Population genetic variation and genetic structure of widely distributed Japanese endemic freshwater crustaceans: Geothelphusa dehaani and Gammarus nipponensis

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Population genetic variation and genetic structure of widely distributed Japanese endemic freshwater crustaceans: *Geothelphusa dehaani* and *Gammarus nipponensis*

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2024

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General Introduction

1.1 Freshwater biodiversity

The Japanese archipelago is considered as one of the world's biodiversity hotspot, owing to its strategic location in the Western Pacific and its extensive archipelagic formation, which spans a diverse array of climatic zones, encompassing from subarctic to subtropical biomes (Fujikura et al. 2010). Similarly, the high biodiversity in Japan can be attributed to the archipelago's fragmented nature, which is caused by biogeographic barriers such as seas and straits. The very complex geological history of the archipelago also significantly contributed to its highly diverse biota (Yonekura et al. 2001; Motokawa 2017). The diverse array of biological specimens found within this archipelago provides a compelling subject for scientific study.

Freshwater ecosystems play a crucial role as habitats for nearly 10% of the described species in the world despite covering about 0.01% of the world's aquatic environment (Dudgeon et al. 2006; Balian et al. 2008; Reid et al. 2018). Numerous studies have demonstrated that freshwater habitats exhibit lower levels of species diversity compared to terrestrial or marine environments. This reduced diversity can be attributed to a variety of factors, including habitat degradation, impacts of climate change, overexploitation of organisms for human consumption, introduction of invasive species and spread of diseases (Dudgeon et al. 2006; Clausnitzer et al. 2009; Cumberlidge et al. 2009; Collen et al. 2014; He et al. 2017). Hence, evaluating the status of species distributions, population dynamics and intraspecific genetic variations is imperative to preserve and protect the freshwater biodiversity and habitats. Due to this, researchers have focused their efforts on population genetics and phylogeographic studies of freshwater species.

Freshwater species provides valuable insights into the relationships between geological events and the formation of fauna due to their inherently limited dispersal abilities (Avise 2000). The geological isolation of any organism causes a disruption in gene flow and contributes to the establishment of differences between its fragmented populations. In Japan, most of the genetic population and phylogeographic studies has predominantly centered on freshwater fishes among the various freshwater species. These investigations have yielded significant findings, including the identification of cryptic lineages (Tominaga et al. 2016; Miranda et al. 2018), the detection of genetic disturbances resulting from artificial introductions (Katsumura et al. 2012; Uemura et al. 2018; Miyake et al. 2021) and contributions to our understanding of the formation of freshwater fish fauna (Watanabe et al. 2017; Tominaga et al. 2020). Likewise, these studies have provided significant information of their genetic structures and phylogeographic patterns. However, the magnitude of gene flow, influenced by the dispersal capability in freshwater fish and responsible for determining the degree of genetic variations among populations, may vary from that observed in other freshwater organisms. Consequently, these findings may not accurately reflect the outcomes observed in other organisms. Due to this, it is important to determine the extent of dispersal ability and its corresponding effects on population and phylogeographic structures from other organisms.

Population genetic studies provide indirect methods to estimate patterns of dispersal. The level of genetic differentiation among populations is dependent on the degree of gene flow among habitats and the extent of habitat fragmentation. These levels of gene flow among populations can be inferred using molecular markers such as mitochondrial DNA (Han et al. 2022; Shih et al. 2022; Hardianto et al. 2023; Wei et al.

2023; Xia et al. 2023). Inferring patterns of population dispersal involves estimating haplotype frequencies at various genes and determine the extent of dispersal required to account for the variance in haplotype frequencies among populations (Hughes et al. 2009). In situations where dispersal is limited among populations, haplotype frequencies are more likely to diverge due to random processes or natural selection, leading to a greater proportion of genetic variation occurring among populations (Slatkin 1985). Conversely, when dispersal is widespread, haplotype frequencies tend to be homogenous due to the mixing of haplotypes from different populations and a significant portion of genetic diversity is observed within populations (Hughes et al. 2009).

1.2 Mitochondrial DNA as molecular markers

The assessment of genetic diversity or variance plays a crucial role in the interpretation and management of both populations and individuals. As organisms continuously undergo evolutionary processes, it is inherent to anticipate genetic diversity within natural populations (Avise 2000). Currently, the available methods for evaluating the biodiversity and evolutionary progression of organisms differ in terms of the information produced and the portion of the genome being analyzed (Harrison 1989). These molecular markers can be categorized into two main groups: nuclear DNA markers and mitochondrial DNA (mtDNA) markers. This categorization is based on how they are inherited and their modes of evolutionary change (Park and Moran 1994).

In this study, mtDNA markers were chosen for several compelling reasons: Firstly, mtDNA is relatively straightforward to amplify since it exists in multiple copies within the cell. Moreover, the mitochondrial gene content remains highly conserved

across animal species, characterized by minimal duplications, the absence of introns and short intergenic regions. Additionally, mtDNA exhibits significant variability in natural populations due to its elevated mutation rate, allowing it to provide insights into population history over short periods of time (Galtier et al. 2009). Furthermore, mtDNA proves to be an effective marker for examining population structure and patterns of intraspecific geographic variation due to its substantial variation among individuals within and between populations. Numerous studies analyzing the distribution of mtDNA haplotypes have yielded evidence for the geographical structuring of populations (Hrbek et al. 2005; Elderkin et al. 2008; Watanabe et al. 2014; Arai and Taha 2021). Maternal inheritance and the absence of recombination also make mtDNA a suitable marker for tracing recent evolutionary history, including colonization (founder) events, introductions and population bottlenecks as exemplified in the studies of Tominaga et al. (2006), Fatsi et al. (2020), Ogasawara et al. (2021) and Cho and Mukai (2023). Assuming a constant rate of mtDNA sequence divergence, estimates of existing divergence can be employed to estimate the time since two individuals (or species) last shared a common maternal ancestor (Harrison 1989). Thus, mtDNA has already demonstrated its utility as a marker for defining relationships among closely related species and a convenient and cost-effective option when a new species has to be genetically explored in its natural habitat.

1.3 Study species

1.3.1 Japanese freshwater crab (*Geothelphusa dehaani*)

The freshwater crab (*Geothelphusa dehaani*) is a decapod, found only in Japan. In the genus *Geothelphusa*, *G*. *dehaani* is unique due to its extensive geographic range, extending from the Southern Kyushu Islands to the northernmost part of Honshu (Suzuki and Tsuda 1991). Notably, there have been recent documented sightings of this species in Hokkaido, however, further validation is required to determine the origin of this population whether it is native or introduced (Sugime et al. 2022). Among the potamid and true freshwater crabs in Japan, only *G*. *dehaani* inhabits more temperate locations since majority of other species are concentrated in Ryukyus (also called the Nansei Islands) . *G. dehaani* occupies a variety of environments, including streams at elevations of up to 1500 m (Suzuki 1992) and rivers with elevations of less than 200 m (Suzuki and Tsuda 1991). This distribution pattern demonstrates its ability to adapt to a wide range of habitats, thus explain its extensive distribution. *G*. *dehaani* typically forms isolated local populations within its extensive range, however, there are reports of sympatric coexistence with *Geothelphusa exigua* in the Ohsumi Peninsula (Suzuki and Tsuda 1994) and *Geothelphusa marmorata* in Yakushima Island (Suzuki and Okano 2000).

G. *dehaani* can attain a carapace size of approximately 25 mm within four years and their estimated lifespan extends to around 7 to 8 years (Araki and Matsuura 1995). The molting frequency for this species typically ranges between 2 to 3 times per year (Yamaguchi and Takamatsu 1980). As an omnivorous feeder, this crab demonstrates a broad and varied feeding preference. Its diet comprises a variety of plant matter,

although a significant portion consists of animal tissues, including aquatic insects. In addition to being omnivorous, there is evidence suggesting cannibalistic behavior in this crab. Instances have been documented where the tissues of *G*. *dehaani* were found in its stomach, likely originating from young crablets. The occurrence of crabs in the stomach contents increases during the autumn and winter seasons, which coincides with the time when young crabs become abundant in their habitat following their release from maternal care, typically occurring in August or September (Yamaguchi and Takamatsu 1980; Oshima et al. 1994; Araki and Matsuura 1995).

This crab is amphibious, and can be found beneath underwater stones and on the forest floor near streams (Shimotsukasa and Wada 1995). Normally, this freshwater crab emerges from the water to graze on land. *G*. *dehaani* was reported to survive desiccation for two days in the laboratory and its overland migrations in the field can stretch far from rivers during the wet season (Batang and Suzuki 2003). Unlike the marine brachyuran crabs, freshwater crabs lack planktonic larval stages. Instead, their females produce a limited number of relatively large-sized eggs, which undergo direct development into crablets (Hartnoll 1988). Adult *Geothelphusa* crabs can grow up to 40.0 mm in size, with a minimum reproductive size of 17.0 mm. Ovigerous females move into aquatic environments when the eggs reach the eye stage and remain submerged until all crablets have been released (Minei 1981). According to Minei (1976), the eggs hatch in late summer and juvenile crabs averaging around 4.0 mm in size detach from the mother crab a week or more after hatching. The released crablets continue to reside in the water then transition to terrestrial habitats once they reach a carapace width of 5.0 mm.

G. *dehaani* presents another interesting feature of distinct color variations (Figure 1). However, various studies have conflicting findings regarding the occurrence and the corresponding genetic correlations behind this variable coloration (Suzuki and Tsuda 1991; Ikeda et al. 1998). Suzuku and Tsuda (1991) conducted a study on *G*. *dehaani* in Kagoshima, where they observed that regional color differentiation is evident only in mature crabs (carapace width >14 mm) and is not apparent in younger crabs (carapace width <14 mm). In a related study, Ikeda et al. (1998) found that BL-colored populations in Honshu Island were genetically distant from other color types (RE and DA). The authors further proposed that the differentiation between RE and DA was not influenced by genetic factors, as indicated by electrophoretic analysis. However, the underlying genetic and biological mechanisms governing these differences remain poorly understood.

Within this species, several distinct color types have been identified (Chokki 1976):

BL-type: Carapace is graying clue or bluish green. Thoracic legs are light grayish.
RE-type: Dark brown carapace, with occasional orange or brown hues in the posterolateral region. The thoracic legs are light reddish-orange.
DA-type: Both the carapace and thoracic legs exhibit a dark purplish-brown coloration.

Figure 1. Freshwater crab *Geothelphusa dehaani* and the different color classification (a. BL, b. RE, c. DA). The taxonomic classification is shown below:

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Order: Decapoda
Infraorder: Brachyura
Family: Potamidae
Genus: *Geothelphusa*
Species: *Geothelphusa dehaani* (White 1847)
Japanese name: Sawagani

The distinct characteristics of *G. dehaani* including its extensive geographic range and limited dispersal capabilities due to the absence of a planktonic stage and the presence of population boundaries, offer valuable insights for investigating the extent of gene flow between geographically isolated populations distributed across a wide range. Molecular investigations related to clarifying the genetic relationships among populations of *G. dehaani* were only conducted in short geographic ranges using allozyme markers as genetic tool: Honshu (Aotsuka et al. 1995; Ikeda et al. 1998) and Kagoshima in Kyushu (Okano et al. 2000a). In Kanagawa Prefecture and Tokyo, Aotsuka et al. (1995)

reported a substantial genetic differentiation among different color-type populations, as well as within the BL populations. A larger geographic scale in Honshu was investigated by Ikeda et al. (1998), revealing that only the BL-colored populations were distantly related to other color types (RE and DA). While, Okano et al. (2000a) conducted a study in Southern Kyushu and the neighboring islands, indicating the absence of genetic differentiation among color types.

This is the first study to look at the population genetics and phylogeny of *G. dehaani* representing populations from Honshu to Southern Kyushu Islands using mitochondrial DNA markers.

1.3.2 Freshwater gammarid (*Gammarus nipponensis*)

Amphipods of the genus *Gammarus* are crustaceans found in a diverse range of freshwater habitats and are widespread throughout the world (Väinölä et al. 2008). The Nippon-yokoebi (*Gammarus nipponensis*) is a freshwater gammarid endemic to Japan, that belongs to the order Amphipoda, family Gammaridae (Figure 2). *G. nipponensis* was initially documented as *G.* (*Rivulogammarus*) *nipponensis* by Uéno 1940, based on specimens collected from Kiyotaki in Kyoto, Japan. This species is most commonly found in western Japan, exhibiting a wide distribution across western Honshu, Shikoku and Kyushu while other *Gammarus* species have a more restricted geographic range (Tomikawa 2017). However, despite the dense number of *G. nipponensis* in rivers and lakes, there is limited knowledge about its physiology, behavior and ecology. As a result, the subsequent descriptions are based on information from other *Gammarus* species.

The life cycle of gammarids is characterized by direct development, lacking a planktonic larval stage, similar to the Japanese freshwater crabs. Female gammarids carry their eggs in a brood chamber between their thoracic legs (pereiopods) (Väinölä et al. 2008). Within this chamber, eggs are brooded until the juveniles hatch. Upon release, these juveniles undergo several molts to reach maturity. These amphipods are known for their high reproductive capacity, characterized by the rapid production of numerous successive broods, several broods per female per year, a high number of offspring and relatively longer lifespans of 1 to 2 years (Gledhill et al. 1993). Under laboratory conditions, a female gammarid, maturing at a body length of 9 mm, can produce about 7 broods (Sheader 1983). The number of eggs produced by females in each brood is directly related to body size. Gammarids display reproductive activity throughout most of the year, with a notable peak during the spring and early summer, while breeding activity diminishes in the autumn (Pöckl et al. 2003).

Amphipods exhibit a wide range of feeding habits, encompassing herbivores, detritivores, carnivores and omnivores. While much of the research has emphasized the herbivorous nature of *Gammarus* spp. and their classification as shredder (Friberg and Jacobsen 1994; Maltby 1994; Nelson 2011), although there are reports to their significant role as predators in the macroinvertebrate community (MacNeil et al. 1997; Kelly et al. 2002). Gammarids are known for their sensitivity to various contaminants, with reports indicating they are among the most sensitive to metals and organic compounds (Wallace and Estephan 2004; De Lange et al. 2006; Alric et al. 2019). This sensitivity makes them valuable test organisms in ecotoxicological studies and reliable species for assessing water quality.

Figure 2. *Gammarus nipponensis* specimens. The taxonomic classification is shown below:

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Order: Amphipoda
Infraorder: Gammarida
Family: Gammaridae
Genus: *Gammarus*
Species: *Gammarus nipponensis* (Uéno 1940)
Japanese name: Nippon-yokoebi

Gammarus spp. typically exhibit passive dispersal. Amphipods drift downstream particularly during flood periods. However, some gammarids have developed behaviors to counteract or resist the effects of drift. For instance, female gammarids may venture

upstream to deposit their offspring in higher reaches of streams where resources are more abundant (Minckley 1964). Additionally, gammarids may undertake upstream journeys to seek mates. Nonetheless, their dispersal is still limited due to geological barriers such as seas, straits and high mountains.

Populations of *Gammarus* spp. are likely to be highly structured genetically based on their dispersal ability and fragmented habitat. However, research on genetic variations within and among *G*. *nipponensis* populations remains scarce. With only one investigation using a segment of mtDNA and nuclear genes, *G*. *nipponensis* monophyly is not adequately supported due to moderate statistical values (Tomikawa et al. 2014). Furthermore, the authors also shown a substantial genetic variation among the populations studied suggesting the presence of sibling species. With this report, it is recommended to reassess the validity of the previous reports using multiple genes or more advance molecular tool.

1.4 Research objectives

The general objective of this study is to provide insights on the population genetics and formation of freshwater crustacean biota characterized by a broad distribution range and the absence of a planktonic larval stage.

The specific objectives are to determine population genetic variations and the evolutionary history of *G*. *dehaani* and *G*. *nipponensis* throughout the Japanese Archipelago. Also, to investigate the impact of intrinsic and extrinsic causes on the formation of these patterns, as well as their relationship to the geological history of the Japanese Archipelago.

To carry out these experiments, a part of *G*. *dehaani*'s mitochondrial DNA and the mitochondrial genome of *G*. *nipponensis* were studied.

This thesis is divided into three chapters as follows:

Chapter 1: Assessment of genetic variation in the Japanese endemic freshwater crab, *Geothelphusa dehaani*, based on mitochondrial DNA sequences

Chapter 2. The complete mitochondrial genome of freshwater gammarid *Gammarus nipponensis* (Crustacea: Amphipoda: Gammaridae)

Chapter 3: Phylogeny of *Gammarus nipponensis* across Honshu to Kyushu regions based on the mitochondrial genome sequences

Chapter 2

Assessment of genetic variation in the Japanese endemic freshwater crab, *Geothelphusa dehaani*, based on mitochondrial DNA sequences

2.1 Abstract

Geothelphusa dehaani, a freshwater crab species endemic to Japan, has the largest distribution range amongst the 19 known species in the country. Due to its low dispersal capability and restricted habitat to freshwater, it serves as an excellent model for understanding gene flow between geographically isolated populations. In this study, we analyzed the genetic relationships of 34 *G. dehaani* populations collected from different locations in the Japanese archipelago using two mitochondrial DNA regions - cytochrome oxidase subunit I (COI*)* and cytochrome b (cytB). Our results from the analysis of molecular variance (AMOVA) revealed high genetic variation amongst populations and the phylogenetic analysis identified four geographical groups: Clade I - Honshu and Shikoku, Clade II - Eastern Kyushu, Clade III - Southern Kyushu and a part of Eastern Honshu and Clade IV - Western Kyushu. Notably, Clade IV exhibited the most genetic distance amongst the observed groupings. Regarding color variations, no correlations between body coloration and genetic differences were observed.

Thus, these initials findings highlight the need for further examination of *G. dehaani* in Kyushu, including morphological and behavioral traits, to better understand the observed diversity within the species in the region. Breeding experiments using juveniles from the tested localities are also suggested to investigate the color patterning and confirm its respective relation to genetic variations.

2.2 Introduction

Freshwater crabs can be found in a wide range of aquatic environments, including rivers and streams. Certain species require specific habitat conditions that may limit their range, whilst others are more tolerant of various habitat types, which may result in their widespread geographic distribution (Cumberlidge and Ng 2009). Freshwater crabs of the genus *Geothelphusa* are found in the East Asian Island Arc, which stretches from Taiwan, Ryukyus and the main islands of Japan (Shih and Ng 2011; Shy et al. 2020). Nineteen species of *Geothelphusa* have been identified in Japan, including *G. dehaani* (White 1847), *G. obtusipes* (Stimpson 1858), *G. levicervix* (Rathbun 1898), *G. sakamotoana* (Rathbun 1905), *G. tenuimanus* (Miyake and Minei 1965), *G. aramotoi* (Minei 1973), *G. exigua* (Suzuki and Tsuda 1994), *G. minei* (Shy and Ng 1998), *G. shokitai* (Shy and Ng 1998), *G. marmorata* (Suzuki and Okano 2000), *G. miyakoensis* (Shokita et al. 2002), *G. marginata fulva* (Naruse et al. 2004), *G. marginata maruginata* (Naruse et al. 2004), *G. grandiovata* (Naruse et al. 2006), *G. iheya* (Naruse et al. 2006), *G. kumejima* (Naruse et al. 2006), *G. amagui* (Naruse and Shokita 2009), *G. koshikiensis* (Suzuki and Kawai 2011) and *G. mishima* (Suzuki and Kawai 2011). From these, only five species are known north of the Ryukyus: *G. exigua* in Osumi Peninsula, *G. marmorata* in Yakushima Island, *G. koshikiensis* in Koshiki Islands, *G. mishima* in Mishima Islands and *G. dehaani* from northernmost Ryukyus to Hokkaido (Suzuki and Tsuda 1994; Suzuki and Okano 2000; Suzuki and Kawai 2011; Sugime et al. 2022). *G. dehaani* has the most widespread distribution and is the only known species that can be found in localities with colder climates as other species are concentrated in warmer freshwater habitats (Shih and Ng 2011).

