize habitats if other lineage(s) are already distributed there, so dispersion events via ocean currents are considered to be an extremely rare phenomenon. In fact, Clade 4 detected on the Izu Peninsula, the Miura Peninsula and the Boso Peninsula was found to have become significantly genetically differentiated from the other clades.

The highlights of this study were the discovery of several cryptic species/lineages or undescribed species, and the completely different heterogeneous dual dispersal pathways detected within a single species; i.e., both land and ocean routes. This is the first observation of such a unique mode of expansion of a species’ distribution area, providing new knowledge and insight regarding the development of biodiversity in Japanese organisms.

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Conflict of Interest Statement:

The authors declare that they have no competing interests.

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Biosketches

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Author contributions: M.T., K.Y. and K.T. designed, managed the study, and performed sample collection; M.T. and K.Y. mainly performed laboratory work and phylogenetic analyses; M.T., K.Y. and K.T. wrote and reviewed the manuscript.

Figure Legends

Figure 1. A) Sampling localities and the distribution area of each detected clade (clades 1-10) of the *Geothelphusa* crabs based on data from the mtDNA COI region. Please refer to Table 1 for specific locality numbers, sample numbers, and GenBank accession numbers. The color and number of each clade correspond to the estimated phylogenetic tree shown in Figures 2, 3. B – G) Photos of Sawa crabs on each island. B) Kyushu, C) Honshu, D – E) the same site (st. 116) in Shikoku, F) Izu Peninsula, G) Takara-jima Island.

Figure 2. The estimated phylogenetic relationships [Bayes tree] of the *Geothelphusa* crabs based on the sequenced data of the combined dataset (mt DNA COI, 16S, and nDNA ITS1, histone h3: 1,796 bp) using 58 specimens. The number of major nodes indicates posterior probabilities and bootstrap values. The color and number of each clade correspond to the map of sampling localities shown in Figure 1.

Figure 3. The estimated phylogenetic relationships [Bayes tree] of the *Geothelphusa* crabs based on the sequenced data of the mtDNA COI region (569 bp) using 283 specimens. The number major nodes indicates posterior probabilities. The color and number of each clade correspond to the map of sampling localities shown in Figure 1.

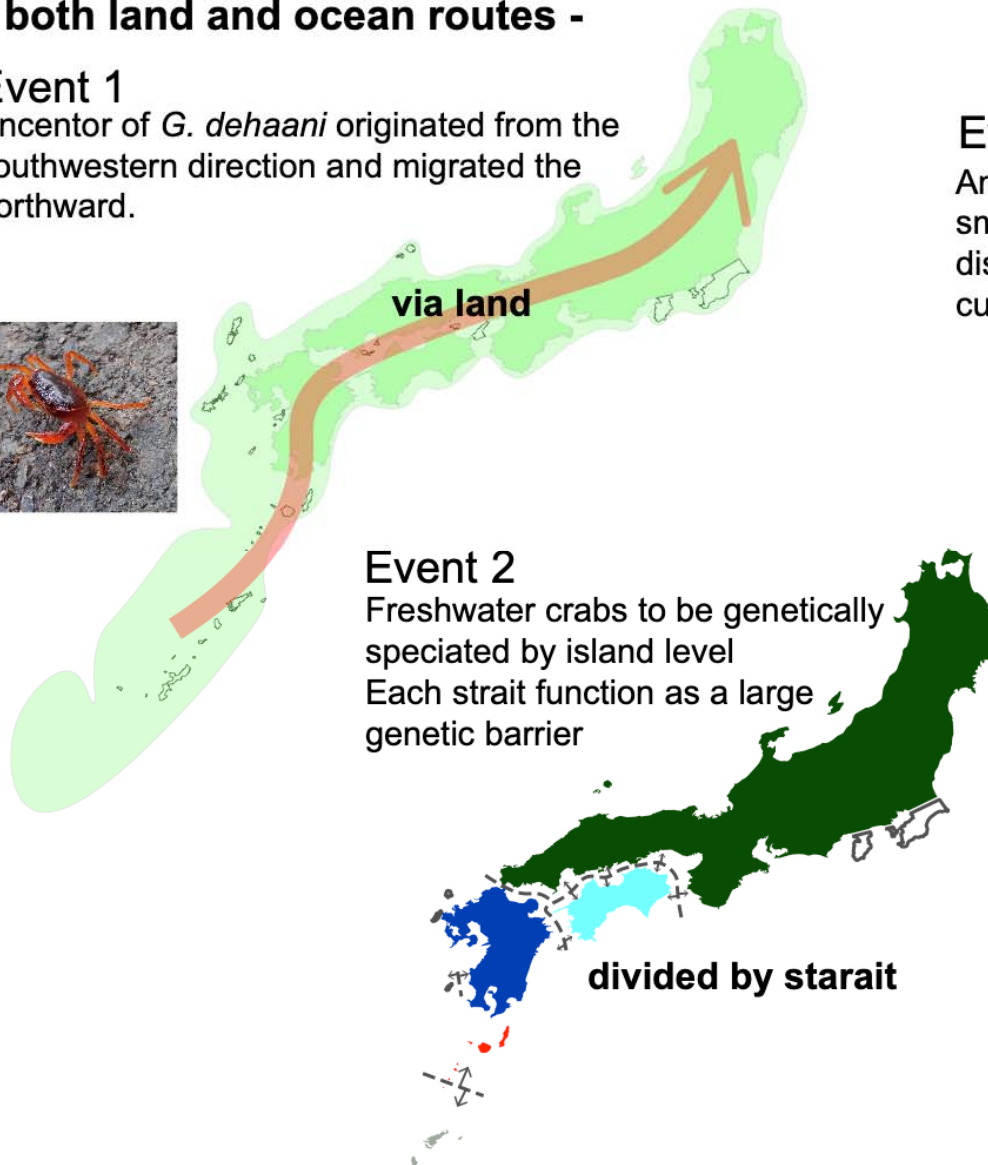
Figure 4. Survival rate of *Geothelphusa dehaani* of Clades 1 and 4 for 15 days in water with different salinity levels (freshwater, seawater, twice concentration seawater). The colors of survival curves correspond to each clade color in Figure 1.

Figure 5. LGM coastlines and major currents around the Japanese Islands. LGM coastlines (Gent et al., 2011), and Sverdrup (Sv: a non-SI metric unit of flow) value of each ocean current are indicated based on previous research (Takikawa, 2005; Andres et al., 2015; Qiu, 2019).

The completely different heterogeneous dual dispersal pathways - both land and ocean routes -

Event 1

Ancentor of *G. dehaani* originated from the southwestern direction and migrated the northward.