These crabs only produce a few large eggs and once fertilized, the eggs develop directly into juvenile crabs, without a planktonic larval stage (Yamaguchi and Takamatsu 1980; Hartnoll 1988). Likewise, the migratory abilities of freshwater crabs are relatively weak since they are intolerant of brackish water and marine environments (Shy et al. 2020). With such characteristics, *G*. *dehaani* has low dispersal capability and geographical isolation amongst its populations can be expected. These factors also indicate low levels of genetic variation within populations and high levels of genetic variation between populations. As a result, *G*. *dehaani* can be a good model to study levels of gene flow between geographically isolated populations with a wide distribution range. Similarly, freshwater decapods are good indicators to reflect the connection between genetic isolation and past geological events (Okano et al. 2000a; Suzuki 2001; Shih et al. 2004; Shih et al. 2006; Shih et al. 2011).

Previous studies to clarify the genetic relationships amongst *G*. *dehaani* populations used allozyme markers as a genetic tool and were only conducted in short geographic ranges: Okano et al. (2000a) worked in Kagoshima mainland in Kyushu and its neighbouring islands, Ikeda et al. (1998) studied populations in Honshu and Aotsuka et al. (1995) investigated populations from Kanagawa and Tokyo prefectures in Honshu.

The current study aims to determine the degree of genetic variability and population structure of *G*. *dehaani* populations using partial sequences of mitochondrial DNA (mtDNA) from cytochrome oxidase I (COI) and cytochrome B (cytB) regions from wide geographically distinct localities across the Japanese archipelago from southern Kyushu to northern Honshu.

2.3 Material and methods

2.3.1 Sample collection

Two hundred ninety-six *G. dehaani* specimens used for the analysis of COI and cytB were obtained from 34 localities covering the Japanese mainland from Honshu to Kyushu and the neighbouring islands of Kyushu (Figure 1). The areas investigated in the present work include the known distribution range of *G*. *dehaani* (see Suzuki and Tsuda (1991)). Another species of the same genus *G. marmorata* from Yakushima Island were also sequenced (two specimens) and used as an outgroup.

The *Geothelphusa* specimens used were identified based on its morphological characters (Chokki 1976; Suzuki and Okano 2000). Body color types for *G*. *dehaani* are described as follows: RE – dark brown carapace and reddish legs; DA – dark purplish carapace and legs; and BL – greyish-blue carapace and light-grey legs (see Chokki 1976). Freshly preserved or live samples were utilized for DNA extraction.

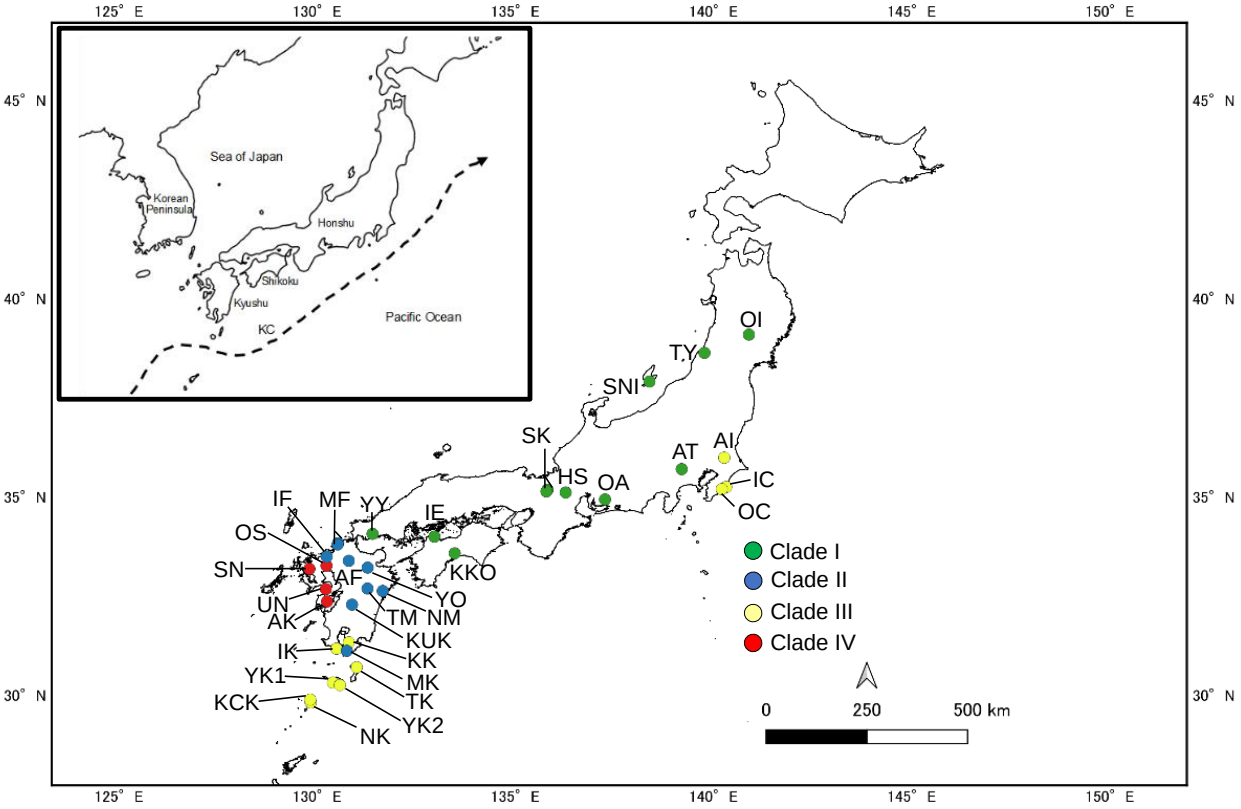

Figure 1. Map showing the locations of *Geothelphusa dehaani* populations used in the study and the respective distribution of four mitochondrial clades. Inset map shows the path of Kuroshio current (KC). Details of the population codes and the corresponding sampling sizes are presented in Table 1.

2.3.2 DNA extraction, amplification and sequencing

Total DNA was extracted from the leg muscles of each crab using the Quick DNA^{TM} Miniprep Plus Kit (Zymo Research, USA) following the manufacturer's instructions. Fragments of COI and cytB genes were amplified using the following primers: COI (COIspF: 5´-ATT AGG AGC CCC AGA TAT GGC C-3´ and COIorR: 5´-TGG TGA GCT CAT ACT ACA AAT CC-3´) and cytB (cytBorF: 5´-ATG ATT TCT CCT ATT CGA AAA TCC C-3´ and cytBotR: 5´-GAT AAA ACA AGG GCT ACA ACT CC-3´).

The primers were designed based on the published mitochondrial genome of *G. dehaani* ((GenBank Accession no. AB187570: Segawa and Aotsuka (2005)). Amplification of the target genes were carried out in a final reaction volume of 20 µl consisting of 2 µl of each 10 µM primers, 10 µl of EmeraldAmp® GT PCR Master Mix (TaKaRa Bio Inc., Japan), 5 µl milliQ water and 1 µl of DNA template. Amplification was performed in a Bio-RAD T100^{TM} thermal cycler (Bio-Rad Laboratories, USA) under the following conditions: 30 s initial denaturation step at 95°C, followed by 30 cycles of 15 s denaturation at 95°C, 15 s annealing at 56°C and 40 s extension at 72°C, then by final extension step of 1 min at 72°C.

All PCR amplification results were visualized to confirm the presence of the target size product using 1.0% agarose gel stained with ethidium bromide. Prior to sequencing, the PCR products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, USA). The sequence primers used for COI and cytB were similar to the primers in the PCR reaction. Sequencing of the purified PCR products were performed by FASMAC sequencing service (FASMAC, Japan) in an Applied Biosystems 3730xl DNA Analyzer (Thermo Fisher Scientific, USA) and the resulting chromatograms were assessed in Finch TV ver. 1.4.0 Geospiza Inc. (Patterson et al. 2006).

The sequences obtained from each gene were verified and aligned with Clustal W implemented in MEGA X software (Kumar et al. 2018). The identical haplotypes from the sequence data of COI and cytB regions were identified and collapsed using FaBox ver. 1.61 (https://users-birc.au.dk/palle/php/fabox/).

2.3.3 Data analysis

The two gene alignments were combined into concatenated data for use in the Phylogenetic analysis performed in IQ-TREE ver. 1.6.12 (Nguyen et al. 2015). Phylogenetic tree was constructed by maximum likelihood (ML) method and the reliability of internal branches were assessed by 10,000 ultrafast bootstrap replicates (Hoang et al. 2018). A partition file was prepared and edge-proportional model was applied for the analysis (Chernomor et al. 2016). The best-fit substitution model for each gene partition was selected using ModelFinder under the Bayesian Information Criterion (Schwarz 1978; Kalyaanamoorthy et al. 2017). The suggested best-fit models in each partition were TN+F+R2 and TN+F+G4 for COI and cytB, respectively.

To investigate the genetic variation within the species of *Geothelphusa* in Japan, the COI sequence data of *G. dehaani* and *G. marmorata* from this study and seven other species available in GenBank (accession numbers: AB266313, AB266312, AB625763, AB625762, AB625760, AB625728, LC743300) were used for the ML analysis. The *Geothelphusa* dataset was aligned and analyzed following similar steps above were applied to the COI dataset, but the K3Pu+F+I+G4 model was used as the best-fit model. The endemic potamid of mainland China, *Longpotamon (Sinopotamon) xiushuiense* (GenBank accession number: NC 029226) was used as an outgroup as it is sister to *Geothelphusa* in the molecular analysis of Wang et al. (2016). The cytB gene was not evaluated because there was no available sequence data from other *Geothelphusa* species in the database.

For genetic analyses, the genetic diversity indices i.e. the number of haplotypes (Hn), haplotype diversity (Hd) and nucleotide diversity (π) of each *G. dehaani* population

and the four identified clades based on the combined datasets, were calculated using Arlequin ver. 3.5 (Excoffier and Lischer 2010). Pairwise FST values, neutrality tests (Tajima's D; Fu's FS), mismatch distribution analysis based on the sudden expansion model and analysis of molecular variance (AMOVA) were also calculated in Arlequin ver. 3.5 (Excoffier and Lischer 2010).

The estimates of the time to the most recent common ancestor (TMRCA) were analyzed using the Bayesian Skyline with HKY (+G) substitution model performed in BEAST ver. 1.10.4 (Suchard et al. 2018). Rate variation was based on established mutation rates of mitochondrial regions in decapods at 2.33% per million of years (Schubart et al. 1998). The Markov Chain Monte Carlo (MCMC) analysis was run for 4 x 10^7 steps and logging parameters every 4,000 steps. Convergence of the parameters were assessed using the TRACER ver. 1.7 (Rambaut et al. 2018).

2.4 Results

2.4.1 Sequence analysis

The aligned sequence lengths of the COI region comprised of 547 base positions (bp) from the 296 *G. dehaani* samples. Eighty-five positions were variable and 65 were parsimony informative. Amongst the total number of sequences, 70 different haplotypes were recorded. The sequence of haplotypes were deposited in DDBJ database (Accession numbers LC735417 to LC735485). The nucleotide composition of the COI region was AT-rich (63.3%) (A:27.4%, T:35.9%, C:20.5%, G:16.3%). For the cytB gene, 592 bp fragment was amplified, resulting in 53 unique haplotypes. The

fragment of cytB sequences was also AT-rich (68.2%) (A:27.5%, T:40.7%, C:18.0%, G:13.9%). In this region, 85 sites were variable and 66 were parsimony informative. The haplotypes for this gene were also deposited in DDBJ database (Accession numbers LC735488 to LC735533).

When considering the combined fragments of COI and cytB, a total of 1,139 bp were generated and resulted in 96 haplotypes. Notably, the crab samples collected in Kyoto (SK1 and SK2) exhibited a shared haplotype, D2. Similarly, the populations in Fukuoka (MF1 and MF2) shared the haplotype D86, while the populations in Ibaraki and Chiba shared haplotype D91. In contrast, the remaining populations were characterized by unique haplotypes, as shown in Table 1.

2.4.2 Phylogenetic analysis

The phylogenetic tree of the combined COI and cytB datasets, along with the respective bootstrap values are shown in Figure 2. The *G dehaani* populations are monophyletic with four geographical groups: Clade I – Honshu and Shikoku; Clade II – Eastern Kyushu; Clade III – Southern Kyushu and a part of Eastern Honshu; and Clade IV – Western Kyushu. All of these clades had high bootstrap values (bs, 87-99%). The freshwater crab populations in Kyushu showed distinct clades (II-IV), while Honshu (except for AI, OC, IC populations) and Shikoku populations formed a single clade (I). For ease in group classifications, the Kyushu mainland was divided into two clades: the eastern clade (II) encompassing the populations of Fukuoka, Oita and Kumamoto located in the northeastern Kyushu, and the populations of Miyazaki and Minamiosumi in southeastern Kyushu. Additionally, the western clade (IV) was identified, consisting of

Nagasaki, Saga and Amakusa populations. The Kagoshima mainland and the Osumi island group consisting of Tanegashima, Yakushima, Nakanoshima and Kuchinoshima were collectively classified as Clade III. The haplotype of Minamiosumi population from the Kagoshima mainland did not correspond to Clade III but was more closely related to Clade II. Furthermore, despite the significant geographic distance, the crab populations in Chiba and Ibaraki in eastern Honshu exhibited a stronger genetic affinity towards Clade III than Clade I.

The monophyly of *G*. *dehaani* was also demonstrated based on the COI gene with high support value (99%) (Figure 3). The ML tree clearly indicated that this species was distinctly different from other *Geothelphusa* species found in Japan. *Geothelphusa dehaani* was sister clade to *G*. *marmorata* collected from Yakushima and *G*. *sakamatoana* from Okinawa. Majority of the observed geographical clades were consistent with the result from the combined gene analysis in Figure 2, but there were some haplotypes that did not correspond to the detected clades.

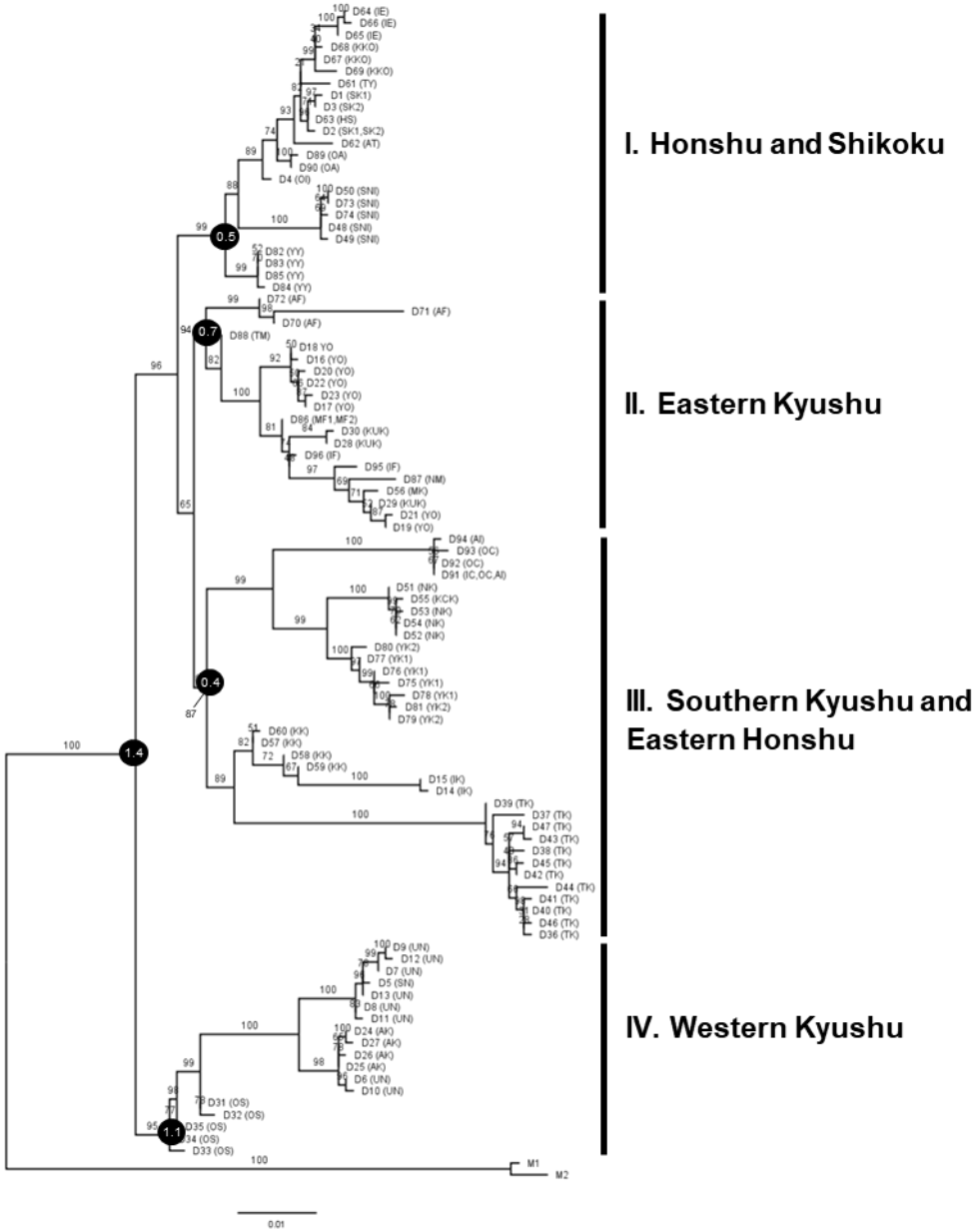

Figure 2. Maximum likelihood tree of the 96 haplotypes of *Geothelphusa dehaani* (D1 to 96) based on the combined COI and cytB genes. The support of each branch is indicated by percentages on each node. Numbers in black circles indicate the node age estimates (mya). Bar signifies 1% nucleotide sequence difference. Letters in parentheses refer to population code as shown in Table 1. *Geothelphusa marmorata* (M1 and M2) is used as the outgroup.

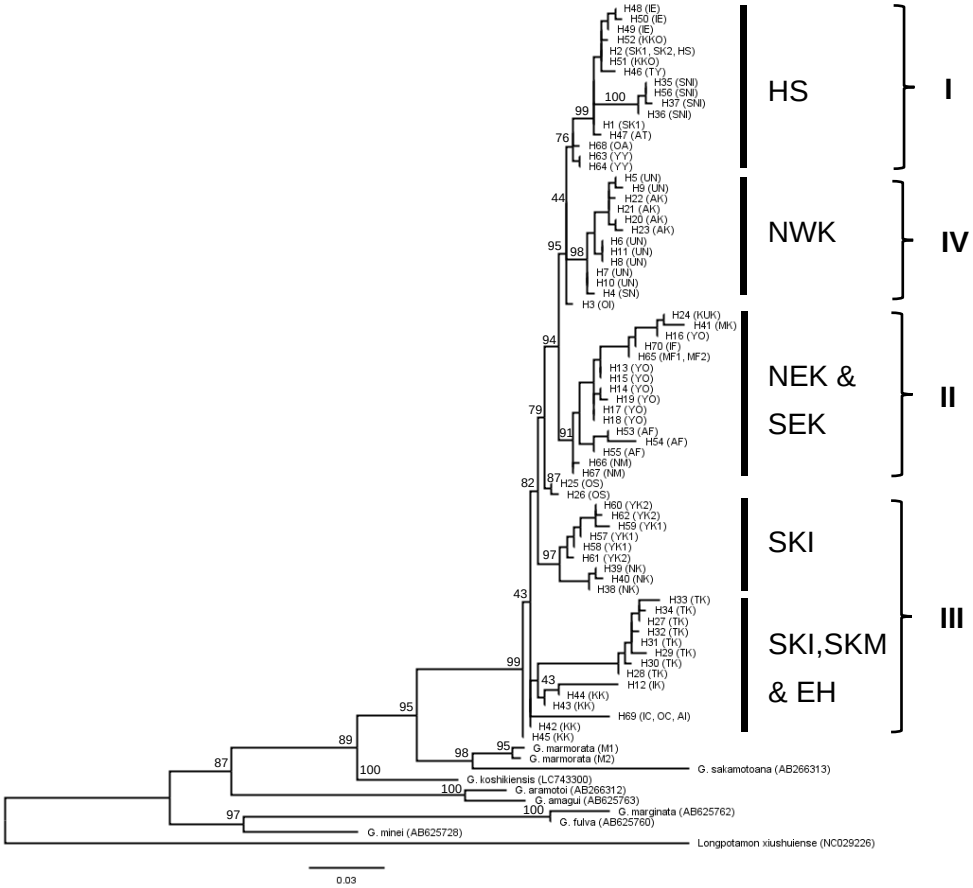

Figure 3. Maximum likelihood tree showing the relationships between haplotypes of *Geothelphusa dehaani* populations and other *Geothelphusa* species in Japan based on COI gene. The support of each branch is indicated by percentages on each node and bar signifies nucleotide sequence difference. Letters in parentheses refer to population code as shown in Table 1. H, haplotypes of *G. dehaani*; M, haplotypes of *G. marmorata*; *Longpotamon* (*Sinopotamon*) *xiushuiense* (outgroup); HS, Honshu and Shikoku; NWK, Northwestern Kyushu; NEK, Northeastern Kyushu; SEK, Southeastern Kyushu; SKM, Southern Kyushu Mainland; SKI, Southern Kyushu Island; EH, Eastern Honshu.

2.4.3 Genetic diversity

Tables 1 and 2 present the estimated gene diversity indices for each population and the observed clades, respectively. When all populations were considered, UN had the highest haplotype diversity (Hd) (0.972±0.064), followed by TK (0.962±0.040) and OS (0.933±0.122). In terms of nucleotide diversity (π), AF had the highest (0.009±0.005), followed by UN and IF (0.006), and KUK (0.005±0.003). These results indicate that Kyushu populations exhibited higher genetic diversity than Honshu and Shikoku populations. When assessed by clade, the Western Kyushu clade (Hd: 0.946±0.031; π: 0.011±0.005) had the highest genetic diversity.

Results of the neutrality test and mismatch distributions of the detected clades are shown in Table 3. Tajima's D was positive in all Clades. Clade I had negative value in Fu's FS test (-1.960) supported with statistical significance ($P<0.05$). In all clades, mismatch distribution analysis revealed that the estimated effective population size after population growth (Θ_1) was greater than the estimated effective population size before population growth (Θ_0). Sum of the square deviations between observed and expected mismatch (SSD) and raggedness index (Rag) were low in all clades and were not statistically significant ($P>0.05$). Clade III (49.760) had the highest Tau (τ) estimate compared to other clades (Clade I: 17.770; Clade II: 9.580; Clade IV: 19.609).

The AMOVA results revealed a significant genetic structure across all levels, with 43.26% of the variation occurred from the differences amongst clades, 51.79% of the variation were observed amongst populations within clades and 4.95% of the variation occurred within populations examined (Table 4). The pairwise FST comparisons also revealed significant differentiation (P=0.000) between each clade

(Table 5), with Clade IV notably the most distant from the rest. Clades I and IV had the highest FST value of 0.654, while Clades II and III had the lowest FST value of 0.375. Similarly, regardless of clade classification, high FST values are recorded between each population, indicating a significant degree of differentiation (Table 6). Low FST values were only observed between SK1 and SK2 (-0.215), MF1 and MF2 (0.000), YK1 and YK2 (0.200), IC and OC (-0.064), AI and OC (0.052), and AI and IC (0.063) with no statistical difference ($P>0.05$). The estimated distance between most sites ranges from 5.0 to 11.0 km with the exception of the Ibaraki (AI) and Chiba (IC and OC) populations, which have a distance of approximately 89 km.

The TMRCA estimates, with 95% highest posterior densities (HPD), were determined as follows: 0.50 million years ago (0.67-0.34 mya) for Clade I, 0.67 mya (0.88-0.46 mya) for Clade II, 0.45 mya (0.61-0.31 mya) for Clade III and 1.07 mya (1.30-0.85 mya) for Clade IV. While at 1.38 mya (1.73-1.04 mya), the *G. dehaani* populations collectively shared a most recent common ancestor. These estimates align with the Pleistocene periods.

Table 1. Genetic diversity indices of *Geothelphusa dehaani* collected from 26 populations using the combined COI and cytB genes.

Population code	Location	Region	Body color	N	Hn	Haplotype number	Hd (sd)	π (sd)
Clade I								
OI	Oshu, Iwate	Honshu	DA	13	1	D4	0	0
TY	Tsuruoka, Yamagata	Honshu	DA	3	1	D61	0	0
AT	Akiruno, Tokyo	Honshu	DA	7	1	D62	0	0
SNI	Sado, Niigata	Honshu	BL	18	5	D48,49, 50,73,74	0.791±0.052	0.001±0.001
OA	Okazaki, Aichi	Honshu	RE	8	2	D89,90	0.571±0.094	0.0005±0.0005
HS	Higashiomi, Shiga	Honshu	RE	10	1	D63	0	0
SK1	Sakyo, Kyoto	Honshu	RE	6	2	D1,2	0.533±0.172	0.001±0.001
SK2	Sakyo, Kyoto	Honshu	RE	3	2	D2,3	0.667±0.314	0.001±0.001
YY	Yamaguchi	Honshu	-	6	4	D82,83, 84,85	0.867±0.129	0.001±0.000
KK0	Kochi	Shikoku	RE	12	3	D67,68,69	0.600±0.130	0.001±0.001
IE	Imabari, Ehime	Shikoku	RE	16	3	D64,65,66	0.425±0.133	0.0004±0.0004
Clade II								
AF	Asakura, Fukuoka	Northern Kyushu	RE	8	3	D70,71,72	0.679±0.122	0.009±0.005
IF	Itoshima, Fukuoka	Northern Kyushu	RE	3	2	D95,96	0.667±0.314	0.006±0.005
MF1	Munakata, Fukuoka	Northern Kyushu	RE	8	1	D86	0	0
MF2	Munakata, Fukuoka	Northern Kyushu	RE	8	1	D86	0	0
YO	Yufu, Oita	Northern Kyushu	RE	12	8	D16,17, 18,19,20, 21,22,23	0.909±0.065	0.004±0.002

KUK	Kuma, Kumamoto	Northern Kyushu	RE	9	3	D28,29,30	0.556±0.165	0.005±0.003
TM	Takachiho, Miyazaki	Southern Kyushu	RE	10	1	D88	0	0
NM	Nobeoka, Miyazaki	Southern Kyushu	RE	10	1	D87	0	0
MK	Minamiosumi, Kagoshima	Southern Kyushu	BL	5	1	D56	0	0
Clade III								
AI	Ami, Ibaraki	Honshu	BL	9	2	D91,94	0.389±0.164	0.0003±0.0004
OC	Otaki, Chiba	Honshu	BL	11	3	D91,92,93	0.345±0.172	0.0004±0.0004
IC	Isumi, Chiba	Honshu	BL	6	1	D91	0	0
YK1	Yakushima Island, Kagoshima	Southern Kyushu	-	6	4	D75,76, 77,78	0.800±0.172	0.002±0.002
YK2	Yakushima Island, Kagoshima	Southern Kyushu	-	6	3	D79,80,81	0.600±0.215	0.002±0.002
NK	Nakanoshima Island, Kagoshima	Southern Kyushu	DA	16	4	D51,52, 53,54	0.725±0.074	0.001±0.001
KCK	Kuchinoshima Island, Kagoshima	Southern Kyushu	DA	9	1	D55	0	0
KK	Kanoya, Kagoshima	Southern Kyushu	-	6	4	D57,58, 59,60	0.900±0.161	0.003±0.002
IK	Ibusuki, Kagoshima	Southern Kyushu	DA	10	3	D14,15	0.200±0.154	0.0002±0.0003
TK	Tanegashima Island, Kagoshima	Southern Kyushu	DA	15	12	D36,37, 38,39,40, 41,42,43, 44,45,46, 47	0.962±0.040	0.004±0.002

Clade IV								
UN	Unzen, Nagasaki	Northern Kyushu	RE	9	8	D6,7,8,9, 10,11,12, 13	0.972±0.064	0.006±0.004
SN	Sasebo, Nagasaki	Northern Kyushu	RE	6	1	D5	0	0
AK	Amakusa, Kumamoto	Northern Kyushu	RE	6	4	D24,25, 26,27	0.867±0.129	0.001±0.001
OS	Ogi, Saga	Northern Kyushu	RE	6	5	D31,32, 33,34,35	0.933±0.122	0.003±0.002

N: number of specimens; Hn: number of observed haplotypes; Hd: haplotype diversity; π: nucleotide diversity; sd: standard deviation; Hyphen (-) indicates no data

Table 2. Genetic diversity indices of the clades detected in *Geothelphusa dehaani* using the combined COI and cytB genes.

Clade	**N**	**Hn**	**Hd (sd)**	**π (sd)**
I	102	24	0.940±0.010	0.009±0.005
II	73	20	0.910±0.016	0.012±0.006
III	94	34	0.918±0.018	0.027±0.013
IV	27	18	0.946±0.031	0.011±0.005

N: number of specimens; Hn: number of observed haplotypes; Hd: haplotype diversity; π: nucleotide diversity; sd: standard deviation

Table 3. Neutrality data and mismatch distribution analysis of the clades detected in *Geothelphusa dehaani* using the combined COI and cytB genes.

Clade	Tajima's D	Fu's FS	τ	Θ_0	Θ_1	SSD	Rag
I	0.281	0.583	17.770	0.047	16.773	0.010	0.013
II	0.931	2.718	9.580	6.333	41.943	0.018	0.032
III	1.801	4.351	49.760	0.000	55.122	0.025	0.014
IV	1.556	-1.960*	19.609	0.100	24.202	0.019	0.013
Mean	1.142	1.423	24.180	1.620	34.510	0.018	0.018

τ: Tau; Θ_0: Θ before population growth; Θ_1: Θ after population growth; SSD: sum of the square deviations between the observed and expected mismatch; Rag: raggedness index; Asterisk (*) indicates P<0.05

Table 4. Analysis of molecular variance (AMOVA) for the combined COI and cytB sequences of *Geothelphusa dehaani*.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indices	P-value
Among clades	3	1838.544	7.636 Va	43.26	Fsc=0.913	0.000±0.000
Among populations within clades	30	2339.191	9.143 Vb	51.79	Fst=0.950	0.000±0.000
Within populations	257	224.406	0.873 Vc	4.95	Fct=0.432	0.000±0.000
Total	290	4402.141	17.652			

Table 5. Matrix of pairwise differences of FST of the four clades detected in *Geothelphusa dehaani* using the combined COI and cytB genes.

Clade	I	II	III	IV
I	0.000			
II	0.599*	0.000		
III	0.458*	0.375*	0.000	
IV	0.654*	0.625*	0.452*	0.000

Asterisk (*) indicates P=0.000

Table 6. Matrix of pairwise differences of FST amongst populations of *Geothelphusa dehaani* using the combined COI and cytB sequences.

Population	SK1	SNI	TY	AT	HS	SK2	IE	KKO	YY	OA	OI	MF2	MK	AF	MF1	NM	TM	IF	YO	KUK	OS	SN	UN	AK	TK	NK	KCK	KK	YK1	YK2	OC	IC	AI	IK
SK1		0.000																																
SNI		0.920	0.000																															
TY		0.825	0.949	0.000																														
AT		0.904	0.958	1.000	0.000																													
HS		0.508	0.951	1.000	1.000	0.000																												
SK2		**-0.215***	0.927	0.889	0.957	0.675	0.000																											
IE		0.908	0.959	0.960	0.973	0.959	0.928	0.000																										
KKO		0.742	0.933	0.872	0.926	0.852	0.755	0.846	0.000																									
YY		0.902	0.932	0.954	0.971	0.968	0.915	0.965	0.927	0.000																								
OA		0.859	0.945	0.954	0.968	0.961	0.900	0.957	0.893	0.931	0.000																							
OI		0.913	0.953	1.000	1.000	1.000	0.967	0.975	0.930	0.966	0.961	0.000																						
MF2		0.974	0.974	1.000	1.000	1.000	0.988	0.989	0.975	0.979	0.987	1.000	0.000																					
MK		0.977	0.978	1.000	1.000	1.000	0.989	0.991	0.980	0.982	0.990	1.000	1.000	0.000																				
AF		0.769	0.886	0.770	0.834	0.835	0.726	0.886	0.823	0.764	0.797	0.846	0.748	0.799	0.000																			
MF1		0.968	0.971	1.000	1.000	1.000	0.985	0.987	0.971	0.975	0.985	1.000	**0.000***	1.000	0.714	0.000																		
NM		0.981	0.978	1.000	1.000	1.000	0.992	0.991	0.982	0.984	0.991	1.000	1.000	1.000	0.826	1.000	0.000																	
TM		0.969	0.968	1.000	1.000	1.000	0.987	0.987	0.970	0.972	0.983	1.000	1.000	1.000	0.712	1.000	1.000	0.000																
IF		0.905	0.951	0.899	0.951	0.958	0.874	0.964	0.930	0.909	0.931	0.966	0.800	0.771	0.675	0.752	0.888	0.916	0.000															
YO		0.868	0.921	0.875	0.903	0.905	0.854	0.926	0.890	0.858	0.876	0.906	0.696	0.767	0.671	0.667	0.814	0.763	0.617	0.000														
KUK		0.886	0.937	0.885	0.916	0.924	0.870	0.939	0.906	0.887	0.902	0.933	0.765	0.501	0.715	0.736	0.796	0.854	0.472	0.579	0.000													
OS		0.894	0.935	0.905	0.941	0.945	0.881	0.954	0.917	0.892	0.912	0.950	0.933	0.947	0.752	0.920	0.956	0.925	0.841	0.831	0.857	0.000												
SN		0.977	0.977	1.000	1.000	1.000	0.989	0.991	0.981	0.981	0.989	1.000	1.000	1.000	0.846	1.000	1.000	1.000	0.949	0.908	0.924	0.913	0.000											
UN		0.847	0.917	0.840	0.889	0.893	0.822	0.923	0.883	0.845	0.869	0.902	0.886	0.898	0.768	0.870	0.918	0.885	0.827	0.835	0.850	0.732	0.234	0.000										
AK		0.949	0.966	0.970	0.982	0.983	0.955	0.981	0.963	0.958	0.972	0.986	0.984	0.984	0.832	0.981	0.989	0.984	0.927	0.894	0.906	0.883	0.957	0.583	0.000									
TK		0.914	0.945	0.917	0.935	0.935	0.906	0.946	0.924	0.914	0.926	0.939	0.926	0.932	0.840	0.918	0.944	0.930	0.894	0.894	0.898	0.901	0.934	0.891	0.926	0.000								
NK		0.969	0.974	0.979	0.984	0.983	0.972	0.983	0.974	0.972	0.977	0.985	0.981	0.983	0.897	0.979	0.985	0.979	0.958	0.922	0.932	0.952	0.985	0.931	0.977	0.946	0.000							
KCK		0.983	0.981	1.000	1.000	1.000	0.993	0.993	0.985	0.987	0.992	1.000	1.000	1.000	0.877	1.000	1.000	1.000	0.963	0.918	0.928	0.960	1.000	0.920	0.989	0.941	0.641	0.000						
KK		0.882	0.945	0.900	0.941	0.945	0.868	0.950	0.904	0.895	0.918	0.955	0.932	0.943	0.619	0.918	0.954	0.918	0.821	0.801	0.818	0.837	0.957	0.835	0.935	0.875	0.952	0.963	0.000					
YK1		0.933	0.956	0.948	0.967	0.969	0.929	0.973	0.952	0.940	0.955	0.975	0.964	0.966	0.805	0.957	0.975	0.963	0.899	0.881	0.891	0.896	0.970	0.879	0.954	0.917	0.913	0.938	0.891	0.000				
YK2		0.936	0.958	0.949	0.968	0.970	0.932	0.974	0.954	0.942	0.958	0.975	0.964	0.966	0.812	0.957	0.976	0.965	0.899	0.881	0.891	0.897	0.970	0.879	0.954	0.917	0.918	0.941	0.896	**0.200***	0.000			
OC		0.976	0.978	0.989	0.992	0.992	0.982	0.989	0.980	0.978	0.985	0.993	0.991	0.992	0.884	0.990	0.993	0.991	0.962	0.927	0.937	0.961	0.993	0.930	0.984	0.944	0.976	0.991	0.956	0.960	0.959	0.000		
IC		0.978	0.978	1.000	1.000	1.000	0.990	0.992	0.983	0.983	0.991	1.000	1.000	1.000	0.852	1.000	1.000	1.000	0.953	0.914	0.923	0.955	1.000	0.912	0.987	0.936	0.978	1.000	0.952	0.957	0.957	**-0.064***	0.000	
AI		0.977	0.978	0.992	0.995	0.995	0.984	0.990	0.981	0.980	0.987	0.996	0.994	0.995	0.874	0.993	0.996	0.994	0.960	0.923	0.932	0.959	0.995	0.925	0.985	0.942	0.977	0.994	0.955	0.960	0.959	**0.052***	**0.063***	0.000
IK		0.978	0.977	0.995	0.996	0.997	0.987	0.989	0.979	0.983	0.988	0.997	0.995	0.995	0.847	0.994	0.997	0.996	0.947	0.884	0.907	0.955	0.997	0.916	0.985	0.939	0.985	0.998	0.934	0.972	0.972	0.991	0.997	0.993	0.000

Asterisk (*) indicates P>0.05

2.5 Discussion

The results of phylogenetic analysis of *Geothelphusa* freshwater crabs in Japan, based on the COI gene, showed that *G. dehaani* is monophyletic and has the closest relationship with *G. marmorata* from Yakushima Island and *G. sakamotoana* in Okinawa (Figure 3). Together with *G. koshikiensis* from Koshiki Island, they then formed another monophyletic group. This finding indicates that *G. dehaani* has more genetic affinity with other *Geothelphusa* species near the Kyushu mainland. Based on mitochondrial and nuclear DNA markers, *G. sakamotoana* has been reported as a sister species of *G. dehaani* (Lee and Kim 2020). The remaining five species, which are more genetically distant from *G. dehaani*, are mainly concentrated in the Ryukyus.