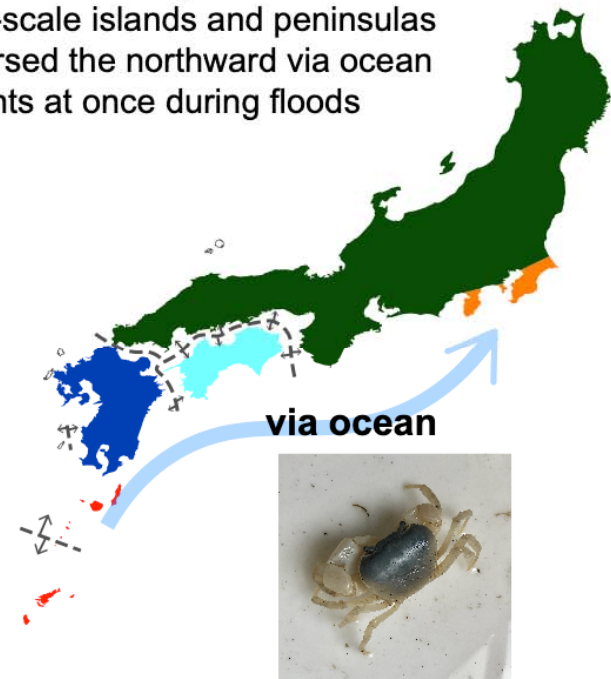


Event 2

Freshwater crabs to be genetically speciated by island level
Each strait function as a large genetic barrier

Event 3

An ancestor lineage of southwestern small-scale islands and peninsulas dispersed the northward via ocean currents at once during floods



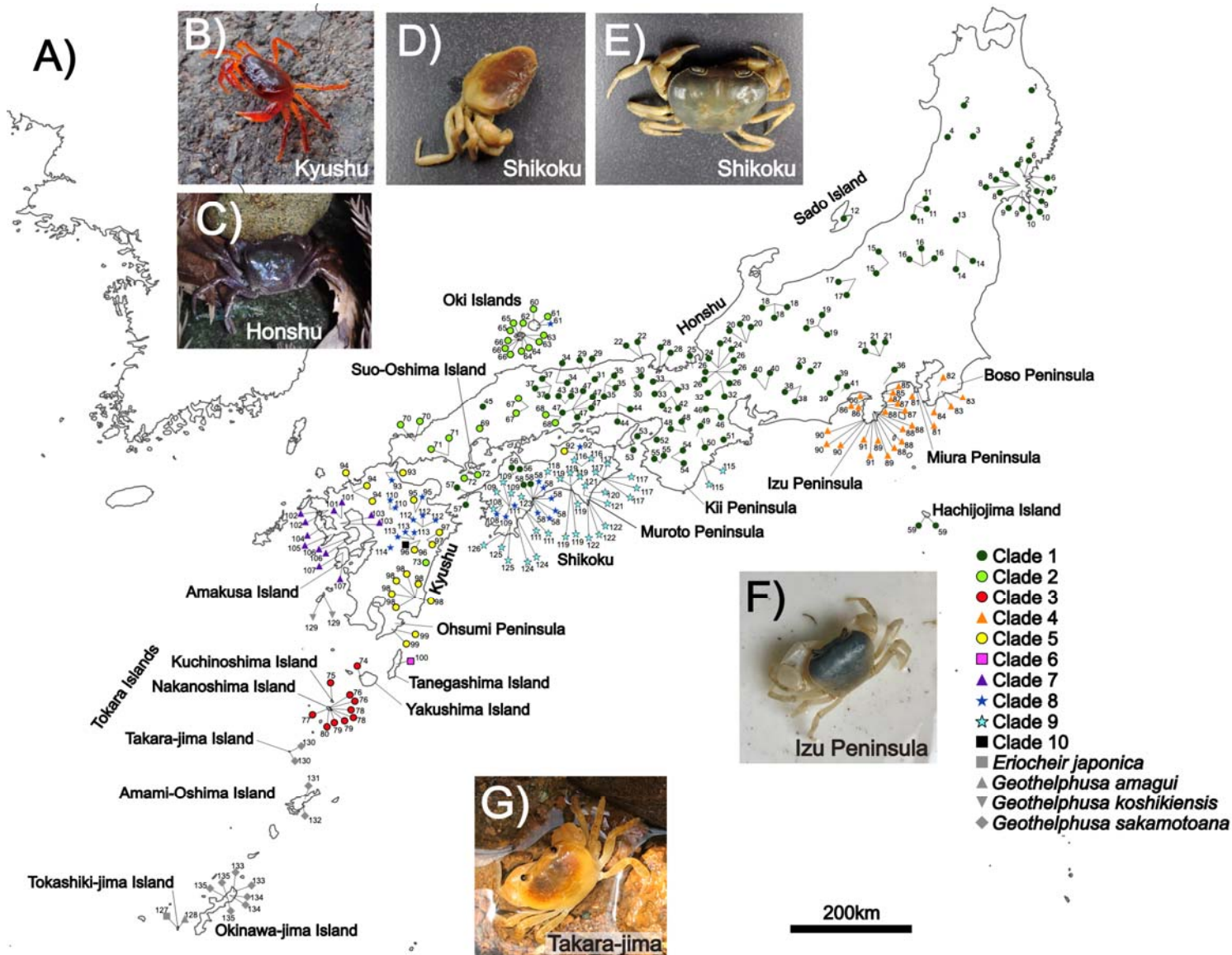


Figure 1. A) Sampling localities and the distribution area of each detected clade (clades 1-10) of the *Geothelphusa* crabs based on data from the mtDNA COI region. Please refer to Table 1 for specific locality numbers, sample numbers, and GenBank accession numbers. The color and number of each clade correspond to the estimated phylogenetic tree shown in Figures 2, 3. B–G) Photos of Sawa crabs on each island. B) Kyushu, C) Honshu, D–E) the same site (st. 116) in Shikoku, F) Izu Peninsula, G) Takara-jima Island.