Using the mtDNA COI and cytB gene sequences, four distinct geographic groups were identified in the Japanese freshwater crab (Clade I - Honshu and Shikoku; Clade II – Eastern Kyushu; Clade III – Southern Kyushu and Eastern Honshu; and Clade IV – Western Kyushu). Previous analyses have suggested that the ancestor of genus *Geothelphusa* originated from the southern region of continental East Asia and dispersed northwards (Shih and Ng 2011). Based on these findings, this study suggests that the original *G. dehaani* stock first made contact in Kyushu and subsequently migrated northwards to Honshu and Shikoku. We deduced that the population expansion event of *G. dehaani* in Japan might have occurred between 1.38-0.45 mya which coincides during the Pleistocene when glaciations and interglaciations caused changes in the dispersal and isolation of freshwater crustaceans in East Asia (Shih and Ng 2011; Shih et al. 2011). During periods of glaciation, the main islands of the Japanese archipelago (Honshu, Shikoku and Kyushu including the Osumi island group) were connected by land bridges

to form the Paleo-Honshu which allowed for the continuous dispersal of freshwater crab (Yonekura et al. 2001; Iwase et al. 2012). However, geographical features, such as marine waters have been demonstrated to restrict the dispersal of *Geothelphusa* crabs, leading to the confinement of crab populations to certain areas and resulting in regional differentiation (Cumberlidge and Ng 2009; Shih et al. 2011; Shih and Ng 2011). The Seto Inland Sea, may have influenced the genetic separation of Clade I (Honshu and Shikoku) from the Kyushu group (Clades II to IV) (Yashima 1994). These marine waters are known to act as barriers to dispersal in freshwater fish and have been implicated in intraspecific divergences in various Japanese freshwater fish (Watanabe et al. 2017). Despite being separated by the Seto Inland Sea, Honshu and Shikoku maintain closer genetic relationships in the present study. While the estimated time periods for the divergence of these clades predate the formation of the Seto Inland Sea, it is plausible that the ancient wetlands associated with the present inland sea may have served as a barrier.

In Kyushu, three geographical group were found representing the Eastern (Clade II), Western (Clade IV) and Southern (Clade III) group. This division apparently resulted from the uneven submergence of areas during the interglacial period and/or major geological events. The Chikushi and Fukuoka plains, currently located between the Sefuri and Sangun Mountains in Northern Kyushu, may represent a potential boundary between Clades II and IV. These plains were likely submerged during the interglacial period, which may have contributed to the vicariance of these clades (Sugitani 1983; Tominaga et al. 2006). Subsequently, the sustained isolation in Clade IV over a long period resulted to its significant differentiation from other clades (Avise 2000). On the other hand, this study did not clearly establish the geographical boundary with respect to the genetic variation between Eastern Kyushu (Clade II) and Southern Kyushu

(Clade III). The clustering of the MK population in the Osumi Peninsula with Clade II suggests that this population may have been able to maintain habitable areas during the Pleistocene period. This could have led to the preservation of genetic characters similar to those found in Clade II. The areas encompassing the populations of IK and KK may have been exposed to various volcanic events since the late Pleistocene that could potentially lead to the extinction of these populations (Nagaoka 1988; Kobayashi and Tameike 2002; Nishihara et al. 2022). On the other hand, evidence showed that Minamiosumi area (MK) retained geological features (e.g. granitic plutons) that were assumed to exist prior to the Miocene period suggesting possible areas that facilitated the survival of freshwater crab populations during pyroclastic flows resulting from the series of volcanic eruptions in Southern Kyushu (Yanagi et al. 1971). In addition, it is possible that the population in the Osumi Peninsula (KK) and in Satsuma Peninsula (IK), were submerged during the interglacial periods in the Pleistocene (Ota and Omura 1991). These sites were situated in areas with lower topography, such as the Kimotsuki plains in Kanoya, and IK is positioned near the coast with a flat topology, in contrast to MK. This could potentially contribute to the local extinction of freshwater crabs in KK and IK and not in MK. Due to the limited number of populations that were studied in the two main peninsulas of southern Kyushu and the inconsistent findings obtained, more sampling is necessary to clarify this result.

The clustering of populations in Eastern Honshu (AI, OC, IC) into Southern Kyushu (Clade III) can be attributed to migration events such as rafting among the distant populations facilitated by the Kuroshio current. This current follows a path along the western part of Okinawa and reaches the south islands of Kyushu and then the eastern coast of Honshu. It is plausible that this current had a similar course during both glacial

and interglacial periods (Saito 2019). Shih and Ng (2011) suggest that ocean currents are possible dispersal routes in freshwater crabs. In a similar way, the presence of freshwater crabs in remote islands can be attributed to overseas rafting such as floating vegetation (Shih et al. 2004; Cumberlidge 2008; Esser and Cumberlidge 2011). The Kerama and Tokara Straits, which have depths exceeding 1000 m along the Ryukyus, act as obstacles for the movement of land-locked animals to the main Japanese island during glacial periods (Okano 2013). This observation provides additional evidence supporting the potential involvement of ocean currents in the dispersal of freshwater crabs, despite the presence of seawater barriers between populations. In a recent study by Takenaka et al. (2023), similar genetic trend was observed in *G*. *dehaani* populations from Izu, Miura and Boso Peninsulas situated in the easternmost portion of Honshu and those from the Southern Kyushu Islands.

The AMOVA results provided support for the high levels of structure observed between clades. The total variance analysis indicated that a large genetic variation was present amongst clades (43.26%), populations within clades (51.79%) and a few within populations (4.95%). A previous study on *G*. *dehaani* revealed that high genetic diversity was observed amongst populations, with a percentage of 63.7%, which is slightly higher than the current results (Ikeda et al. 1998). This pattern of larger genetic differences amongst populations than within them has also been observed in other species of freshwater crabs using a mtDNA marker. For example, in the Chinese potamid *Longpotamon yangtsekiense*, over 50% of the genetic variations are found amongst groups while 32.23% of the variations amongst populations within groups, and 13.58% variations within populations (Shi et al. 2021). Similarly, for the Chinese *Longpotamon acutum*, AMOVA showed 72.52% genetic variance between the main groups and only

9.01% was explained by within population variance (Fang et al. 2015). High FST values amongst clades and amongst populations also indicate a strong degree of genetic differentiation (Tables 5 and 6).

The analysis of mitochondrial DNA sequence data showed that majority of *G*. *dehaani* populations had high haplotype diversity, low nucleotide diversity and a large number of rare haplotypes (Table 1). This pattern is typical of species with limited dispersal ability, which is supported by previous studies on other freshwater species, such as prawns (Liu et al. 2011), fish (Dodson et al. 1995; Garg and Mishra 2018) and crabs (Shih et al. 2004; Shih et al. 2006; Fang et al. 2015; Shi et al. 2021). The presence of unique haplotypes suggests that *G*. *dehaani* populations are geographically isolated, with little gene flow occurring between them (Avise 1994). The presence of shared haplotypes and low FST values between SK1 and SK2, MF1 and MF2, as well as IC and OC populations can be attributed to the close proximity of the collection site. This could be attributed to the amphibious behavior of *G*. *dehaani*, wherein they are capable of surviving desiccation for several days and are able to disperse over short distances across land, particularly during the rainy season (Okano et al. 2000b; 2003; Batang and Suzuki 2003). The presence of a shared haplotype (D91) and low FST values in both Ibaraki (AI) and Chiba (IC and OC) populations, despite their considerable distance, might be attributed to a rapid population expansion subsequent to a bottleneck period. This local extinction can possibly triggered due to the submergence of the Kanto plain by seawater during the interglacial periods (Ishihara et al. 2012; Tanabe et al. 2015).

The Japanese freshwater crab *G*. *dehaani* has three different color types: RE, which has a dark brown carapace and reddish legs; DA, which has a dark purplish carapace and legs; and BL, which has a greyish blue carapace and light-grey legs

(Chokki 1976). Apparently, the causative genetic or biological mechanisms behind these color variations are still not well understood. Aotsuka et al. (1995) reported that DA and BL color populations in Kanagawa Prefecture of Honshu existed with substantial genetic variations, based on allele frequencies. A similar study was also evaluated by Ikeda et al. (1998) in Honshu, but with a wider coverage area where they found that only the BL type was distantly related to other types, while the genetic differentiation of RE and DA was not supported as stipulated by electrophoretic analysis (Ikeda et al. 1998). Okano et al. (2000a) noted that there was no genetic differentiation observed amongst the different color types in Kagoshima mainland. In this study, we also observed that there is no relationship between body colorations and genetic differences in *G. dehaani*. All three color types were found in Clade I, RE and BL types were found in Clade II, DA and BL types were recorded in Clade III and only the RE type in Clade IV (Table I). We suggest that morphological differentiation based on color does not relate to speciation but may indicate possible plasticity.

In conclusion, this study found that *G. dehaani* can be divided into four distinct geographical clades, based on COI and cytB datasets. High genetic variations were observed amongst the populations examined as a result of low gene flow. The geologically dynamic history of the Japanese archipelago appears to have sporadically and repeatedly facilitated discontinuity in freshwater habitats over evolutionary time, thereby inducing strong divergence amongst populations of *G. dehaani*. The findings highlight the need for further examination of morphological and behavioral characteristics, particularly in the populations studied in Kyushu.

Chapter 3

The complete mitochondrial genome of freshwater gammarid *Gammarus nipponensis* (Crustacea: Amphipoda: Gammaridae)

3.1 Abstract

This study presents the complete mitochondrial genome sequence of *Gammarus nipponensis*, a freshwater crustacean found primarily in the western regions of Honshu, Shikoku and Kyushu in Japan. The entire genome is 16,429 bp in length, encoding a standard set of 13 protein-coding genes, two ribosomal RNA genes and 22 transfer RNA genes, as well as the putative control regions. Notably, the mitochondrial genome of *G. nipponensis* contains long TATTTTA repeats (approximately 690 bp long) and is characterized by a high concentration of A and T nucleotides (67.1%). This newly available genome information will be useful for studying the evolutionary relationships within the genus *Gammarus* and for understanding diversification among *G. nipponensis* populations.

3.2 Introduction

The genus *Gammarus* Fabricius 1775, which includes over 200 species, is considered one of the most diverse genera of crustaceans (Väinölä et al. 2008). In Japan, three *Gammarus* species have been recorded: *G. nipponensis* Uéno 1940, *G. koreanus* Uéno 1940 and *G. mukudai* Tomikawa, Soh, Kobayashi and Yamaguchi 2014. Among these, *G. nipponensis* has the widest distribution, inhabiting western Honshu, Shikoku and Kyushu, while other species have limited distributions, mainly in the islands around Nagasaki prefecture (Tomikawa 2017). These *Gammarus* species are exclusively found in freshwater habitats which limits migration. Moreover, the life cycle of gammarids involves direct development, in which the fertilized eggs are brooded until the juvenile stage (Hyne 2011; Bastos-Pereira and Bueno 2016). This absence of planktonic larval stage further contributes to their limited dispersal capability. Given these characteristics, *G. nipponensis* populations can be used to examine the effect of habitat fragmentation on the degree of genetic variation within and among their populations.

A previous study on the phylogenetics of *G. nipponensis* relies on partial mitochondrial gene (Tomikawa et al. 2014). Similarly, the classification and phylogenetic status of the genus *Gammarus* remains problematic (Hou et al. 2014, Hou and Sket 2016, Macher et al. 2017). The lack of adequate molecular markers diminishes the potential of *G. nipponensis* as a model for studying population genetics and biogeography. As a result, having the full mitochondrial genome of *G. nipponensis* can give useful resources for additional phylogeographic information. Furthermore, this is the first complete mitochondrial genome for the genus *Gammarus* found in Japan.

3.3 Materials and methods

The specimen of *G. nipponensis* (Figure 1) was obtained from Kuro River in Asakura, Fukuoka (33°23'56.8"N, 130°48'19.6"E) and immediately preserved in 95% ethanol. The species identification was based on its morphological characters as described in Tomikawa et al. (2014). This specimen was then stored in Kyushu University facility and registered in the ffish.asia database (https://ffish.asia/?page=specimen&pid=53448) under specimen ID number QUYK-10765. Total genomic DNA was extracted from the pereopod muscles using the Quick DNA™ Miniprep Plus Kit (Zymo Research, USA) following the manufacturer's protocol.

Figure 1. Freshly preserved specimen of *Gammarus nipponensis*.

Subsequently, the extracted DNA was submitted to Bioengineering Lab. Co., Ltd. (Sagamihara, Japan) to conduct the library construction and sequencing. Library preparation was done according to MGIEasy FS DNA Library Prep Set manual, and high-throughput DNA sequencing was performed using the DNBSEQ-G400 system (MGI Tech, Shenzhen, China). Raw reads obtained from the sequencing process were filtered using

Sickle ver. 1.33 (Joshi and Fass 2011) to remove adapter sequences and reads with lower quality score than 30. The resulting high-quality reads were assembled using metamode of SPAdes ver. 3.14.0 (Nurk et al. 2017), then annotated using the MITOS web server (Bernt et al. 2013) and sequentially manually corrected to improve accuracy. Results of the short read assembly revealed the presence of tandem repeats of TATTTTA in the mitochondrial genome of this species. PCR was conducted across the repeats using primers (Gnip CR2F: 5'-GTGATTACAATAACATTTGGCGGC-3' and Gnip CR2R: 5'-GCTATAAGGCTGACCTCAAGGC-3') and showed that the length of repeats was approximately 690 bp. Since its precise length could not be determined by Sanger sequencing, the number of repeats was confirmed using MinION sequencer (Oxford Nanopore Technologies) equipped with a FLO-MIN106D flow cell and SQK-RAD004 kit. The long reads were assembled with Flye ver. 2.9.1 (Kolmogorov et al. 2019), then the assembled contig was polished with Pilon ver. 1.22 using the short reads (Walker et al. 2014).

The read coverage depth and circular map of the mitochondrial genome was visualized using the integrative genomics viewer (Thorvaldsdóttir et al. 2013) and the Proksee server at https://proksee.ca/projects (Grant et al. 2023), respectively. The annotated sequence was submitted to NCBI GenBank and is available with accession number OR268765.2.

Phylogenetic analysis was performed using nucleotide sequences from 13 protein-coding genes, encompassing amphipods within the superfamily Gammaroidea. Additionally, the isopod *Ligia oceanica* (Linnaeus 1767) was included as an outgroup. The nucleotide sequences of each gene were aligned using MAFFT ver. 7 in "auto" strategy settings (Katoh et al. 2019) and filtered in "strict" mode using trimAL ver. 1.2

(Capella-Gutiérrez et al. 2009). Phylogenetic trees were then generated using maximum likelihood (ML) method using the concatenated dataset with 10,000 ultrafast bootstrap replicates in IQ-TREE ver. 1.6.12 (Nguyen et al. 2015). To determine the best-fit substitution model, ModelFinder in IQ-TREE ver. 1.6.12 was employed under the Bayesian Information Criterion (Kalyaanamoorthy et al. 2017). The selected model for ML was GTR+F+I+G4.

3.4 Results

The mitochondrial genome of *G. nipponensis* is 16,429 bp in length and contains 13 protein-coding genes (PCGs: cox1-3, nad1-6, nad4L, CytB, atp6 and atp8), two ribosomal RNAs (rRNA: 16S and 12S), 22 transfer RNAs (tRNAs: one for each amino acid and two for Leu and Ser) and putative control regions (CR) (Figure 2; Table 1). The organization of the mitochondrial genome in *G. nipponensis* and its gene arrangements, with reference to the pancrustacean ground pattern and other *Gammarus* species is illustrated in Figure 3. The CR in *G. nipponensis* are found in two positions: CR1 – located after the 12S gene and CR2 – situated between tRNA-Tyr and tRNA-Ile, with a combined length of about 2,200 bp. Interestingly, the mitochondrial genome of *G. nipponensis* contained 98 TATTTTA repeats in CR2. Among the genes, nine of the 13 PCGs and 14 of the 22 tRNAs are encoded by the forward (+) strand, while the remaining genes are encoded by the reverse (-) strand.

The read coverage depth obtained for the assembled mitogenome generated in DNBSEQ short reads was about 400x, with the lowest coverage depth (1x) at the

repetitive region of CR2 (Figure 4). Improved coverage depth of above 1000x in CR2 was achieved from Oxford Nanopore long reads, suggesting the reliability of the final assembly.

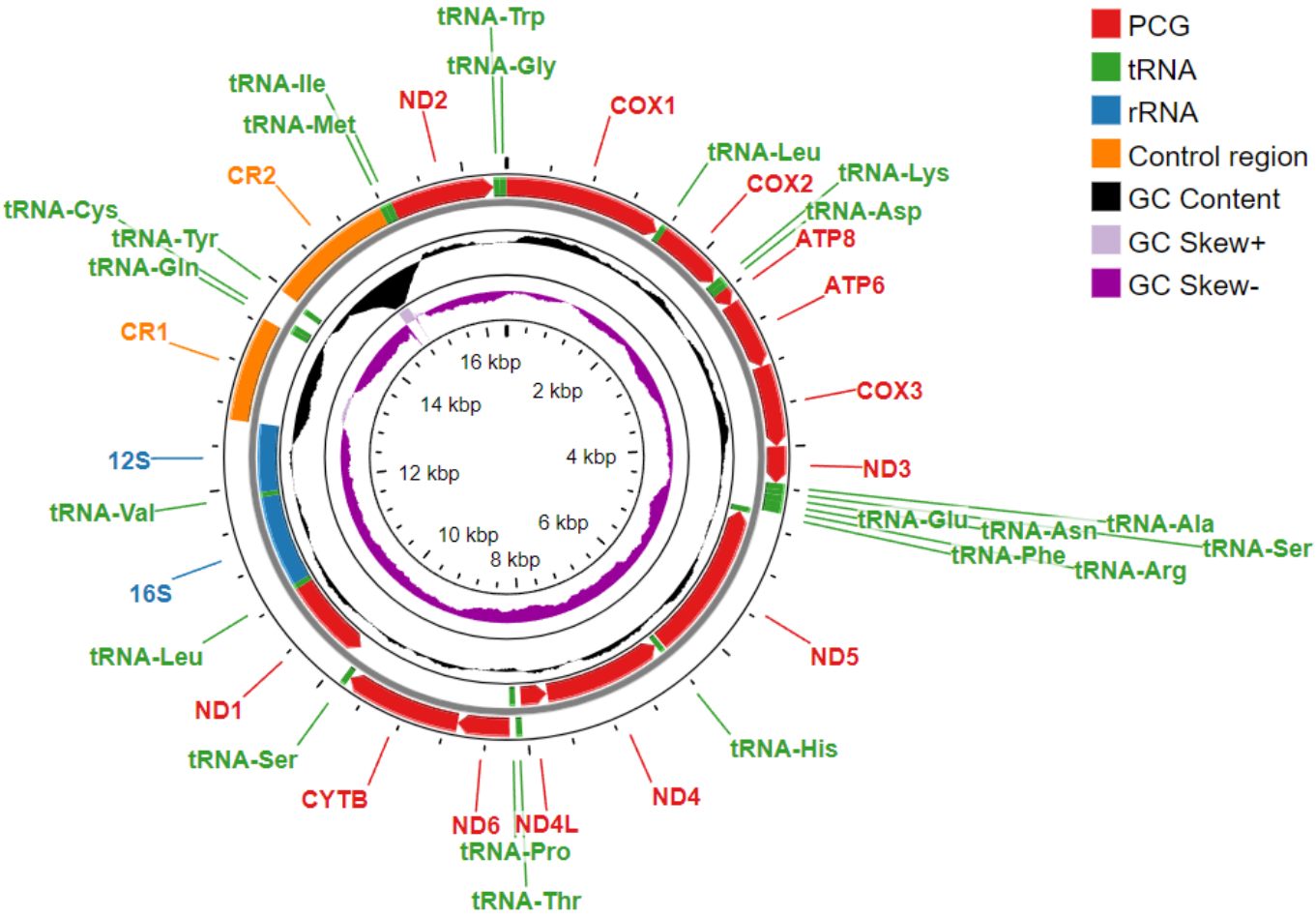

Figure 2. Mitochondrial genome map of *Gammarus nipponensis* showing the arrangement of PCGs, tRNAs, rRNAs and putative control regions.