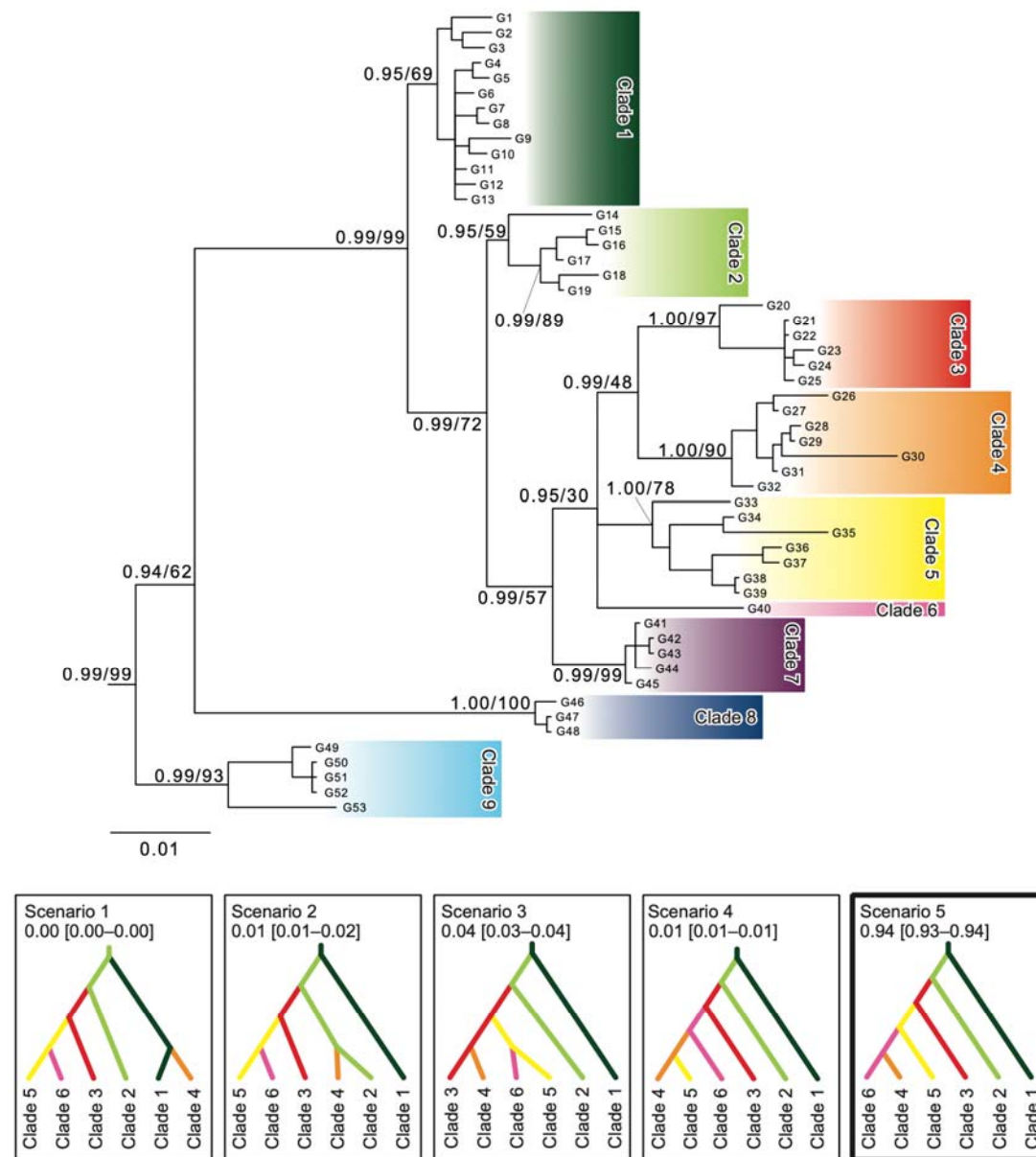


Figure 2. The estimated phylogenetic relationships [Bayes tree] of the *Geothelphusa* crabs based on the sequenced data of the combined dataset (mt DNA COI, 16S, and nDNA ITS1, histone h3: 1,796-bp) using 58 specimens. The number major nodes indicate posterior probabilities and bootstrap values. The color and number of each clade correspond to the map of sampling localities shown in Figures 1.

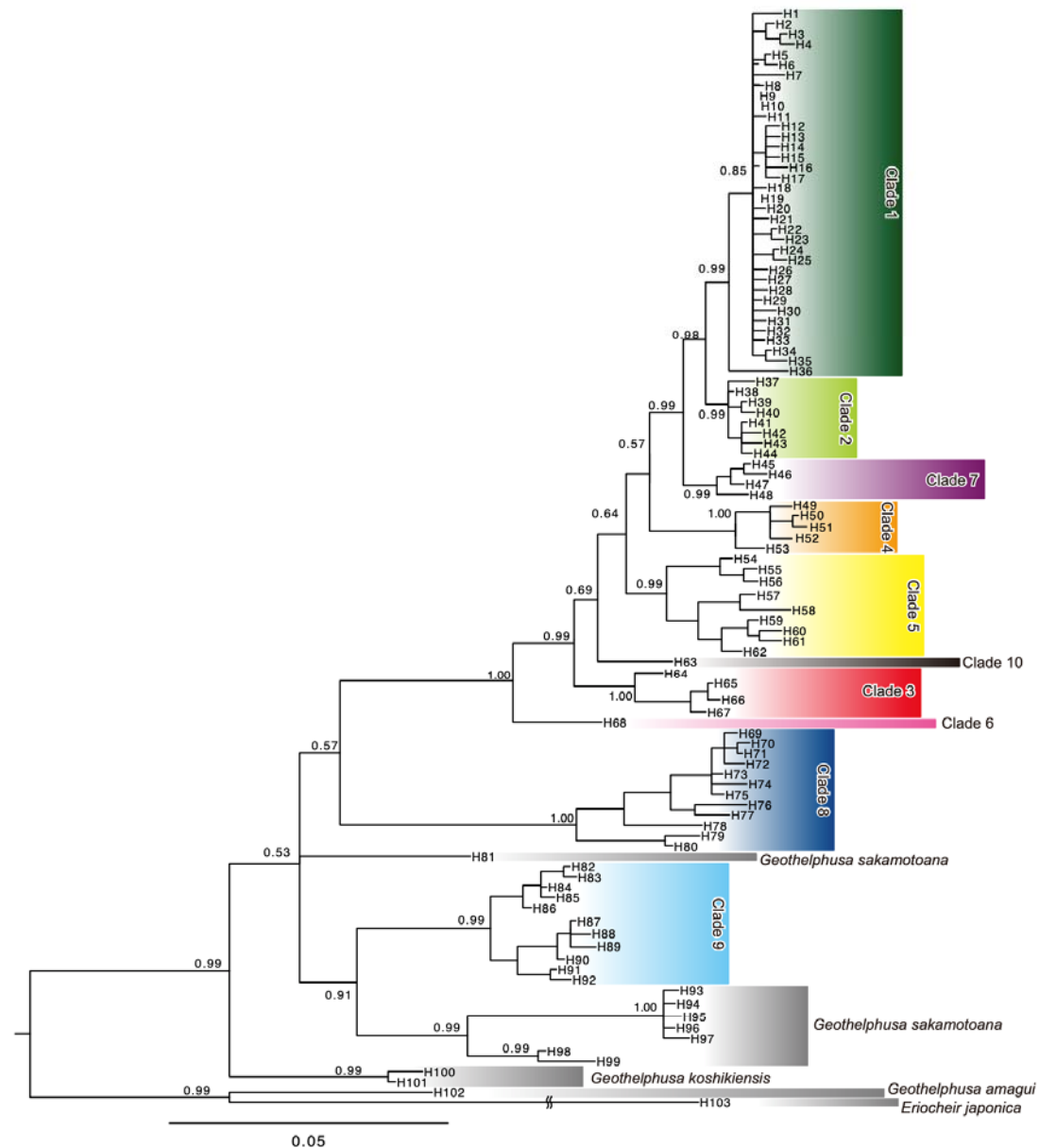


Figure 3. The estimated phylogenetic relationships [Bayes tree] of the *Geothelphusa* crabs based on the sequenced data of the mtDNA COI region (569 bp) using 283 specimens. The number major nodes indicate posterior probabilities. The color and number of each clade correspond to the map of sampling localities shown in Figures 1.

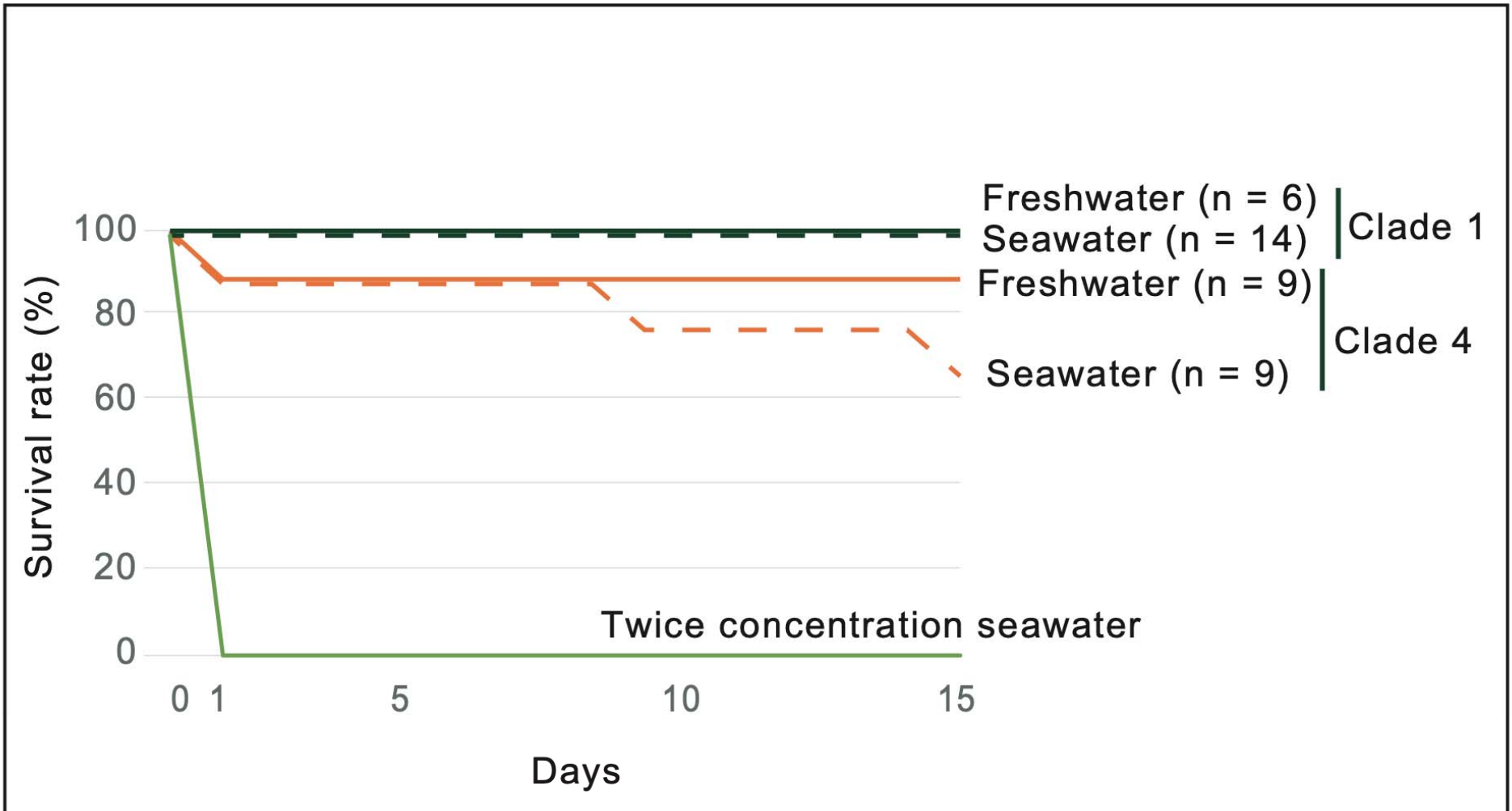


Figure 4. Survival rate of *Geothelphusa dehaani* of Clade 1 and 4 during 15 days in different salinity levels (freshwater, seawater, twice concentration seawater). The colors of survival curve correspond to each clade color in Figures 1.

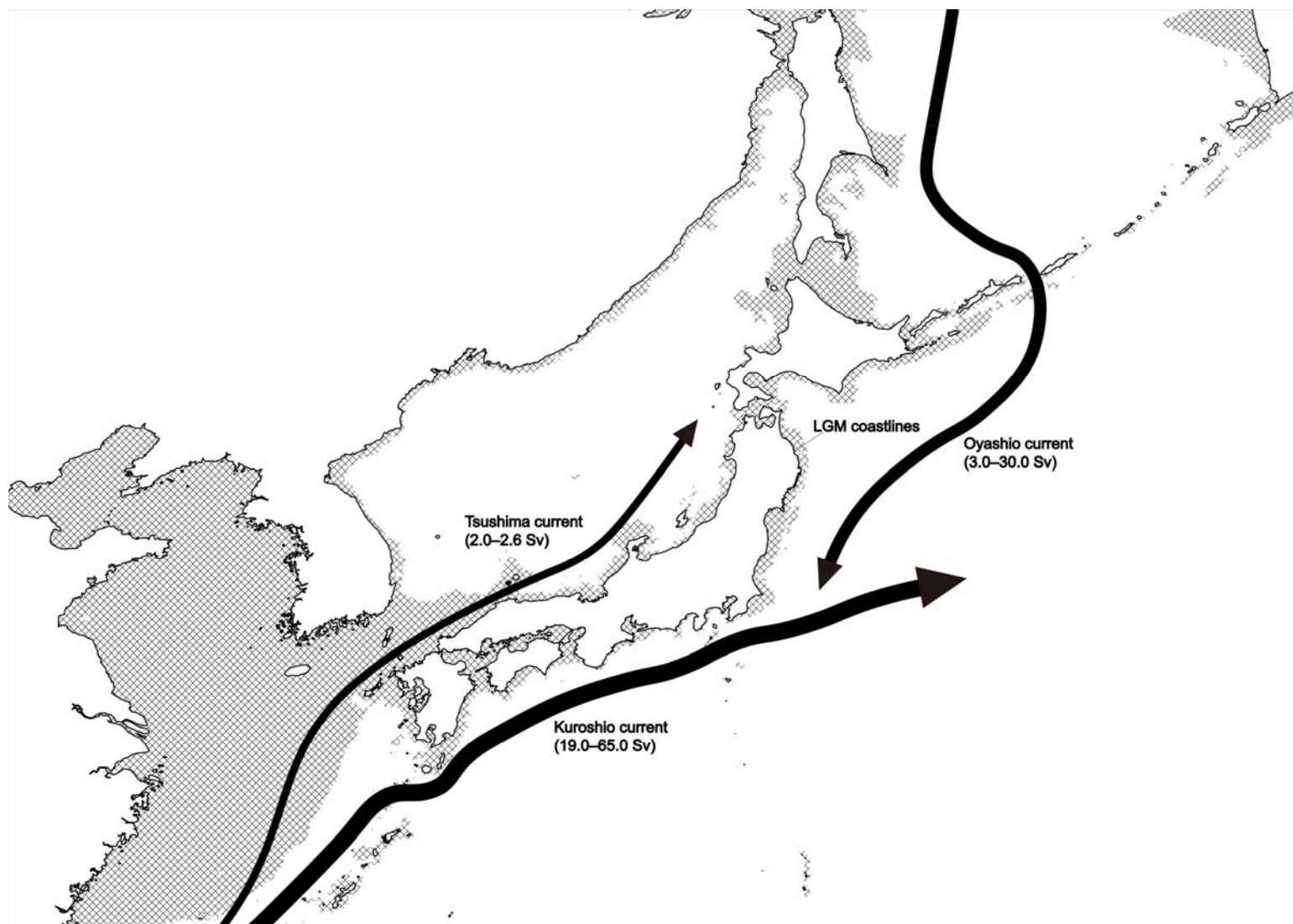


Figure 5. LGM coastlines and major currents around the Japanese Islands. LGM coastlines (Gent et al., 2011), and Sverdrup (Sv) value of each current were indicated based on previous research (Takikawa, 2005; Andres et al., 2015; Qiu, 2019).