Nucleotide composition of the *G. nipponensis* mitogenome is AT-rich at 67.1% (A:31.7%, C:21.8%, G:11.1%, T:35.4%) and the overall AT-skew and GC-skew are -0.055 and -0.325, respectively. The PCGs make up 11,010 bp (67.1%) and encode 3,659 amino acids.

Table 1. Annotation of the *Gammarus nipponensis* mitogenome.

Gene	Position number		Length (bp)	Start codon	Stop codon	Intergenic length	Strand
	Start	End					
COX1	1	1536	1536	att	taa		F
tRNA-Leu	1536	1595	60			-1	F
COX2	1593	2268	676	att	t--	-3	F
tRNA-Lys	2269	2327	59			0	F
tRNA-Asp	2327	2387	61			-1	F
ATP8	2388	2546	159	ata	taa	0	F
ATP6	2540	3211	672	atg	taa	-7	F
COX3	3211	3996	786	atg	taa	-1	F
ND3	3999	4352	354	atg	tag	2	F
tRNA-Ala	4353	4411	59			0	F
tRNA-Ser	4412	4461	50			0	F
tRNA-Asn	4463	4523	61			1	F
tRNA-Glu	4521	4582	62			-3	F
tRNA-Arg	4577	4634	58			-6	F
tRNA-Phe	4633	4692	60			-2	R
ND5	4692	6383	1692	att	taa	-1	R
tRNA-His	6402	6460	59			18	R
ND4	6461	7757	1297	ata	t--	0	R
ND4L	7766	8050	285	atg	taa	8	R
tRNA-Thr	8054	8112	59			3	F
tRNA-Pro	8112	8173	62			-1	R
ND6	8176	8679	504	atg	taa	2	F
CYTB	8679	9809	1131	atg	taa	-1	F
tRNA-Ser	9811	9870	60			1	F
ND1	9909	10838	930	ata	taa	38	R
tRNA-Leu	10839	10898	60			0	R
16S	10900	11877	978			1	R
tRNA-Val	11879	11930	52			1	R
12S	11931	12653	723			0	R
CR1	12654	13653	1000			0	-
tRNA-Gln	13654	13705	52			0	R
tRNA-Cys	13706	13760	55			0	R
tRNA-Tyr	13903	13963	61			142	R

CR2	13964	15195	1232			0	-
tRNA-Ile	15196	15254	59			0	F
tRNA-Met	15255	15316	62			0	F
ND2	15317	16304	988	ttg	t--	0	F
tRNA-Trp	16305	16364	60			0	F
tRNA-Gly	16365	16426	62			0	F

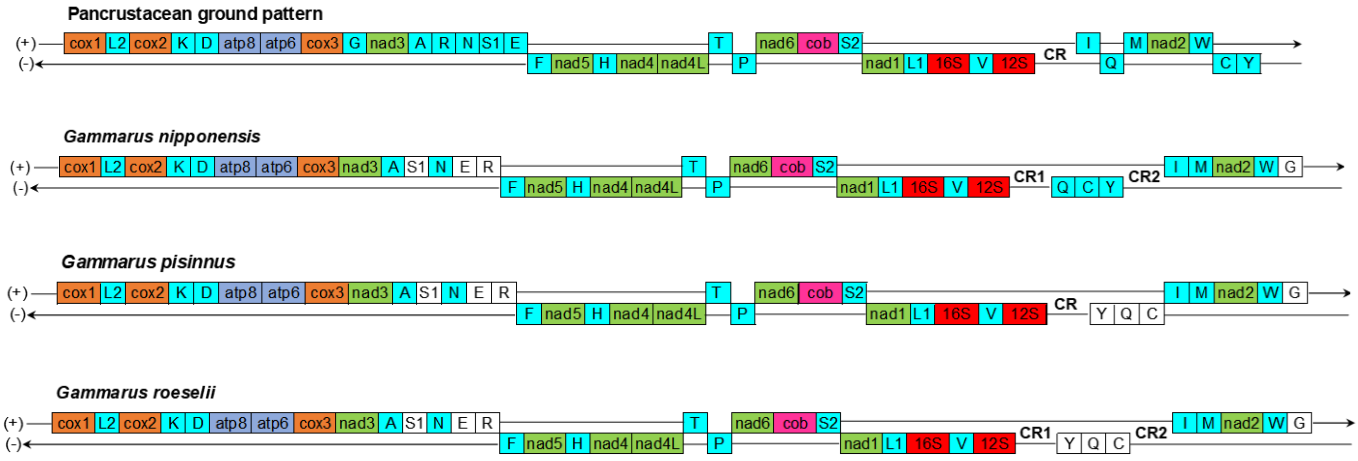

Figure 3. Gene order of *Gammarus nipponensis* in comparison to the pancrustacean ground pattern and other *Gammarus* species. Uncolored blocks in the *Gammarus* mitogenome indicate changes compared to the pancrustacean ground pattern. Label of tRNAs correspond to their single-letter amino acid code. The positions of putative control regions (CR) are also shown. (+) indicates forward strand; (-) indicates reverse strand.

Figure 4. The read coverage data of *Gammarus nipponensis* mitochondrial genome. The upper panel shows the coverage depth of DNBSEQ short reads (~400x), exhibiting a substantial decrease at the repetitive region of control region 2 at 1x. While, the lower panel shows the coverage depth of Oxford Nanopore long reads (>1000x), revealing an increase in coverage within the control region 2. This illustration was generated using the integrative genomics viewer (Thorvaldsdóttir et al. 2013).

The phylogenetic tree results in Figure 5 demonstrated that *G. nipponensis* has closer genetic affinity to *G. pisinnus* than the other *Gammarus* species.

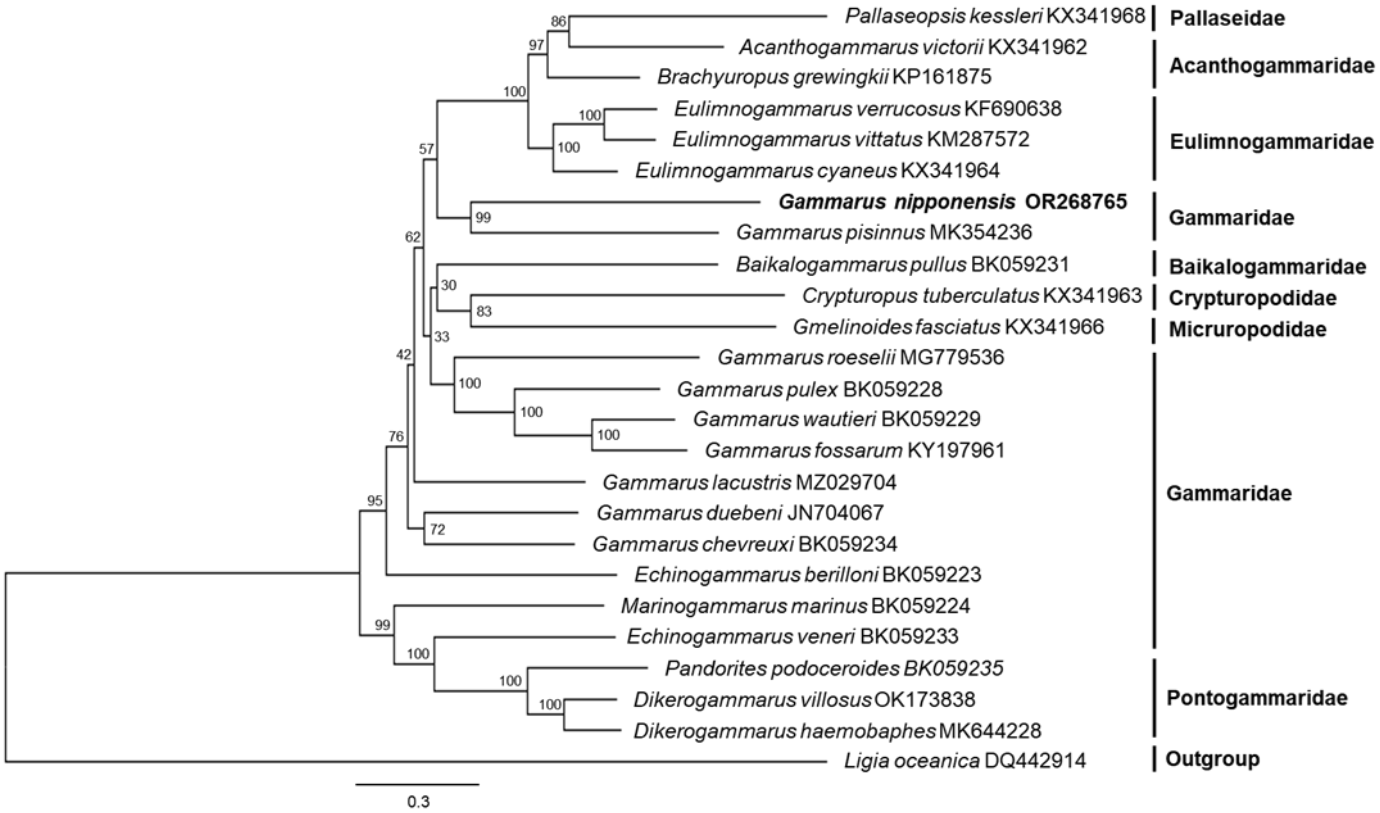

Figure 5. The maximum likelihood phylogenetic tree based on the sequence alignment of 13 protein-coding genes of *Gammarus nipponensis* and amphipods under superfamily Gammaroidea. Numbers on each node indicate the bootstrap support (%) values and the isopod, *Ligia oceanica* is used as outgroup. Scale bar signifies nucleotide sequence difference. The following sequences were used: KX341968, KX341962, KX341964, KX341963, KX341966 (Romanova et al. 2016a); KP161875 (Romanova et al. 2016b); KF690638 (Rivarola-Duarte et al. 2014); KM287572 (Romanova et al. 2016c); OR268765 (this study); MK354236 (Sun et al. 2020); BK059231, BK059228, BK059229, BK059234, BK059223, BK059224, BK059233, BK059235, OK173838 (Mamos et al. 2021); MG779536 (Cormier et al. 2018); KY197961 (Macher et al. 2017); MZ029704 (Li et al. 2021); JN704067 (Krebes and Bastrop 2012); MK644228 (Bojko 2020); and DQ442914 (Kilpert and Podsiadlowski 2006).

3.5 Discussion

Mitochondrial genes of *G*. *nipponensis* are encoded on the similar strand which is consistent with the putative ancestral pancrustacean ground pattern (Boore et al. 1998). However, there are differences in the order of tRNAs (Ser, Glu, Arg and Gly) and the occurrence of dual CR (Figure 3). This translocation of tRNAs is commonly found in gammarids (Krebes and Bastrop 2012; Cormier et al. 2018; Mamos et al. 2021). Two CRs are also reported in *G*. *roeselii*, but these regions displayed nearly identical sequences unlike in our study species (Cormier et al. 2018). Another interesting feature of *G*. *nipponensis* mitogenome is the presence of tandem repeats within the control region. Although tandem repeats are also found in *G. duebeni*, these are less abundant compared to those in *G*. *nipponensis* (Krebes and Bastrop 2012). Phylogenetic analysis results do not support the monophyly of Gammaridae, corroborating with the previous findings by Hou and Sket (2016), Macher et al. (2017), Cormier et al. (2018), Li et al. (2021) and Mamos et al. (2021). Results also revealed a close relationship between *G*. *nipponensis* and *G*. *pisinnus*, both of which are native to East Asia (Sun et al. 2020).

The complete mitogenome of *G*. *nipponensis* contributes to further clarify the phylogenetic relationships within the family Gammaridae. Also, the information from this study may lead to the development of improved tools for phylogenetic analysis among the populations of *G*. *nipponensis*.

Chapter 4

Phylogeny of *Gammarus nipponensis* across Honshu to Kyushu regions based on the mitochondrial genome sequences

4.1 Abstract

Gammarus nipponensis have limited dispersal capability, yet it is highly abundant in freshwater habitats and widely distributed throughout the western portion of the Japanese Archipelago. This study investigated the phylogenetic relationships among the 11 specimens of *G. nipponensis* collected from its known distribution range, using nearly complete mitochondrial genome sequences. These mitogenomes formed three divergent groups: Clade I or the Honshu and Shikoku group; Clade II or the Eastern Kyushu group; and Clade III or the Western Kyushu group. The estimated age of the entire lineage is approximately 24 million years ago, with the emergence of all major clades occurring around 22 to 17 million years ago. These characteristics showed the ancient origin of *G*. *nipponensis*.

A comparison of these mitochondrial genomes revealed differences in number of control regions between individuals from Kyushu to that in the Honshu and Shikoku group. Additionally, high nucleotide variation was observed among the mitogenomes of the specimens. These initial findings provide an important baseline for future molecular population investigations emphasizing the need for employing several species delimitation methods within each identified lineage.

4.2 Introduction

The Japanese Archipelago is considered as one of the global biodiversity and endemism hotspot, owing to its complex geological history as well as its unique geological and climatic conditions (Yonekura et al. 2001; Motokawa 2017). This archipelago serves as a model system for exploring the interplay between biogeography and evolution. The biodiversity dynamics on the islands comprising the archipelago are shaped by two primary processes: dispersal over the ocean and immigration through land bridges, particularly during the interglacial and glacial periods of the Pleistocene (Walter 2004; Cowie and Holland 2006; Taniguchi et al. 2021). The freshwater organisms in the archipelago are ideal candidates for studying the correlation between geological events and fauna development, as they often exhibit distinct population variations attributed to their inherently limited dispersal capabilities.

The freshwater *Gammarus* Fabricius, 1775 are considered land-locked because their entire life cycle is exclusively spent in freshwater environments. Additionally, their life cycle includes direct development, in which fertilized eggs are brooded until they reach the juvenile stage (Hyne 2011; Bastos-Pereira and Bueno 2016). Three species of freshwater *Gammarus* have been described in Japan, namely *Gammarus nipponensis* (Uéno 1940), *G. koreanus* (Uéno 1940) and *G. mukudai* (Tomikawa et al. 2014). In addition to this, the occurrence of *G. sobaegensis* (Uéno 1966) in Japan is still contentious (Tomikawa and Morino 2012). *Gammarus koreanus* originated from Korean peninsula and its occurrence is also reported in Gotô, Fukue and Nakadôri Islands in Nagasaki prefecture (Tomikawa et al. 2012). *G. nipponensis* and *G. mukudai* are considered endemic to Japan, however, only the former is widely distributed and can be

abundantly found in freshwater habitats of West Honshu, Shikoku and Kyushu, while the latter is limited to narrow areas and is located in the Iki and Tsushima Islands in Nagasaki prefecture (Uéno 1940; Tomikawa et al. 2014).

Despite its wide distribution range and popularity as one of the most common freshwater crustacean species in Japan, *G. nipponensis* lacks comprehensive phylogenetic and genetic investigations. Only one investigation using nuclear 28S rRNA and the mitochondrial COI genes has been reported wherein *G. nipponensis* monophyly is not adequately supported due to moderate statistical values (Tomikawa et al. 2014). Furthermore, the authors highlighted a substantial genetic variation among the populations studied suggesting the presence of sibling species. In an effort to analyze genetic variations among *G. nipponensis* populations, the initial work of this current study employed species-specific primers designed from the complete mitogenome of *G. nipponensis* to amplify sections of the mitochondrial DNA (e.g. COI and cytB genes). However, this attempt yielded inconsistent amplification success among localities. Thus, to further elucidate this observation, we utilized the nucleotide sequences from 11 nearly complete mitogenomes of *G. nipponensis*, acquired from various locations within its known distribution range.

The objectives of this research are to examine the phylogenetic relationships of *G. nipponensis* mitogenomes and to assess the differences in the compositional properties exhibited by these mitogenomes. Furthermore, based on the mitogenomic information obtained from each locality, molecular markers will be developed for a more detailed analysis of population genetic structure.

4.3 Materials and methods

4.3.1 Sample collection and DNA extraction

Specimens of *G*. *nipponensis* were collected from 11 distinct localities, as illustrated in Figure 1. The reference locality for Asakura, Fukuoka was obtained from Chapter 3, along with its associated sample description in NCBI accession number: OR268765.2. These specimens were collected from rivers and streams and immediately preserved in 95% ethanol. The preserved specimens were then stored at the Fishery Research Laboratory, Kyushu University until DNA extraction.

Genomic DNA was subsequently extracted from the pereopod muscles of each specimen using NucleoSpin® Tissue kit (Machery-Nagel, Germany), following the manufacture's protocol. DNA quality and concentration were examined using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA).

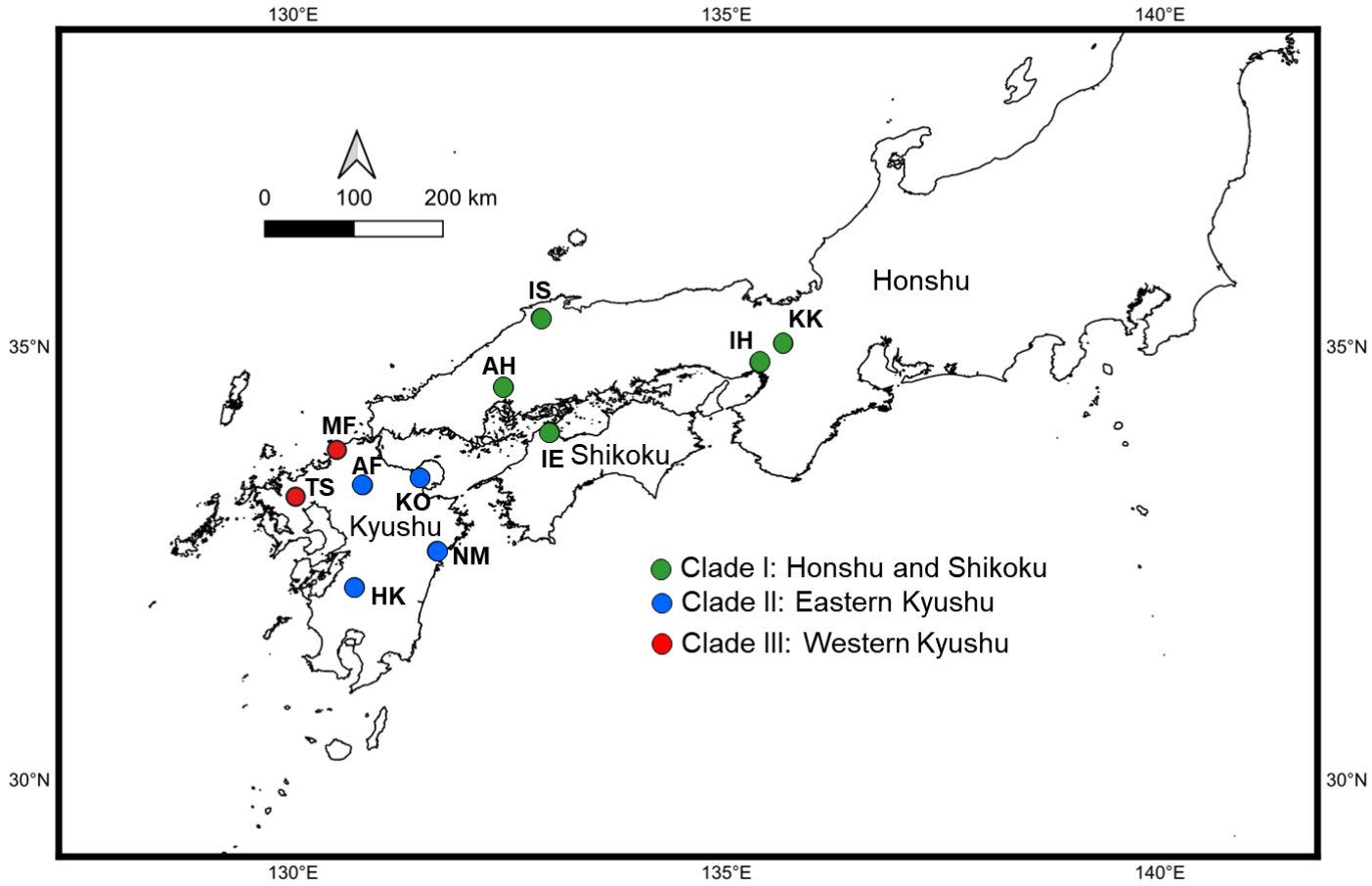

Figure 1. Map showing the sampling locality of *Gammarus nipponensis* used in the study and the respective phylogenetic groupings. The following are the locality codes: KK - Kiyotaki, Kyoto; IH - Itami, Hyogo; IS - Izumo, Shimane; AH - Asakita, Hiroshima; IE - Imabari, Ehime; KO - Kitsuki, Oita; NM - Nobeoka, Miyazaki; HK - Hitoyoshi, Kumamoto; AF - Asakura, Fukuoka; MF - Munakata, Fukuoka and TS - Taku, Saga.

4.3.2 Sequencing, assembling, and annotation

The genomic DNA extracts (>30 ng/µl) from all specimens were sent to Bioengineering Lab. Co., Ltd. in Sagamihara, Japan, for library construction and sequencing. Library preparation followed the MGIEasy FS DNA Library Prep Set manual, and high-throughput DNA sequencing was conducted using the DNBSEQ-G400 system (MGI Tech, Shenzhen, China). Raw sequencing reads were subjected to initial quality

assessment with FastQC (Andrews 2010). Low-quality reads and adapter sequences at both ends were trimmed and filtered using Trimmomatic (Bolger et al. 2014). After trimming, another round of data quality checking was performed with FastQC. The clean reads were then subjected to de novo assembly using metamode of SPAdes ver. 3.14.0 (Nurk et al. 2017). Blast alignment of the assembled mitochondrial sequences was done by BLASTN ver. 2.11.0+ (Zhang et al. 2000) and then compared with those of *G. nipponensis* (NCBI accession number: OR268765) to verify their correctness. The resulting consensus sequences were subsequently annotated using the mitochondrial genome annotation web server MITOS (Bernt et al. 2013). Furthermore, the predicted genomic features and annotation results from MITOS were manually corrected to enhance accuracy.

4.3.3 Data analysis

The phylogenetic analysis was conducted using partial mitogenome sequences, including 10 protein-coding genes (PCGs) and 2 rRNA genes, obtained from various *G. nipponensis* specimens. Additionally, the recently available mitogenome sequence of *G. nipponensis* (NCBI accession no. OR268765.2) was included in the analysis. Species from the same genus, *G. pisinnus* (NCBI accession no. MK354236) and *G. lacustris* (NCBI accession number no. MZ029704), both originating from China were chosen as outgroups. The nucleotide sequences of each gene were aligned using the “auto” strategy in MAFFT ver. 7 (Katoh et al. 2019) and filtered using trimAL ver. 1.2 on “strict” mode (Capella-Gutiérrez et al. 2009) and then adjusted manually in MEGA X. Phylogenetic trees were then generated using maximum likelihood (ML) method with

10,000 ultrafast bootstrap replicates in IQ-TREE ver. 1.6.12 (Nguyen et al. 2015). To determine the best-fit substitution model, ModelFinder implemented in IQ-TREE ver. 1.6.12 was employed under the Bayesian Information Criterion (Kalyaanamoorthy et al. 2017). The selected model was TVM+F+I+G4. The COI phylogenetic tree was also constructed in order to establish the connections between the newly sequenced *G. nipponensis* specimens with the previously published data within the genus *Gammarus* particularly those inhabiting the Japanese Archipelago (see Tomikawa et al. 2014). The COI data was aligned, trimmed and analyzed following similar steps as described in the mitogenome dataset, but the selected substitution model applied was TIM2+F+I+G4. The resulting phylogenetic trees were then visualized using FigTree ver. 1.4.4 (Rambaut 2018).

The estimation of the times to the most recent common ancestor (TMRCA) was conducted through Bayesian skyline analysis, implemented in BEAST ver. 1.10.4 (Suchard et al. 2018) and XML files were prepared using BEAUti ver. 1.7.5 (Drummond et al. 2012). The Markov Chain Monte Carlo analysis ran for 40,000,000 generations, with samples taken every 4000 iterations. The HKY nucleotide-substitution model was applied and rate variations among sites were modeled using a gamma distribution. The mutation rate was set at 1.77% per million years, based on the average mutation rate previously employed for the mitochondrial gene in amphipods (Copilaş-Ciocianu et al. 2019; Mamos et al. 2021). Convergence of the parameters were assessed using the TRACER ver 1.7 (Rambaut et al. 2018).

Computation of the nucleotide composition of the mitogenomes was performed in MEGA X (Kumar et al. 2018). Compositional skews were also estimated in MEGA X using the following formulas: AT-skew = (A − T) / (A + T) and GC-skew = (G − C) / (G +

C). To measure the genetic similarity, nucleotide sequences of the PCGs and rRNAs were aligned in pairs using CLUSTAL W (Thompson et al. 1994) and the conserved sites (%) were determined in MEGA X.

4.4 Results

4.4.1 Phylogenetic Analysis

The phylogenetic relationships within *G*. *nipponensis*, as determined by the partial mitogenome sequences, are illustrated in Figure 2. These mitogenomes formed into three divergent groups: Clade I or Honshu and Shikoku group; Clade II or Eastern Kyushu group; and Clade III or Western Kyushu group. The robustness of these groupings was supported by high bootstrap values (95-100%).

Clade I include Asakita, Hiroshima (AH); Imabari, Ehime (IE); Itami, Hyogo (IH); Kiyotaki, Kyoto (KK) and Izumo, Shimane (IS). Clade II consists of Asakura, Fukuoka (AF); Nobeoka, Miyazaki (NM); Hitoyoshi, Kumamoto (HK) and Kitsuki, Oita (KO). Lastly, Clade III include Munakata, Fukuoka (MF) and Taku, Saga (TS).

In the phylogenetic tree derived from the COI gene (Figure 3), the *G*. *nipponensis* specimens from this study, along with previously reported sequences, constitute a distinct clade, distinguishing them from other *Gammarus* species in Japan.

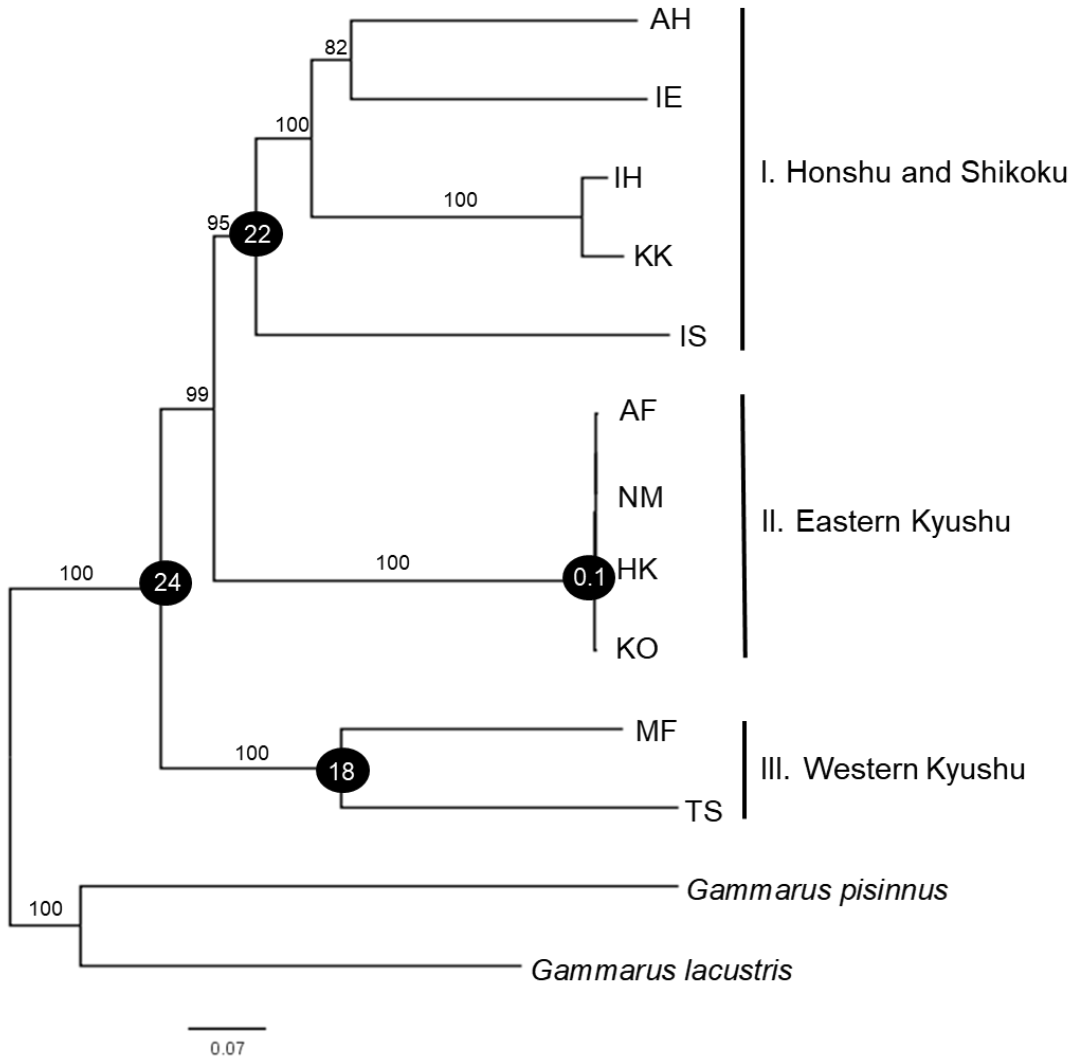

Figure 2. Maximum likelihood tree based on the partial mitogenome sequences (9,548 bp: 11 PCGs and 2 rRNA) from the 11 *Gammarus nipponensis* specimens. Bootstrap probabilities (%) are indicated beside the internal branches, and the scale bar represents a 7% nucleotide sequence difference. Numbers in black circles indicate the node age estimates (mya). Taxon letters correspond to the population codes shown in Figure 1. The outgroups are freshwater gammarids, *Gammarus pisinnus* and *Gammarus lacustris*.

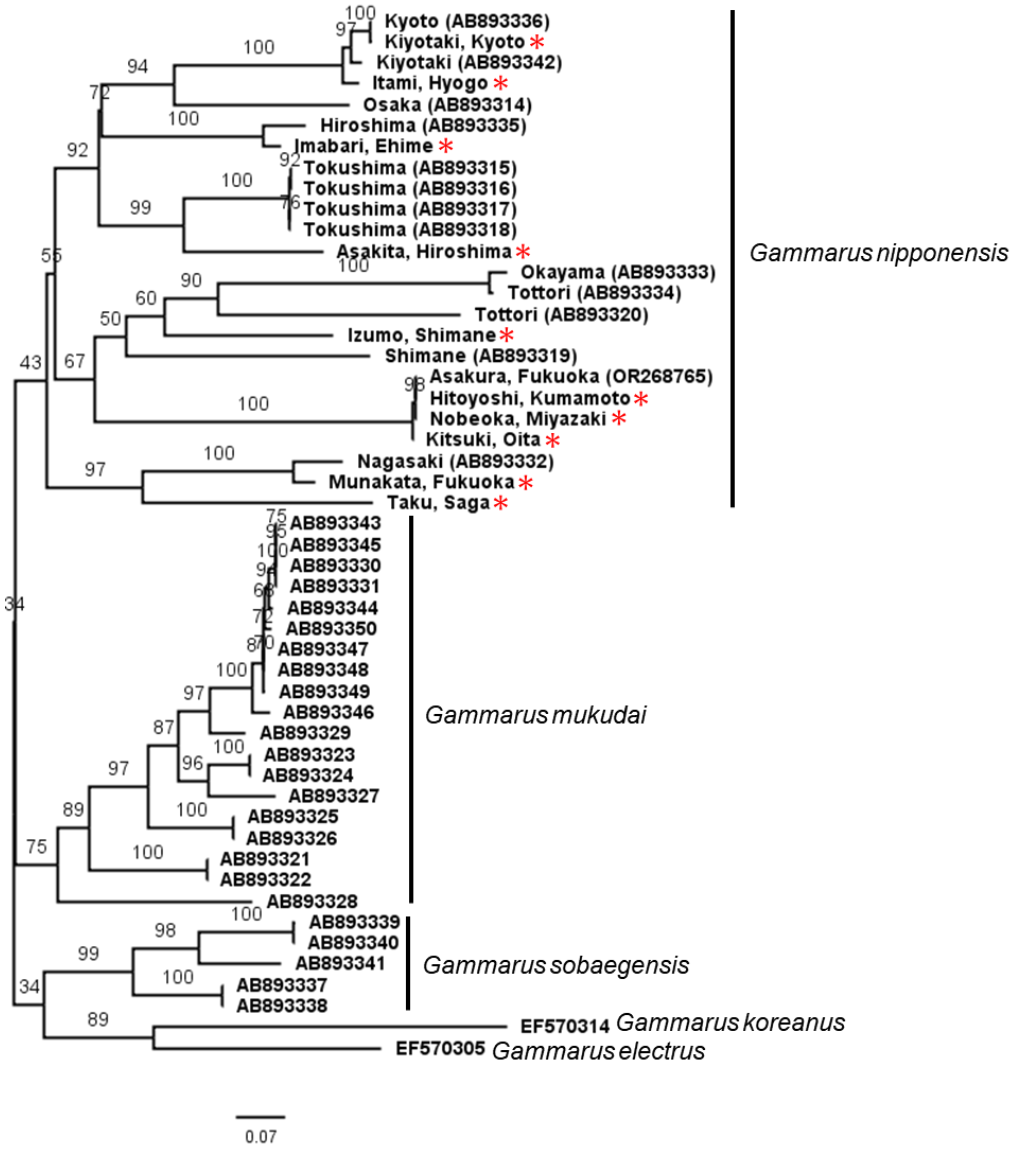

Figure 3. Maximum likelihood tree based on COI gene (676 bp) showing the relationships between *Gammarus nipponensis* and other *Gammarus* species in Japan, including their respective NCBI accession numbers and sampling locality. The newly sequenced gene in this study is marked with an asterisk (*). The support for each branch is indicated by percentages on the respective nodes. Bar signifies the nucleotide sequence differences.

The TMRCA estimates with 95% highest posterior densities (HPD), proposed that the *G*. *nipponensis* populations collectively shared a most recent common ancestor approximately 24.3 mya (HPD: 26.1-22.4 mya), corresponding to the late Oligocene period. Specifically, TMRCA for Clade I was around 22.3 mya (HPD: 24.0-20.6 million years ago), for Clade II was approximately 0.13 mya (HPD: 0.16-0.10 mya) and for Clade III was roughly 17.7 mya (HPD: 19.3-16.3 mya).

4.4.2 Mitogenome organization and nucleotide composition

From the mitogenome data, only the specimens from Asakura (AF) and Munakata (MF) had complete sequences with lengths of 16,429 and 16,395 bp, respectively (Table 1). Both specimens possess the typical closed-circular mitochondrial genome structure. However, certain specimens (AH, IH, KK and HK) will require Sanger sequencing at the ends of the contig to confirm their circular genome status. Currently, the mitogenome length of other specimens varies between 13,891 and 15,860 bp which was lower compared to AF and MF, primarily due to the presence of missing data or gaps in the sequences.

The mitogenome of *G*. *nipponensis* encompasses a total of 37 genes, comprising 13 protein-coding genes (PCGs: cox1-3, nad1-6, nad4L, CytB, atp6 and atp8), two ribosomal RNAs (rRNA: 16S and 12S), 22 transfer RNAs (tRNA: one for each amino acid and two for Leucine and Serine) and putative control regions (CRs) as outlined in Table 1. The majority of these genes were encoded on the forward (+) strand, with the exception of 4 PCGs (nad5, nad4, nad4L and nad1), 8 tRNAs (trnF, trnH, trnP, trnL1, trnV, trnQ, trnC and trnY) and 2 rRNAs situated on the reverse (-) strand. AH and TS, however,

exhibit an incomplete set of genes, necessitating interventions such as Sanger sequencing to address these deficiencies.

An interesting feature in the mitogenome that distinguishes certain localities from others was the presence of dual control regions (CRs). CR1 is situated between 12S and trnQ. Localities in Clade I had a CR1 length ranging from 858 to 934 bp, while Clade II localities exhibit nearly identical lengths at ~1000 bp. MF had the longest size at 1,451 bp, while TS had a length of 870 bp; both belong to Clade III. CR2 was exclusively identified in localities from Kyushu. Notably, only the localities in Clade II (AF, KO, NM and HK) exhibited tandem repeats. Both CR1 and CR2 possess a high AT content.

The nucleotide compositions of the *G. nipponensis* mitogenome sequence were highly biased toward A and T. The AT content of PCGs, tRNAs and rRNAs ranges from 60 to 67% (Table 2). The GC-skew of the *G. nipponensis* mitogenome was negative, while the AT-skew varies. Generally, AT skew was negative except for tRNAs of Clade I and III.

The average similarity of mitogenome sequences was only 75.04% (see Table 3). Higher sequence similarity was observed among mitogenomes belonging to the Clade II group, ranging from 98.97 to 99.47%. Notably, among the localities, TS exhibited the greatest differentiation with only 70.24% similarity to the others. The nucleotide variability of mitochondrial genes ranged from 38.64 to 76.27% with the highest record in ATP8 at 76.27% followed by nad6 at 73.16% and nad5 at 61.80%.

Table 1. Summary of the mitogenome characteristics of *Gammarus nipponensis* collected from various localities.