Table 1 List of specimens of *Geothelphusa* crabs examined in this study, sequence types, and the GenBank accession numbers

Species name	Genetic Clade		Locality		Station No.	COI haplotype No.	Whole data genotype No.	GPS		GenBank Acc. No.			
	COI	Whole dataset	City / Town / Village	Prefecture				Latitude	Longitude	COI	16S	ITS1	Histone h3
<i>Geothelphusa dehaani</i>													
	Clade 1	-	Kuji	Iwate	1	H13		40.1667	141.5645				
	Clade 1	-	Kitaakita	Akita	2	H16		39.9139	140.4387				
	Clade 1	-	Misato	Akita	3	H14		39.3984	140.5890				
	Clade 1	-	Yurihonjo	Akita	4	H17		39.3842	140.1657				
	Clade 1	-	Sumita	Iwate	5	H13		39.2397	141.5281				
	Clade 1	-	Minamisanriku	Miyagi	6	H13		38.7499	141.4488				
	Clade 1	-	Minamisanriku	Miyagi	6	H13		38.7499	141.4488				
	Clade 1	-	Minamisanriku	Miyagi	6	H13		38.7499	141.4488				
	Clade 1	-	Kesennuma	Miyagi	7	H13		38.7472	141.4209				
	Clade 1	-	Kesennuma	Miyagi	7	H13		38.7472	141.4209				
	Clade 1	-	Ishimaki	Miyagi	8	H13		38.5881	141.3675				
	Clade 1	-	Ishimaki	Miyagi	8	H13		38.5881	141.3675				
	Clade 1	-	Ishimaki	Miyagi	8	H13		38.5881	141.3675				
	Clade 1	-	Ishimaki	Miyagi	8	H13		38.5881	141.3675				
	Clade 1	-	Ishimaki	Miyagi	9	H13		38.5020	141.4064				
	Clade 1	-	Ishimaki	Miyagi	9	H13		38.5020	141.4064				
	Clade 1	-	Ishimaki	Miyagi	9	H13		38.5020	141.4064				
	Clade 1	-	Ishimaki	Miyagi	10	H13		38.2897	141.5542				
	Clade 1	-	Ishimaki	Miyagi	10	H13		38.2897	141.5542				

Clade 1	-	Murakami	Niigata	11	H13		38.2393	139.6286
Clade 1	-	Murakami	Niigata	11	H13		38.2393	139.6286
Clade 1	-	Murakami	Niigata	11	H13		38.2393	139.6286
Clade 1	Clade 2	Sado	Niigata	12	H36	G14	38.0196	138.4372
Clade 1		Shichikashuku	Miyagi	13	H1		37.9987	140.3036
Clade 1	Clade 1	Motomiya	Fukushima	14	H12	G5	37.5016	140.3260
Clade 1	-	Motomiya	Fukushima	14	H12		37.5016	140.3260
Clade 1	-	Uonuma	Niigata	15	H13		37.3080	139.1800
Clade 1	-	Uonuma	Niigata	15	H18		37.3080	139.1800
Clade 1	-	Minamiaizu	Fukushima	16	H12		37.2328	139.7656
Clade 1	-	Minamiaizu	Fukushima	16	H12		37.2328	139.7656
Clade 1	Clade 1	Minamiaizu	Fukushima	16	H12	G4	37.2328	139.7656
Clade 1	-	Tsunan	Niigata	17	H19		37.0205	138.6310
Clade 1	-	Tsunan	Niigata	17	H15		37.0205	138.6310
Clade 1	-	Toyama	Toyama	18	H24		36.6056	137.2905
Clade 1	-	Toyama	Toyama	18	H25		36.6056	137.2905
Clade 1	-	Toyama	Toyama	18	H24		36.6056	137.2905
Clade 1	-	Matsumoto	Nagano	19	H19		36.2479	138.0113
Clade 1	-	Matsumoto	Nagano	19	H19		36.2479	138.0113
Clade 1	-	Matsumoto	Nagano	19	H19		36.2479	138.0113
Clade 1	-	Gujo	Gifu	20	H24		35.9619	136.8187
Clade 1	-	Gujo	Gifu	20	H24		35.9619	136.8187
Clade 1	-	Gujo	Gifu	20	H13		35.9619	136.8187
Clade 1	-	Hinohara	Tokyo	21	H7		35.7401	139.0389
Clade 1	-	Hinohara	Tokyo	21	H7		35.7401	139.0389
Clade 1	-	Hinohara	Tokyo	21	H7		35.7401	139.0389

Clade 1	-	Kyotango	Kyoto	22	H5		35.6815	135.1106
Clade 1	-	Kyotango	Kyoto	22	H5		35.6815	135.1106
Clade 1	-	Iida	Nagano	23	H34		35.5597	137.6958
Clade 1	-	Ibigawa	Gifu	24	H9		35.5425	136.3466
Clade 1	-	Ibigawa	Gifu	24	H9		35.5425	136.3466
Clade 1	-	Ibigawa	Gifu	24	H25		35.5425	136.3466
Clade 1	-	Wakasa	Fukui	25	H9		35.5317	135.9209
Clade 1	-	Nagahama	Shiga	26	H3		35.4884	136.3140
Clade 1	-	Nagahama	Shiga	26	H9		35.4884	136.3140
Clade 1	-	Nagahama	Shiga	26	H20		35.4884	136.3140
Clade 1	-	Nagahama	Shiga	26	H9		35.4884	136.3140
Clade 1	-	Nagahama	Shiga	26	H20		35.4884	136.3140
Clade 1	-	Takagi	Nagano	27	H35		35.4843	137.8847
Clade 1	-	Maizuru	Kyoto	28	H3		35.4481	135.3630
Clade 1	-	Maizuru	Kyoto	28	H3		35.4481	135.3630
Clade 1	-	Tottori	Tottori	29	H19		35.4420	134.1836
Clade 1	-	Tottori	Tottori	29	H19		35.4420	134.1836
Clade 1	-	Asago	Hyogo	30	H5		35.3710	134.9075
Clade 1	-	Asago	Hyogo	30	H9		35.3710	134.9075
Clade 1	-	Yazu	Tottori	31	H33		35.3480	134.2952
Clade 1	Clade 1	Maibara	Shiga	32	H30	G2	35.3334	136.3994
Clade 1	Clade 1	Maibara	Shiga	32	H3	G12	35.3334	136.3994
Clade 1	Clade 1	Nantan	Kyoto	33	H22	G6	35.3311	135.5662
Clade 1	-	Nantan	Kyoto	33	H22		35.3311	135.5662
Clade 1	-	Nantan	Kyoto	33	H22		35.3311	135.5662
Clade 1	-	Maniwa	Okayama	34	H19		35.2998	133.6483

Clade 1	-	Maniwa	Okayama	34	H19		35.2998	133.6483
Clade 1	-	Wakasa	Tottori	35	H6		35.2754	134.4692
Clade 1	-	Wakasa	Tottori	35	H9		35.2754	134.4692
Clade 1	Clade 1	Wakasa	Tottori	35	H6	G9	35.2754	134.4692
Clade 1	-	Odawara	Kanagawa	36	H7		35.2706	139.1011
Clade 1	-	Shinjyo	Okayama	37	H21		35.2465	133.5714
Clade 1	-	Shinjyo	Okayama	37	H19		35.2465	133.5714
Clade 1	-	Shinjyo	Okayama	37	H21		35.2465	133.5714
Clade 1	-	Anan	Nagano	38	H8		35.2447	137.7336
Clade 1	Clade 1	Anan	Nagano	38	H8	G1	35.2447	137.7336
Clade 1	-	Shizuoka	Shizuoka	39	H2		35.2197	138.3487
Clade 1	-	Shizuoka	Shizuoka	39	H2		35.2197	138.3487
Clade 1	-	Toyota	Aichi	40	H10		35.1820	137.1355
Clade 1	-	Toyota	Aichi	40	H10		35.1820	137.1355
Clade 1	-	Shizuoka	Shizuoka	41	H2		35.1229	138.4269
Clade 1	-	Kameoka	Kyoto	42	H31		35.0656	135.5746
Clade 1	-	Kameoka	Kyoto	42	H31		35.0656	135.5746
Clade 1	-	Misaki	Okayama	43	H21		34.9431	133.8003
Clade 1	-	Misaki	Okayama	43	H21		34.9431	133.8003
Clade 1	-	Himeji	Hyogo	44	H22		34.9381	134.6850
Clade 1	-	Himeji	Hyogo	44	H23		34.9381	134.6850
Clade 1	-	Ohnan	Shimane	45	H10		34.8842	132.4315
Clade 1	-	Kameyama	Mie	46	H9		34.8516	136.3216
Clade 1	-	Kameyama	Mie	46	H20		34.8516	136.3216
Clade 1	-	Okayama	Okayama	47	H21		34.8432	133.9241
Clade 1	-	Okayama	Okayama	47	H21		34.8432	133.9241