Gene	Position (Intergenic length)											Strand
	AH*	IE*	IH*	KK*	IS*	AF	NM*	HK*	KO*	MF	TS*	
cox1	1-1560 (0)	1-1560 (0)	1-1563 (0)	1-1563 (0)	1-1560 (0)	1-1536 (0)	4-1539 (0)	1-1563 (0)	4-1539 (0)	4-1569 (0)	4-1540 (0)	F
trnL2	1535-1594 (-26)	1535-1594 (-26)	1538-1597 (-26)	1538-1597 (-26)	1535-1594 (-26)	1536-1595 (-1)	1539-1598 (-1)	1536-1595 (-28)	1539-1598 (-1)	1538-1597 (-32)	1538-1597 (-3)	F
cox2	1610-2294 (15)	1610-2261 (15)	1613-2270 (15)	1613-2270 (15)	1610-2297 (15)	1593-2268 (-3)	1614-2312 (15)	1611-2268 (15)	1614-2312 (15)	1613-2320 (15)	1613-2270 (15)	F
trnK	2268-2326 (-27)	2268-2325 (6)	2271-2328 (0)	2271-2328 (0)	2268-2325 (-30)	2269-2327 (0)	2272-2330 (-41)	2269-2327 (0)	2272-2330 (-41)	2277-2333 (-44)	2277-2334 (6)	F
trnD	2327-2387 (0)	2326-2386 (0)	2329-2389 (0)	2329-2389 (0)	2326-2386 (0)	2327-2387 (-1)	2330-2390 (-1)	2327-2387 (-1)	2330-2390 (-1)	2334-2394 (0)	2335-2395 (0)	F
atp8	2388-2546 (0)	2387-2545 (0)	2390-2548 (0)	2390-2548 (0)	2396-2545 (9)	2388-2546 (0)	2391-2549 (0)	2388-2546 (0)	2391-2549 (0)	2404-2553 (9)	2396-2554 (0)	F
atp6	2543-3211 (-4)	2542-3210 (-4)	2542-3213 (-7)	2542-3213 (-7)	2551-3210 (5)	2540-3211 (-7)	2537-3214 (-13)	2534-3211 (-13)	2537-3214 (-13)	2550-3218 (-4)	2548-3219 (-7)	F
cox3	3211-3996 (-1)	3210-3995 (-1)	3213-3998 (-1)	3213-3998 (-1)	3210-3995 (-1)	3211-3996 (-1)	3214-3999 (-1)	3211-3996 (-1)	3214-3999 (-1)	3218-4003 (-1)	3219-4004 (-1)	F
nad3	3999-4352 (2)	4002-4346 (6)	4000-4353 (1)	4001-4354 (2)	3998-4351 (2)	3999-4352 (2)	4002-4355 (2)	3999-4352 (2)	4002-4346 (2)	4006-4359 (2)	4006-4359 (1)	F
trnA	4351-4411 (-2)	4350-4409 (3)	4352-4411 (-2)	4353-4412 (-2)	4357-4416 (5)	4353-4411 (0)	4354-4412 (-2)	4351-4409 (-2)	4354-4412 (7)	4358-4418 (-2)	4358-4418 (-2)	F
trnS1	4411-4461 (-1)	4409-4458 (-1)	4412-4463 (0)	4413-4463 (0)	4416-4466 (-1)	4412-4461 (0)	4415-4464 (2)	4412-4461 (2)	4415-4464 (2)	4418-4467 (-1)	4418-4467 (-1)	F
trnN	4463-4525 (1)	4460-4520 (1)	4465-4525 (1)	4465-4525 (1)	4466-4526 (-1)	4463-4523 (1)	4466-4526 (1)	4463-4523 (1)	4466-4526 (1)	4469-4530 (1)	4469-4526 (1)	F
trnE	4523-4582 (-3)	4518-4577 (-3)	4523-4584 (-3)	4523-4584 (-3)	4526-4580 (-1)	4521-4582 (-3)	4524-4585 (-3)	4521-4582 (-3)	4524-4585 (-3)	4528-4591 (-3)	4527-4587 (0)	F
trnR	4577-4636 (-6)	4572-4632 (-6)	4579-4641 (-6)	4579-4641 (-6)	4582-4641 (1)	4577-4634 (-6)	4580-4637 (-6)	4577-4634 (-6)	4580-4637 (-6)	4586-4645 (-6)	4582-4640 (-6)	F
trnF	4635-4693 (-2)	4631-4690 (-2)	4640-4698 (-2)	4640-4698 (-2)	4640-4697 (-2)	4633-4692 (-2)	4636-4695 (-2)	4633-4692 (-2)	4636-4695 (-2)	4644-4703 (-2)	4639-4698 (-2)	R
nad5	4693-6384 (-1)	4691-6382 (0)	4699-6390 (0)	4699-6390 (0)	4701-6384 (3)	4692-6383 (-1)	4695-6386 (-1)	4692-6383 (-1)	4695-6386 (-1)	4705-6402 (1)	4669-6382 (-30)	R
trnH	6403-6464 (18)	6401-6461 (18)	6409-6469 (18)	6409-6469 (18)	6403-6463 (18)	6402-6460 (18)	6405-6464 (18)	6402-6461 (18)	6405-6464 (18)	6421-6482 (18)	6401-6461 (18)	R
nad4	6465-7764 (0)	6462-7752 (0)	6458-7748 (-12)	6458-7748 (-12)	6476-7775 (12)	6461-7757 (0)	6424-7737 (-41)	6450-7749 (-12)	6424-7737 (-41)	6442-7755 (-41)	6457-7737 (-5)	R
nad4L	7770-8054 (5)	7767-8054 (14)	7775-8062 (26)	7775-8062 (26)	7769-8056 (-7)	7766-8050 (8)	7770-8054 (32)	7767-8051 (17)	7770-8054 (32)	7788-8081 (32)	7767-8060 (29)	R
trnT	8061-8120 (6)	8058-8118 (3)	8066-8125 (3)	8066-8124 (3)	8059-8118 (2)	8054-8112 (3)	8058-8116 (3)	8055-8113 (3)	8058-8116 (3)	8084-8143 (2)	8063-8123 (2)	F
trnP	8120-8181 (-1)	8118-8179 (-1)	8125-8185 (-1)	8124-8184 (-1)	8118-8178 (-1)	8112-8173 (-1)	8116-8177 (-1)	8113-8174 (-1)	8116-8177 (-1)	8143-8204 (-1)	8123-8184 (-1)	R
nad6	8184-8687 (2)	8184-8687 (4)	8188-8691 (2)	8187-8690 (2)	8190-8681 (11)	8176-8679 (2)	8180-8683 (2)	8177-8680 (2)	8180-8683 (2)	8219-8707 (14)	8187-8681 (2)	F
cytb	8687-9820 (-1)	8673-9806 (-15)	8691-9824 (-1)	8690-9823 (-1)	8681-9814 (-1)	8679-9809 (-1)	8683-9813 (-1)	8680-9810 (-1)	8683-9813 (-1)	8712-9851 (4)	-	F
trnS2	9819-9878 (-2)	9805-9864 (-2)	9823-9881 (-2)	9822-9880 (-2)	9813-9872 (-2)	9811-9870 (1)	9815-9874 (1)	9812-9871 (1)	9815-9874 (1)	9850-9909 (-2)	9900-9957	F
nad1	9920-10858 (41)	9911-10843 (46)	9976-10914 (94)	10365-11303 (484)	9913-10851 (40)	9909-10838 (38)	9913-10848 (38)	9910-10845 (38)	9913-10848 (38)	9949-10884 (39)	-	R
trnL1	10853-10912 (-6)	10838-10897 (-6)	10909-10968 (-6)	11298-11357 (-6)	10849-10908 (-3)	10839-10898 (0)	10843-10902 (-6)	10840-10899 (-6)	10843-10902 (-6)	10879-10938 (-6)	10063-10122 (105)	R
16S	10913-11896 (0)	10899-11876 (1)	10970-11948 (1)	11359-12338 (1)	10909-11886 (0)	10900-11877 (1)	10904-11882 (1)	10901-11880 (1)	10904-11881 (1)	10939-11918 (0)	10124-11101 (1)	R
trnV	11895-11944 (-2)	11875-11928 (-2)	11947-12000 (-2)	12337-12390 (-2)	11885-11936 (-2)	11879-11930 (1)	11883-11934 (0)	11880-11931 (-1)	11883-11934 (1)	11919-11969 (0)	11100-11154 (-2)	R
12S	11944-12678 (-1)	11920-12640 (-9)	11992-12728 (-9)	12382-13122 (-9)	11936-12658 (-1)	11931-12653 (0)	11927-12653 (-8)	11931-12656 (-1)	11927-12651 (-8)	11962-12713 (-8)	11146-11882 (-9)	R
CR1	-	12641-13574 (0)	12729-13586 (0)	13123-13981 (0)	12659-13520 (0)	12654-13653 (0)	12654-13659 (0)	12657-13656 (0)	12652-13657 (0)	12714-14164 (0)	11883-12752 (0)	
trnQ	-	13575-13632 (0)	13587-13642 (0)	13982-14037 (0)	13521-13572 (0)	13654-13705 (0)	13660-13711 (0)	13657-13708 (0)	13658-13709 (0)	14165-14215 (0)	12753-12808 (0)	R
trnC	-	13629-13686 (-4)	13643-13698 (0)	14038-14093 (0)	13563-13617 (-10)	13706-13760 (0)	13712-13766 (0)	13709-13763 (0)	13710-13764 (0)	14216-14271 (0)	12809-12864 (0)	R
trnY	-	13655-13707 (-32)	13823-13876 (124)	14274-14333 (180)	13618-13678 (0)	13903-13963 (142)	13909-13969 (142)	13906-13966 (142)	13907-13967 (142)	14272-14333 (0)	12865-12926 (0)	R
CR2	-	-	-	-	-	13964-15195 (0)	13970-14649 (0)	13967-14177 (0)	13968-14629 (0)	14334-15172 (0)	12927-13189 (0)	
trnI	12663-12721 (-16)	13785-13843 (77)	13905-13963 (28)	14362-14420 (28)	13767-13825 (88)	15196-15254 (0)	14650-14708 (0)	14177-14235 (0)	14630-14688 (0)	15173-15230 (0)	13190-13247 (0)	F
trnM	12722-12782 (0)	13844-13904 (0)	13964-14024 (0)	14421-14481 (0)	13826-13886 (0)	15255-15316 (0)	14709-14769 (0)	14236-14297 (0)	14689-14750 (0)	15231-15291 (0)	13248-13309 (0)	F
nad2	12804-13793 (21)	13904-14889 (-1)	14046-15009 (21)	14503-15466 (21)	13887-14874 (0)	15317-16304 (0)	14770-15757 (0)	14298-15285 (0)	14751-15738 (0)	15292-16279 (0)	13334-14324 (24)	F
trnW	13768-13826 (-26)	14890-14948 (0)	15010-15068 (0)	15467-15526 (0)	14875-14934 (0)	16305-16364 (0)	15758-15817 (0)	15286-15345 (0)	15739-15798 (0)	16274-16334 (-6)	14295-14355 (-30)	F
trnG	13827-13888 (0)	14949-15010 (0)	15069-15130 (0)	15527-15588 (0)	14935-14995 (0)	16365-16426 (0)	15818-15879 (0)	15346-15407 (0)	15799-15860 (0)	16335-16395 (-6)	14356-14416 (0)	F
Mitogenome length	13,891	15,013	15,130	15,588	14,998	16,429	15,879	15,410	15,860	16,395	14,416	

Asterisk (*) denotes an incomplete mitogenome sequence, hyphen (-) indicates missing data from Next-Generation Sequencing (NGS)

Table 2. Nucleotide composition and skewness of *Gammarus nipponensis* mitogenome from the 11 localities.

Pop code	A+T%				G+C%				AT-skew				GC-skew			
	All	PCGs	tRNAs	rRNAs	All	PCGs	tRNAs	rRNAs	All	PCGs	tRNAs	rRNAs	All	PCGs	tRNAs	rRNAs
Clade I																
AH*	-	60.839	-	65.340	-	39.161	-	34.660	-	-0.067	-	-0.001	-	-0.379	-	-0.332
IE*	-	61.245	64.901	65.219	-	38.755	35.099	34.781	-	-0.076	0.015	-0.011	-	-0.421	-0.228	-0.405
IH*	-	64.021	66.210	65.312	-	35.979	33.790	34.688	-	-0.072	0.007	-0.023	-	-0.400	-0.187	-0.420
KK*	-	63.511	66.376	67.654	-	36.489	33.624	32.346	-	-0.068	0.018	-0.015	-	-0.388	-0.217	-0.365
IS*	-	60.430	62.979	64.429	-	39.570	37.021	35.571	-	-0.089	0.006	-0.005	-	-0.385	-0.201	-0.374
Clade II																
AF	67.100	64.363	65.722	67.707	32.900	35.637	34.278	32.293	-0.054	-0.064	-0.006	-0.0003	-0.325	-0.370	-0.190	-0.365
NM*	-	64.332	65.944	67.539	-	35.668	34.056	32.461	-	-0.066	-0.006	-0.0001	-	-0.364	-0.154	-0.370
HK*	-	64.153	66.043	67.670	-	35.847	33.957	32.330	-	-0.065	-0.005	-0.003	-	-0.366	-0.156	-0.360
KO*	-	64.403	66.119	67.495	-	35.597	33.881	32.505	-	-0.064	-0.004	-0.003	-	-0.370	-0.163	-0.366
Clade III																
MF	62.700	60.799	62.757	64.690	37.300	39.201	37.243	35.310	-0.027	-0.077	0.004	-0.003	-0.335	-0.383	-0.192	-0.377
TS*	-	-	61.810	60.933	-	-	38.190	39.067	-	-	0.020	-0.028	-	-	-0.180	-0.359

Asterisk (*) denotes an incomplete mitogenome sequence, hyphen (-) indicates missing data from Next-Generation Sequencing (NGS)

Table 3. Genetic similarity (%) of the 11 *Gammarus nipponensis* partial mitogenome sequences (PCGs and rRNAs).

Pop code	TS	AH	IE	IH	KK	IS	AF	NM	HK	KO
AH	69.85	-								
IE	69.81	75.13	-							
IH	69.69	75.58	75.61	-						
KK	69.53	75.27	75.07	94.36	-					
IS	68.95	72.39	72.05	72.48	72.46	-				
AF	70.14	72.82	72.91	73.05	72.76	72.05	-			
NM	70.18	72.80	72.97	73.16	72.83	72.08	99.16	-		
HK	70.23	72.84	72.94	73.14	72.82	72.11	99.10	99.47	-	
KO	70.17	72.77	72.96	73.12	72.80	72.09	98.98	99.09	98.97	-
MF	73.89	71.04	71.24	71.77	71.70	70.36	71.19	71.27	71.29	71.24

4.5 Discussion

This study estimated the molecular phylogenetic relationships of *G*. *nipponensis* collected from its known distribution range. The monophyly of *G*. *nipponensis* as determined by the partial mitogenome sequences is strongly supported. This species was composed of three distinct groupings: Clade I comprise localities from Honshu and Shikoku, Clade II from Eastern Kyushu, and Clade III from Western Kyushu. Utilizing mitogenome sequences significantly enhanced the monophyletic support for *G*. *nipponensis* reaching 100%, in contrast to the approximately 70% support observed when employing partial gene sequences as reported by Tomikawa et al. (2014). Also, a low support value of 43% was observed for the COI gene (Figure 3). Although, high nucleotide differences can be indicative of evolutionary divergence, it may also introduce noise or saturation in the data leading to insufficient statistical support for the inferred branching pattern thus, concatenating multiple genes yields more accurate trees (Gadagkar et al. 2005; Fleming et al. 2023). Also, relying on a single or few genes has a significant probability of supporting conflicting topologies, while analyses of larger datasets such as the mitogenome dataset resolve species relationships with maximum support (Rokas et al. 2003; Shen et al. 2013). Despite some minor gaps or missing data in the genes on the mitogenome in the present study, the phylogenetic analysis still yielded reliable data for inferring genetic relationships among the representative local populations of *G*. *nipponensis*.

The mitogenomes of *G*. *nipponensis* examined in this study generally display a conserved gene order, however, variations in the number of CR were noted. Specimens from Kyushu had two CRs while those from Honshu and Shikoku have only one. Within

the Kyushu localities, only those associated with Clade II exhibits tandem repeats in CR2. Duplications in CR are not rare in the amphipod mitogenome. A duplication of the CR within superfamily Gammaroidea was identified in the *Garjajewia cabanisii* (Romanova et al. 2016a) and a similar occurrence was reported in the mitogenome of species under Genus *Gammarus*. Among the available eight mitochondrial genomes in the public database, *G*. *pullex* and *G*. *roeselii* were found to possess the duplicated CRs (Cormier et al. 2018, Mamos et al. 2021). Thus, variability in the number of CR among species within the same genus is observed. However, in the present study, differences in the number of CR were observed even within species. Apparently, this occurrence appeared to be unique to *G*. *nipponensis*, as no previous studies documenting similar results are available. The existence of duplicated CRs in certain lineages while absent in others suggest two potential explanations for their evolution: 1) the region underwent duplication in a common ancestor and was subsequently inherited or lost by its descendants, or 2) the duplication independently occurred in specific lineages (Mackiewicz et al. 2019). Similarly, once duplicate CRs manifest in a mitogenome, their evolution may proceed either in concert or independently. Independent evolution results in the divergence of nucleotide sequences between the two CRs (similar to *G*. *nipponensis*), potentially leading to the eventual deletion of one of the CR (Bensch and Harlid 2000). Conversely, concerted evolution maintains high nucleotide sequence similarity between the two CRs (Kumazawa et al. 1996; Li et al. 2015). In most studies, duplicate CRs in mitogenome tend to evolve in concert in each species rather than independently (Shao et al. 2004). Identical or highly similar nucleotide sequences have been observed in invertebrates such as ticks (Campbell and Barker 1999; Shao et al. 2005), ostracods (Ogoh and Ohmiya 2004) and amphipods (Ito et al. 2010; Cormier et al. 2018, Mamos et al. 2021)

while species with heterologous CRs are not extensively documented. The underlying mechanism responsible for the occurrence of tandem repeats in the CR2 of localities within Clade II, and not in other Clades, remains unclear. In a study on Medaka (*Oryzias latipes)*, a freshwater fish commonly found in East Asia, the variation in the number of repeat units within CR among its populations has been correlated with indices reflecting cold environmental conditions and seasonal climatic changes (Hirayama et al. 2010). The distinctive features observed in the CR of *G*. *nipponensis* warrant further investigation to ascertain the ecological or biological factors influencing these variations. Likewise, the observed variations in the lengths of CR1 among *G*. *nipponensis* specimens suggest that this could serve as a valuable molecular marker for conducting population genetic investigations at a finer scale, such as between rivers or between branches of the river system. The nucleotide composition of the mitogenomes, as well as the individual mitochondrial genes, exhibits variations among the specimens. This can be a valuable source for designing new molecular markers for future intraspecific phylogeographic and population genetic studies (Romanova et al. 2016a; Zhang et al. 2018). Considering the species-specific markers with enhanced sensitivity and superior discrimination capabilities will provide for more precise genetic analyses.

The divergence time estimates from this study suggest that *G*. *nipponensis* diverged from other *Gammarus* species in mainland Asia more than 24 mya, prior to the isolation of Japan from the mainland Asia. This time estimates are consistent with the investigation by Hu et al. (2022), suggesting that *Gammarus* species in Northeast Asia likely initiated diversification in the late Oligocene. Subsequently, the common ancestor of *G*. *nipponensis* probably migrated from mainland Asia to the western part of Japan. During the early Miocene (20-15 mya), the Japan Sea underwent gradual opening

process leading to the isolation of Japan as an island arc system (Otofuji et al. 1991; Ma et al. 2003; Isozaki et al. 2010), which have driven the separation between the mainland Asia and Japan. In addition, during the late Miocene, a few seawater bodies probably existed in Eastern Japan because of subsidence of the Tohoku-Hokkaido area (Sato 1994; Yoshida et al. 2013), hindering the continues expansion of *G. nipponensis* to the area.

The relatively old lineage of *G. nipponensis* within Clades I and III demonstrates the potential for the continuous survival of ancient lineages, despite millions of years of significant climatic fluctuations during the Pleistocene. Similar instances of this adaptive capacity has also been documented in other *Gammarus* species (Copilaş-Ciocianu and Petrusek 2015). Moreover, the high reproductive capability of gammarids could play a crucial role in their ability to rapidly expand their population in the case of high mortality and to colonize new territories when conditions are favorable (Pöckl et al. 2003). This adaptive feature enables them to effectively counteract the adverse effects of unfavorable environmental conditions that have recurrently occurred throughout the glacial and interglacial periods of the Pleistocene era. During glacial periods, the Japanese Archipelago's main islands (modern-day Honshu, Shikoku, and Kyushu) formed land bridges, facilitating the continuous dispersal of freshwater species. However, geographical features such as marine waters constrained the movement of these land-locked species, leading to regional differentiation due to restricted gene flow. The Seto Inland Sea likely influenced the genetic separation of Clade I (Honshu and Shikoku) from Clades II and III (Kyushu). Although being separated by the Seto Inland Sea, Honshu and Shikoku still exhibit close genetic relationships, possibly due to their relatively recent separation. The estimates of divergence time between these clades, however, suggest

a period prior to the formation of the Seto Inland Sea. This divergence occurred approximately during the Miocene, aligning with the opening of the Japan Sea. This geological event could have played a role in dividing chains of mountains, subsequently influencing the distribution range of these populations and affects the structuring of the ancient lineage (Iijima and Tada 1990; Yonekura et al. 2001). Following this disruption in distribution, Japan has been divided into multiple islands as observed in the present day, with the Seto Inland Sea functioned as a significant dividing barrier for these populations.