Clade 1	-	Okayama	Okayama	47	H21		34.8432	133.9241
Clade 1	-	Okayama	Okayama	47	H21		34.8432	133.9241
Clade 1	-	Okayama	Okayama	47	H21		34.8432	133.9241
Clade 1	-	Asuka	Nara	48	H26		34.4635	135.8027
Clade 1	-	Asuka	Nara	48	H26		34.4635	135.8027
Clade 1	-	Higashiyoshino	Nara	49	H4		34.3987	135.9936
Clade 1	-	Higashiyoshino	Nara	50	H3		34.3765	136.0691
Clade 1	Clade 1	Taiki	Mie	51	H3	G3	34.3358	136.4378
Clade 1	-	Sennan	Osaka	52	H10		34.3338	135.3251
Clade 1	-	Sumoto	Hyogo	53	H11		34.2928	134.8768
Clade 1	-	Sumoto	Hyogo	53	H11		34.2928	134.8768
Clade 1	Clade 1	Tanabe	Wakayama	54	H10	G10	34.0539	135.5705
Clade 1	Clade 1	Tanabe	Wakayama	54	H27	G11	34.0539	135.5705
Clade 1	Clade 1	Hidakagawa	Wakayama	55	H32	G13	33.9531	135.3833
Clade 1	-	Hidakagawa	Wakayama	55	H32		33.9531	135.3833
Clade 1	-	Toon	Ehime	56	H29		33.7562	132.9235
Clade 1	Clade 1	Toon	Ehime	56	H29	G7	33.7562	132.9235
Clade 1	-	Ikata	Ehime	57	H29		33.4315	132.2257
Clade 1	Clade 1	Ikata	Ehime	57	H28	G8	33.4315	132.2257
Clade 1	-	Kesennuma	Miyagi	58	H13		33.3638	133.2527
Clade 1	-	Kesennuma	Miyagi	58	H19		33.3638	133.2527
Clade 1	-	Hachijo	Tokyo	59	H29		33.0650	139.8163
Clade 1	-	Hachijo	Tokyo	59	H29		33.0650	139.8163
Clade 2	-	Okinoshima	Shimane	60	H41		36.3037	133.3137
Clade 2	-	Okinoshima	Shimane	61	H41		36.2498	133.3128
Clade 2	-	Nishinoshima	Shimane	62	H41		36.0897	133.0257

Clade 2	-	Ama	Shimane	63	H42		36.0841	133.1038
Clade 2	-	Ama	Shimane	63	H43		36.0841	133.1038
Clade 2	-	Ama	Shimane	64	H41		36.0724	133.0879
Clade 2	Clade 2	Ama	Shimane	64	H41	G18	36.0724	133.0879
Clade 2	-	Nishinoshima	Shimane	65	H41		36.0703	133.0313
Clade 2	-	Nishinoshima	Shimane	65	H41		36.0703	133.0313
Clade 2	-	Chiburi	Shimane	66	H41		36.0203	133.0256
Clade 2	-	Chiburi	Shimane	66	H41		36.0203	133.0256
Clade 2	-	Chiburi	Shimane	66	H41		36.0203	133.0256
Clade 2	-	Shobara	Hiroshima	67	H39		34.8881	133.1903
Clade 2	-	Shobara	Hiroshima	67	H39		34.8881	133.1903
Clade 2	-	Soja	Okayama	68	H39		34.7188	133.7318
Clade 2	-	Soja	Okayama	68	H40		34.7188	133.7318
Clade 2	-	Hiroshima	Hiroshima	69	H38		34.5213	132.3784
Clade 2	-	Mine	Yamaguchi	70	H41		34.3159	131.2937
Clade 2	-	Mine	Yamaguchi	70	H44		34.3159	131.2937
Clade 2	Clade 2	Shunan	Yamaguchi	71	H44	G19	34.1091	131.8628
Clade 2	Clade 2	Shunan	Yamaguchi	71	H44	G17	34.1091	131.8628
Clade 2	Clade 2	Suo-oshima	Yamaguchi	72	H37	G15	33.9305	132.2279
Clade 2	Clade 2	Suo-oshima	Yamaguchi	72	H37	G16	33.9305	132.2279
Clade 2	-	Tsuno	Miyazaki	73	H41		32.2859	131.4663
Clade 3	Clade 3	Yakushima	Kagoshima	74	H64	G20	30.3317	130.3989
Clade 3	Clade 3	Toshima	Kagoshima	75	H66	G24	29.9866	129.9226
Clade 3	Clade 3	Toshima	Kagoshima	76	H65	G21	29.8511	129.8448
Clade 3	Clade 3	Toshima	Kagoshima	76	H65	G22	29.8511	129.8448
Clade 3	Clade 3	Toshima	Kagoshima	77	H65	G25	29.8426	129.8478

Clade 3	Clade 3	Toshima	Kagoshima	78	H67	G23	29.8404	129.8904
Clade 3	-	Toshima	Kagoshima	78	H65		29.8404	129.8904
Clade 3	-	Toshima	Kagoshima	79	H65		29.8396	129.8913
Clade 3	-	Toshima	Kagoshima	79	H65		29.8396	129.8913
Clade 3	-	Toshima	Kagoshima	80	H65		29.8367	129.8586
Clade 4	-	Yokosuka	Kanagawa	81	H52		35.2567	139.6347
Clade 4	-	Yokosuka	Kanagawa	81	H52		35.2567	139.6347
Clade 4	-	Kimitsu	Chiba	82	H52		35.2524	139.9978
Clade 4	-	Kimitsu	Chiba	83	H52		35.1852	140.0601
Clade 4	Clade 4	Kimitsu	Chiba	83	H52	G29	35.1852	140.0601
Clade 4	Clade 4	Kimitsu	Chiba	84	H52	G30	35.1763	140.0205
Clade 4	-	Atami	Shizuoka	85	H50		35.1382	139.0580
Clade 4	-	Atami	Shizuoka	85	H50		35.1382	139.0580
Clade 4	-	Shimizu	Shizuoka	86	H50		35.1018	138.9016
Clade 4	-	Shimizu	Shizuoka	86	H50		35.1018	138.9016
Clade 4	-	Shimizu	Shizuoka	86	H51		35.1018	138.9016
Clade 4	-	Ito	Shizuoka	87	H50		34.9337	139.0606
Clade 4	-	Ito	Shizuoka	87	H50		34.9337	139.0606
Clade 4	-	Ito	Shizuoka	87	H50		34.9337	139.0606
Clade 4	-	Izu	Shizuoka	88	H52		34.8373	138.9308
Clade 4	-	Izu	Shizuoka	88	H50		34.8373	138.9308
Clade 4	-	Izu	Shizuoka	88	H52		34.8373	138.9308
Clade 4	Clade 4	Izu	Shizuoka	88	H50	G26	34.8373	138.9308
Clade 4	Clade 4	Izu	Shizuoka	88	H50	G27	34.8373	138.9308
Clade 4	Clade 4	Kawazu	Shizuoka	89	H52	G31	34.8120	138.9076
Clade 4	-	Kawazu	Shizuoka	89	H52		34.8120	138.9076

Clade 4	-	Minamiizu	Shizuoka	90	H49		34.6995	138.8024
Clade 4	-	Minamiizu	Shizuoka	90	H49		34.6995	138.8024
Clade 4	-	Minamiizu	Shizuoka	90	H49		34.6995	138.8024
Clade 4	Clade 4	Shimoda	Shizuoka	91	H52	G28	34.6965	138.9066
Clade 4	Clade 4	Shimoda	Shizuoka	91	H53	G32	34.6965	138.9066
Clade 5	-	Manno	Kagawa	92	H62		34.1279	133.9921
Clade 5	Clade 5	Kitakyushu	Fukuoka	93	H58	G35	33.7905	130.8175
Clade 5	Clade 5	Saga	Saga	94	H57	G34	33.4632	130.2767
Clade 5	-	Saga	Saga	94	H57		33.4632	130.2767
Clade 5	-	Saga	Saga	94	H57		33.4632	130.2767
Clade 5	Clade 5	Yufu	Oita	95	H59	G37	33.1263	131.3083
Clade 5	-	Hinokage	Miyazaki	96	H59		32.6601	131.3901
Clade 5	Clade 5	Nobeoka	Miyazaki	97	H60	G36	32.6597	131.5048
Clade 5	-	Nobeoka	Miyazaki	97	H60		32.6597	131.5048
Clade 5	-	Nichinan	Miyazaki	98	H54		31.7121	131.3082
Clade 5	-	Nichinan	Miyazaki	98	H55		31.7121	131.3082
Clade 5	-	Nichinan	Miyazaki	98	H54		31.7121	131.3082
Clade 5	Clade 5	Nichinan	Miyazaki	98	H54	G33	31.7121	131.3082
Clade 5	-	Nichinan	Miyazaki	98	H54		31.7121	131.3082
Clade 5	-	Nichinan	Miyazaki	98	H56		31.7121	131.3082
Clade 5	Clade 5	Kinko	Kagoshima	99	H61	G38	31.1843	130.9019
Clade 5	Clade 5	Kinko	Kagoshima	99	H61	G39	31.1843	130.9019
Clade 6	Clade 6	Nishinoomote	Kagoshima	100	H68	G40	30.5741	131.0085
Clade 7	Clade 7	Arita	Saga	101	H45	G45	33.2084	129.8750
Clade 7	-	Arita	Saga	101	H45		33.2084	129.8750
Clade 7	-	Kashima	Saga	102	H45		33.0466	130.0772

Clade 7	-	Kashima	Saga	102	H45		33.0466	130.0772
Clade 7	-	Kashima	Saga	102	H45		33.0466	130.0772
Clade 7	-	Omura	Nagasaki	103	H45		32.9762	130.0662
Clade 7	-	Omura	Nagasaki	103	H46		32.9762	130.0662
Clade 7	Clade 7	Nagasaki	Nagasaki	104	H45	G41	32.9258	129.7718
Clade 7	Clade 7	Nagasaki	Nagasaki	105	H45	G44	32.7960	129.9217
Clade 7	Clade 7	Unzen	Nagasaki	106	H47	G42	32.7602	130.2400
Clade 7	Clade 7	Unzen	Nagasaki	106	H47	G43	32.7602	130.2400
Clade 7	-	Amakusa	Kumamoto	107	H48		32.4665	130.0924
Clade 7	-	Amakusa	Kumamoto	107	H48		32.4665	130.0924
Clade 8	-	Okinoshima	Shimane	61	H80		36.2498	133.3128
Clade 8	-	Manno	Kagawa	92	H72		34.1279	133.9921
Clade 8	-	Kitakyushu	Fukuoka	93	H77		33.7905	130.8175
Clade 8	-	Susaki	Kochi	58	H69		33.3638	133.2527
Clade 8	Clade 8	Susaki	Kochi	58	H69	G48	33.3638	133.2527
Clade 8	-	Susaki	Kochi	58	H69		33.3638	133.2527
Clade 8	-	Susaki	Kochi	58	H69		33.3638	133.2527
Clade 8	Clade 8	Susaki	Kochi	58	H69	G47	33.3638	133.2527
Clade 8	-	Susaki	Kochi	58	H69		33.3638	133.2527
Clade 8	Clade 8	Matsuno	Ehime	108	H70	G46	33.2567	132.7700
Clade 8	-	Shimanto	Kochi	109	H70		33.2252	132.8779
Clade 8	-	Yame	Fukuoka	110	H73		33.1816	130.8791
Clade 8	-	Yame	Fukuoka	110	H73		33.1816	130.8791
Clade 8	-	Shimanto	Kochi	111	H71		33.1720	133.1343
Clade 8	-	Yufu	Oita	95	H75		33.1263	131.3083
Clade 8	-	Bungo-ohno	Oita	112	H77		32.8832	131.4314

Clade 8	-	Bungo-ohno	Oita	112	H77		32.8832	131.4314
Clade 8	-	Bungo-ohno	Oita	112	H77		32.8832	131.4314
Clade 8	-	Takachiho	Miyazaki	113	H79		32.6859	131.2831
Clade 8	-	Takachiho	Miyazaki	113	H78		32.6859	131.2831
Clade 8	-	Takachiho	Miyazaki	113	H76		32.6859	131.2831
Clade 8	-	Yatsushiro	Kumamoto	114	H74		32.5345	130.8938
Clade 9	-	Kumano	Mie	115	H91		33.9691	136.0707
Clade 9	-	Kumano	Mie	115	H92		33.9691	136.0707
Clade 9	-	Tsurugi	Tokushima	116	H82		33.8932	134.0716
Clade 9	-	Tsurugi	Tokushima	116	H83		33.8932	134.0716
Clade 9	-	Mugi	Tokushima	117	H85		33.6993	134.4227
Clade 9	-	Mugi	Tokushima	117	H85		33.6993	134.4227
Clade 9	-	Mugi	Tokushima	117	H85		33.6993	134.4227
Clade 9	-	Mugi	Tokushima	117	H85		33.6993	134.4227
Clade 9	-	Mugi	Tokushima	117	H85		33.6993	134.4227
Clade 9	-	Tosa	Kochi	118	H82		33.6839	133.4791
Clade 9	Clade 9	Aki	Kochi	119	H82	G52	33.5687	133.8978
Clade 9	-	Aki	Kochi	119	H82		33.5687	133.8978
Clade 9	Clade 9	Aki	Kochi	119	H82	G51	33.5687	133.8978
Clade 9	-	Aki	Kochi	119	H82		33.5687	133.8978
Clade 9	Clade 9	Aki	Kochi	119	H82	G50	33.5687	133.8978
Clade 9	-	Aki	Kochi	119	H82		33.5687	133.8978
Clade 9	-	Muroto	Kochi	120	H84		33.4604	134.1960
Clade 9	Clade 9	Muroto	Kochi	121	H86	G49	33.4162	134.1115
Clade 9	-	Muroto	Kochi	121	H86		33.4162	134.1115
Clade 9	-	Muroto	Kochi	122	H84		33.3424	134.1743