The presence of two genetically divergent groups in Kyushu (Clade II and III) may be attributed to isolation resulting from the formation of the nearly flat-topology Chikushi and Fukuoka plains, acting as a boundary between the two. The clustering of MF to TS in Clade III suggests that this may have been able to maintain habitable areas and led to their genetic similarity. However, to validate this observation, it is necessary to further clarify the distribution and boundaries of both Clades II and III in greater detail. A comprehensive investigation, perhaps extending to additional localities near to the lowland areas of Western Kyushu should be undertaken. Additionally, Clade II, might experience rapid reduction in population size around ~0.13 mya. This often leads to low sequence variability as only a subset of the original genetic diversity survives (Nei et al. 1975; Sousa et al. 2008; Zimmermann et al. 2018). The potential reduction in population size observed in Clade II may be linked to the historical activity of the Aso Volcano, situated in the west-central region of Kyushu. Between 1.30-0.09 mya, the volcano produced extensive pyroclastic flows after volcanic eruptions, resulting in significant environmental destruction (Watanabe and Ono 1992). This severe habitat destruction is also known to contribute to declines in salamander populations (Nishikawa et al. 2005; Sakamoto et al. 2005).

In summary, the mitogenomes of *G*. *nipponensis* offer valuable insights into both genetic relationships and the geological events that have influenced the observed clustering of representative localities. These initial findings suggest to conduct more extensive population samplings to clarify the boundaries between the observed groupings. Following this, population genetic studies is recommended specifically designed with molecular markers derived from the data of this study such as the CR1. Furthermore, the need for a thorough morphological reexamination among the identified groupings to confirm the presence of a potential cryptic species complex, as well as the necessity for hybridization experiments.

Chapter 5

General discussion

General discussion

Based on the findings presented in this thesis, it is speculated that the ancestral population of *G*. *nipponensis* dispersed from mainland Asia to Western Japan around the Oligocene period (~24 mya), taking advantage of land connections that existed prior to the opening of the Japan Sea around 15 mya (Figure 1A). Also, it can be possible that *G*. *nipponensis* have been inhabiting Japan prior to this period. The subsequent opening of the Japan Sea led to the separation of *G*. *nipponensis* from mainland Asia (Figure 1B). This diversification pattern aligns with similar observations in other invertebrates (Xu et al. 2016; Saito et al. 2018; Li et al. 2020). Following the formation of the Japan Sea, Eastern Japan transformed into a chain of islands and did not undergo uplift until the late Miocene (Iijima and Tada 1990). Moreover, during the middle Miocene (Figure 1C), a sedimentary basin known as the Fossa Magna took shape in central Japan due to back-arc rifting associated with the opening of the Japan Sea (Iijima and Tada 1990). Hence, it can be assumed that the ancestral lineage of *G*. *nipponensis*, existing prior to this event, might have undergone a significant population decline or extermination attributed to the submergence of Eastern Japan by seawater during that period. This geological phenomenon could have hindered the continuous dispersal of *G*. *nipponensis* in Eastern Japan and potentially contributed to the establishment of its distribution boundary. Consequently leading to the restricted distribution of this species observed in Western Japan that persists to the present day (Tomikawa 2017).

During the period when Japan was connected to the mainland, the ancestors of various terrestrial and freshwater organisms colonized Japan via multiple routes (Ota 1998; Motokawa 2017; Miyake et al. 2021). Following colonization, it is well-established

that during the Oligocene and Miocene periods, Japan underwent significant tectonic activities and land movements, leading to mountain uplifts and land submergence and eventually contributing to the division of mountain chains (Lallemand and Jolivet 1986; Taira et al. 2016). These events likely played a crucial role in shaping the distribution patterns of ancient *G. nipponensis* and influencing the structure of its ancestral lineage. Following the distribution expansion, ancient lowlands between mountains could have functioned as barriers to the continuous expansion in this species. Subsequently, the glaciations and interglaciations events of the Pleistocene increased the genetic divergence and species' local adaptation (Fu and Wen 2023).

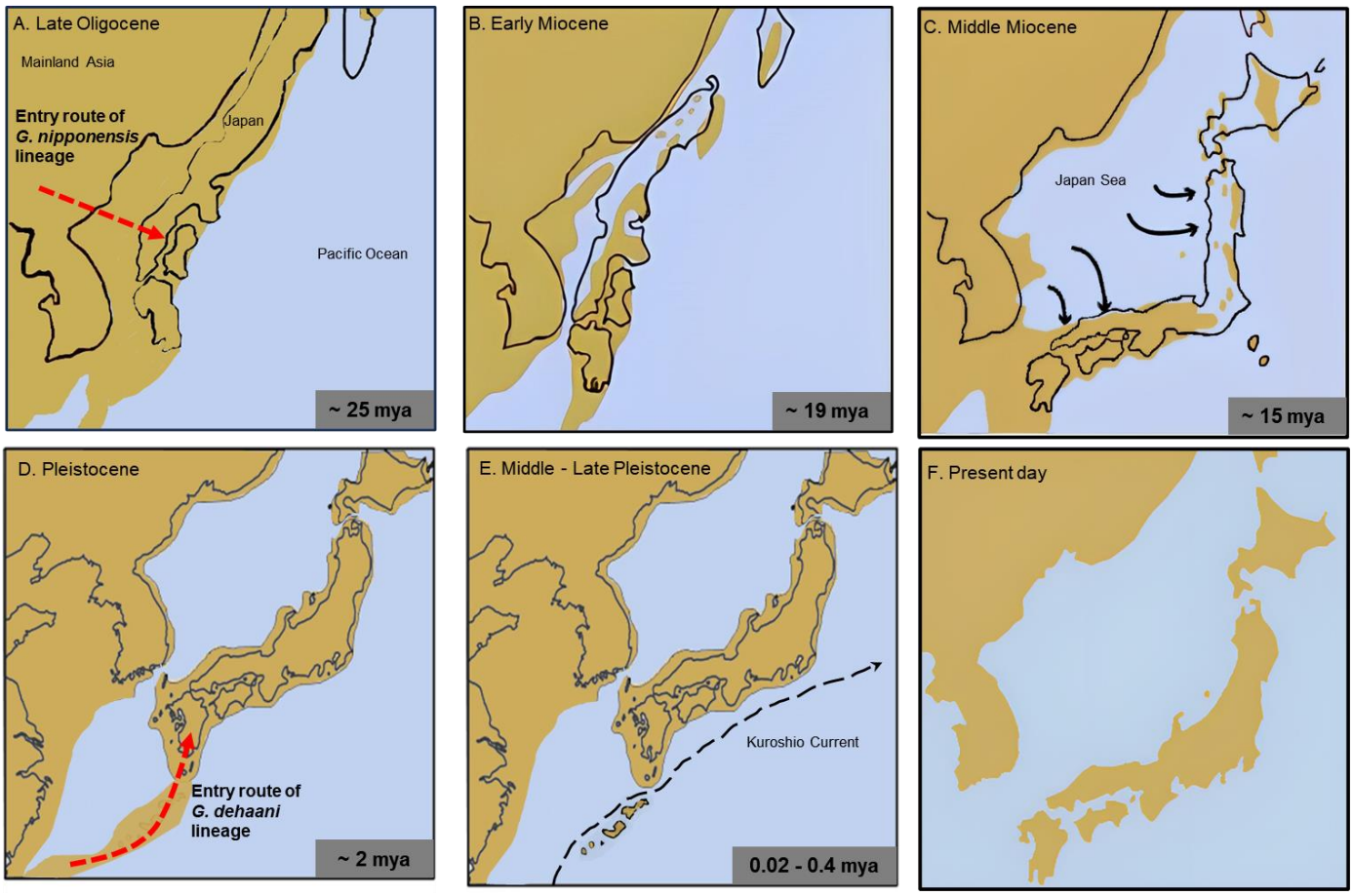

Figure 1. Hypothetical paleogeographic reconstruction for Japanese Archipelago from late Oligocene to present (A-F) depicting its role to the assumed formation and structure of *Gammarus nipponensis* and *Geothelphusa dehaani.* Brown shading (A-F) indicates

land areas not submerged by sea represented in Blue shade. Red arrow signify entry route of *G*. *nipponensis* and *G*. *dehaani* (A&D), while black arrow (E) shows path of the Kuroshio Current (Kimura 1996; Yonekura et al. 2001; Saito 2019).

As mentioned, the divergence patterns among *G*. *nipponensis* group likely originated from late Oligocene to Miocene tectonic activities. This phenomenon aligns with the restricted distribution observed in numerous *Gammarus* species across Northeast Asia where vicariance events were linked to the onset of volcanism starting around the late Oligocene, as discussed by Hu et al. (2022). With this, *G*. *nipponensis* is considered to have been spread over Japan from Miocene until most populations became extinct. Thus, present *G*. *nipponensis* population can be hypothesized to contain relicts of pre-Pleistocene. The survivors were able to colonize newly available habitat thereafter. However, additional phylogeographic investigations are needed to confirm this hypothesis. While pre-Pleistocene relicts have been found in other crustaceans (Grandcolas et al. 2014; Kotov et al. 2022), population genetic studies on pre-Pleistocene relicts are scarce.

As indicated by the findings of this study, the original stock of *G*. *dehaani* was assumed to dispersed from Ryukyus during the Pleistocene where land bridges emerged repeatedly during the Pleistocene around 1.7-2.0 mya (Kimura 1996) (Figure 1D). It is also suggested that the genus *Geothelphusa* have occurred on the Ryukyu Islands since the Miocene (Shokita 1996). Then isolated on the islands after continental separation and subsequently leading to further speciation. At around 0.4-1.0 mya, Kyushu and Ryukyus separated and formed to its present shape due to sea level rise (Kimura 1996). As for primary freshwater fish in Japan, active interactions between Japanese and

mainland Asia freshwater fish fauna occurred during the late Miocene to the early Pleistocene and no evidence of faunal interactions after that (Watanabe et al. 2006).

The *G*. *nipponensis* and *G*. *dehaani* populations in this study were highly monophyletic and the observed genetic groupings in both species appeared to be similar. However, *G*. *nipponensis* populations have a more ancient lineage, a higher degree of isolation and a poorer dispersal ability compared to those of *G*. *dehaani*. Notably, the populations in both species inhabiting the Honshu and Shikoku regions were observed to form a monophyletic group, genetically differentiated from the Kyushu region. The ancient lowlands in the current Seto Inland Sea were identified as an effective boundary in the division of the Honshu-Shikoku and Kyushu groups. While, within the Kyushu region, two distinct geographic groups were identified, with a dispersal boundary around the Chikushi and Fukuoka plains.

In contrast to the observation in the present study, previous research has identified the Suzuka Mountains and Central Highlands in Honshu as significant geographic barriers that segregate populations of widely distributed freshwater species (Yoshikawa et al. 2008; Tominaga et al. 2016; Watanabe et al. 2017; Tominaga et al. 2020). In fact, notable genetic differentiation within species is commonly observed between the western and eastern areas of the Central Highlands (Watanabe et al. 2017). However, the lowland freshwater fish, medaka *Oryzias sakaizumii*, and bitterling *Tanakia lanceolata*, are found to be distributed across the highlands (Takehana et al. 2003; Katsumura et al. 2019). It is suggested that during the glacial periods, the low salinity of the Japan Sea (Tada 1994; Kitamura et al. 2001) may have facilitated the movement of lowland freshwater fishes along the coast. The divergence of *G*. *dehaani* populations does not align with the differentiation associated with these barriers, likely attributable to

the limited number of populations examined near these recognized boundaries. Nonetheless, despite this limitation populations within Honshu showed unique haplotypes, indicating very limited gene flow even between populations. Additionally, in some freshwater fish populations in Western Honshu and Kyushu, there is genetic homogeneity, suggesting some historical gene flow among these regions (Takehana et al. 2003; Komiya et al. 2014; Tominaga et al. 2016). In giant water bugs, *Appasus japonicus*, it was also observed that Kyushu and the westernmost region of Honshu shared common haplotypes (Suzuki et al. 2014). Such gene flow would be possible, at least in part, during periods of lower sea levels when river systems in these regions have reconnected due to the drying of Seto Inland Sea (Yonekura et al. 2001). In freshwater shrimp, *Palaemon paucidens*, close genetic relations were observed between populations from Shikoku and Kyushu (Chow et al. 2019). In this study, the populations of *G*. *dehaani* and *G*. *nipponensis* in Honshu and Shikoku exhibited a closer genetic relationship compared to the populations in Kyushu with Seto Inland Sea assumed to function as a geological boundary. The variation in the patterns of population subdivision may be attributed to differences in ecological and life history factors of each species, such as variations in dispersal abilities and local extinction events (Tominaga et al. 2016; Watanabe et al. 2017). Similarly, the Chikushi and Fukuoka plains proves to be a significant barrier, delineating two distinct genetic groups within these two freshwater crustaceans in Kyushu. Remarkably, this plain does not exhibit a similar barrier effect in the distribution of fishes. In bitterling for example, the isolation of two regional groups in Kyushu (Northern and Middle-southern), was suggested to be due to the mountains of Northern Kyushu (Sefuri mountains) (Tominaga et al. 2020). Similar pattern is found in

some freshwater fish species (*Rhodeus atremius*: Miyake et al. 2011), but not in others (*Tanakia limbata*: Hashiguchi et al. 2006).

This variation among different freshwater organisms may arise from disparities in their abilities and mechanisms of dissemination. Freshwater fish have a high active dispersal potential within well-connected river and wetland systems but a low dispersal potential within isolated aquatic ecosystems (Fagan 2002; Shurin et al. 2009). On the other hand, freshwater crustaceans such as crabs and gammarids may have a limited active dispersal potential but may present a passive dispersal potential (Bilton et al. 2001; Bohonak and Jenkins 2003; Esser and Cumberlidge 2011). For species with passive dispersal potential, the extent to which gene flow is constrained depends on the degree of habitat fragmentation. Greater habitat fragmentation tends to inhibit the movement of individuals, limiting gene flow, while less fragmentation allows for more dispersal and increased gene flow among populations.

In addition, variations in population structures between freshwater organisms may also influenced by effective population size (Avise 1992), ecological traits (Wellborn et al. 1996; Davis et al. 2013) and chance events (Bohonak 1999; De Bruyn and Mather 2007). Furthermore, to better understand the complex distributional pattern of the *G*. *dehaani* and *G*. *nipponensis* mtDNA lineage, a more detailed analysis using additional molecular markers (e.g. nuclear DNA) and expanding the scope of sampling sites are suggested. Future investigations of morphological and behavioral characteristics based on the genetically detected groups from these crustaceans should also be conducted.

Summary

Freshwater crustaceans, *Geothelphusa dehaani* (White 1847) and *Gammarus nipponensis* (Uéno 1940), are commonly found in rivers, streams and lakes in Japan. The distribution range of *G. nipponensis* is concentrated in the Western portion of Japan while *G. dehaani* exhibits a more widespread distribution. Both species undergo direct development from eggs to juveniles and have limited migratory abilities, as they are intolerant to brackish and marine environments. This restricted dispersal capability leads to geographical isolation among populations, making these freshwater crustaceans as ideal models for studying gene flow. Despite their significance, there were scarcity of studies that clarified genetic patterns in these two widely distributed crustaceans. Thus, the objectives of this thesis were to conduct genetic assessments on both *G. dehaani* and *G. nipponensis* to understand their population structure and variation, and to provide insights into past geological events that have contributed to these variations. Here, mitochondrial DNA sequence data were utilized to infer the relationships between the local populations of these species.

Results of this study revealed substantial genetic variations among populations in both crustaceans. In *G. dehaani*, distinct haplotypes were observed in most populations, while *G. nipponensis* exhibited remarkably high mitogenome sequence variability across different localities. These findings implied extremely limited gene flow within each population and a reduced capacity for migration over extended periods, a characteristic that distinguishes them from other strictly freshwater species. The time to most recent common ancestor (TMRCA) estimates proposed that the ancestor lineage of *G. dehaani* was introduced more recently in Japan, approximately around the

Pleistocene (~1 million years ago), while that of *G*. *nipponensis* predates the separation of Japan from mainland Asia around late Oligocene (~24 million years ago). Despite these differences, the structural patterns of populations in both species appeared relatively similar.

Based on the phylogenetic analysis, the populations in both species are considered highly monophyletic with several geographical groups detected. In *G*. *dehaani*, four distinct geographic groups formed: Clade I (Honshu and Shikoku), Clade II (Eastern Kyushu), Clade III (Southern Kyushu and a portion of Eastern Honshu) and Clade IV (Western Kyushu). In *G*. *nipponensis*, the three identified groups were Clade I (Honshu and Shikoku), Clade II (Eastern Kyushu) and Clade III (Western Kyushu). The geological boundaries separating these major clades consisted of ancient lowlands such as the present Seto Inland Sea and the Chikushi and Fukuoka plains, acting as barriers for gene flow. The Seto Inland Sea potentially contributes to the divergence of Clade I from the Kyushu group. Despite the Seto Inland Sea acting as a barrier, Honshu and Shikoku displayed closer genetic relationships, possibly attributed to their more recent separation. Likewise, the Chikushi and Fukuoka plains was a significant barrier, defining distinct genetic groups in Kyushu.

In conclusion, this study emphasized the importance of these two freshwater crustaceans in understanding genetic relationships characterized with limited gene flow and the impact of geological events on their population dynamics in the Japanese Archipelago. The need for further examination of morphological and behavioral characteristics based on the genetically detected groups of *G*. *dehaani* and *G*. *nipponensis* is suggested.

Acknowledgements

I dedicate this significant milestone to my family and friends, whose continuous inspiration and support have been instrumental in driving me to successfully complete my PhD degree.

I would like to express my sincere gratitude to the following who have made an outstanding impact throughout my PhD journey:

To the management of SEAFDEC/AQD and MEXT, who provided me an opportunity to advance and enhance my professional career,

To my supervisor, Dr. Yoshihisa Kurita, who has been exceptionally patient, understanding and kind while guiding me through all of my research activities. I could not have survived this journey without his constant direction and assistance in my research,

To Dr. Norio Onikura, one of my review committees, who supported my laboratory activities from Day 1 and provided valuable comments on this thesis,

To the other members of my review committee, Dr. Yukiko Ogino and Dr. Ka Fai William Tse, who eagerly provided suggestions and constructive comments that markedly enhanced the quality of this thesis,

To our research collaborators, Dr. Norio Miyamoto and Dr. Yuichi Kano, who generously shared their knowledge and expertise,

To Mr. Ando Daiki and my labmates, who provided invaluable help during my initial years in Japan and never hesitated whenever I sought assistance,

To all other members of the Aquatic Field Science Laboratory (Fishery Research Laboratory), both former and current staff, as well as my fellow students, who maintained a respectful and friendly environment,

To my parents, who inspired me to become a better person every day,

To my ever-supportive and loving husband, Fredson, who immensely sacrificed for fulfilling the roles of both mother and father while I am away. Who helped me endure life away from my comfort zone and pushed me to continue despite the difficulties,

To my children, Hans and Euna, who inspired me to pursue better opportunities for our future and to be strong no matter what,

Lastly, to God, who provided me with strength and refuge every day, enabling me to sustain and protect the life bestowed upon me.

Looking forward to a better future! May God continue to shower us with blessings!

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