Clade 9	-	Muroto	Kochi	122	H84		33.3424	134.1743
Clade 9	-	Muroto	Kochi	122	H84		33.3424	134.1743
Clade 9	-	Nakatosa	Kochi	123	H87		33.3087	133.1943
Clade 9	-	Matsuno	Ehime	108	H90		33.2567	132.7700
Clade 9	-	Shimanto	Kochi	109	H87		33.2252	132.8779
Clade 9	-	Shimanto	Kochi	109	H88		33.2252	132.8779
Clade 9	-	Shimanto	Kochi	109	H87		33.2252	132.8779
Clade 9	-	Shimanto	Kochi	111	H87		33.1720	133.1343
Clade 9	Clade 9	Shimanto	Kochi	111	H87	G53	33.1720	133.1343
Clade 9	-	Shimanto	Kochi	124	H87		33.0980	132.9683
Clade 9	-	Shimanto	Kochi	124	H87		33.0980	132.9683
Clade 9	-	Mihara	Kochi	125	H89		32.8949	132.8838
Clade 9	-	Mihara	Kochi	125	H89		32.8949	132.8838
Clade 9	-	Tosashimizu	Kochi	126	H89		32.7902	132.7809
Clade 10	-	Hinokage	Miyazaki	96	H63		32.6601	131.3901
<i>Eriocheir japonica</i>								
Outgroup	-	Tokashiki	Okinawa	127	H103		26.2178	127.3664
<i>Geothelphusa amagui</i>								
Outgroup	-	Tokashiki	Okinawa	128	H102		26.1888	127.3542
<i>Geothelphusa koshikiensis</i>								
Outgroup	-	Satsumasendai	Kagoshima	129	H100		31.8127	129.8313
Outgroup	-	Satsumasendai	Kagoshima	129	H101		31.8127	129.8313
<i>Geothelphusa sakamotoana</i>								
Outgroup	Outgroup	Toshima	Kagoshima	130	H81		29.1487	129.2118
Outgroup	-	Toshima	Kagoshima	130	H81		29.1487	129.2118
Outgroup	Outgroup	Amami	Kagoshima	131	H98		28.3291	129.4816

Outgroup	Outgroup	Amami	Kagoshima	132	H99	28.2659	129.4277
Outgroup	-	Kunigami	Okinawa	133	H95	26.8039	128.2863
Outgroup	Outgroup	Kunigami	Okinawa	133	H96	26.8039	128.2863
Outgroup	-	Kunigami	Okinawa	134	H97	26.7613	128.2192
Outgroup	-	Kunigami	Okinawa	134	H97	26.7613	128.2192
Outgroup	-	Kunigami	Okinawa	135	H93	26.7006	128.1956
Outgroup	Outgroup	Kunigami	Okinawa	135	H93	26.7006	128.1956
Outgroup	-	Kunigami	Okinawa	135	H94	26.7006	128.1956

Table 2 Primers used in this study

	Primer name	Sequence 5'–3'	Reference	Annealing temperature
COI	LCO1490	GGTCAACAAATCATAAAGATATT GG	Folmer et al. (1994)	45°C
	HCOoutout	GTAAATATATGRTGDGCTC	Prendini et al. (2005)	
16S	1471	CCTGTTTANCAAAAACAT	Crandall and Fitzpatrick (1996)	44°C
	1472	AGATAGAAACCAACCTGG	Crandall and Fitzpatrick (1996)	
ITS1	PT1	GGAAGTAAAAGTCGTAACAAGG	Tang et al. (2003)	48°C
	PT2R	ATCGACCCACGAGCCGAGTGAC	Tang et al. (2003)	
histone h3	H3AF	ATGGCTCGTACCAAGCAGACVGC	Terry and Whiting (2005)	48°C
	H3AR	ATATCCTTRGGCATRATRG TGAC	Terry and Whiting (2005)	

Table 3. Genetic distance (p -distance) between each clade of *Geothelphusa dehaani* based on the mtDNA COI (569-bp) region.

	Clade1	Clade2	Clade3	Clade4	Clade5	Clade6	Clade7	Clade8
Clade2	0.015	-	-	-	-	-	-	-
Clade3	0.041	0.041	-	-	-	-	-	-
Clade4	0.032	0.034	0.041	-	-	-	-	-
Clade5	0.031	0.035	0.041	0.042	-	-	-	-
Clade6	0.048	0.049	0.046	0.051	0.046	-	-	-
Clade7	0.021	0.019	0.04	0.037	0.029	0.046	-	-
Clade8	0.090	0.090	0.091	0.087	0.092	0.083	0.084	-
Clade9	0.075	0.079	0.082	0.079	0.081	0.082	0.074	0.077

Table 4 Mismatch distribution analysis and neutrality tests of each clades

	n	Hd	π	Tajima's D	Fu's Fs
All samples	268	0.981	0.042	-0.533	-17.942*
Clade1	120	0.949	0.006	-1.942*	-33.646*
Clade2	22	0.697	0.003	-1.155	-2.305
Clade3	9	0.583	0.002	-1.678	-0.822
Clade4	29	0.810	0.010	-1.298	1.634
Clade5	17	0.912	0.018	0.487	1.649
Clade6	1	-	-	-	-
Clade7	13	0.615	0.004	-0.599	1.283
Clade8	22	0.900	0.018	-0.895	0.492
Clade9	34	0.877	0.012	0.963	0.324

n, sample size; Hd, haplotype diversity; π , nucleotide diversity; *, $p < 0.01$

Table 5. The experiment of the salt tolerance evaluation for *Geothelphusa dehaani*

Ind.	sites	size	first 15 days	survive days	second 15 days	survive days
1	St. 88	23 × 19	Freshwater	15<	Seawater	15<
2	St. 88	23 × 18	Freshwater	15<	Seawater	15<
3	St. 88	6 × 5	Freshwater	15<	Seawater	1
4	St. 88	6 × 5	Freshwater		3 -	
5	St. 88	6 × 5	Freshwater	15<	Seawater	8
6	St. 88	27 × 20	Seawater	15<	Freshwater	15<
7	St. 88	22 × 17	Seawater	15<	Freshwater	15<
8	St. 88	6 × 5	Seawater		14 -	
9	St. 88	6 × 5	Seawater	15<	Freshwater	15<
10	St. 88	6 × 5	Seawater	15<	Freshwater	15<
11	St. 88	22 × 18	Twice seawater	<1	-	
12	St. 88	19 × 15	Twice seawater	<1	-	
13	St. 88	6 × 5	Twice seawater	<1	-	
14	St. 88	6 × 5	Twice seawater	<1	-	
15	St. 88	5 × 4	Twice seawater	<1	-	
16	Nagano*	11 × 9	Seawater	15<	-	
17	Nagano*	4 × 3	Seawater	15<	-	
18	Nagano*	4 × 3	Seawater	15<	-	
19	Nagano*	7 × 6	Seawater	15<	-	
20	Nagano*	6 × 5	Seawater	15<	-	
21	Nagano*	6 × 5	Seawater	15<	-	
22	Nagano*	5 × 4	Seawater		15 -	
23	Nagano*	6 × 5	Seawater	15<	-	
24	St. 19	N/A	Seawater	15<	Freshwater	15<

25	St. 19	N/A	Seawater	15<	Freshwater	15<
26	St. 19	N/A	Seawater	15<	Freshwater	15<
27	St. 19	N/A	Seawater	15<	Freshwater	15<
28	St. 19	N/A	Seawater	15<	Freshwater	15<
29	St. 19	N/A	Seawater	15<	Freshwater	15<

Ind., Individual number; sites, collected sites of Individual sample; size, body size of the shell of each individual (horizontal x vertical); Nagano*, Sugadaira Nagano which is the site not used for genetic analysis