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Past, present, and future of Japanese branchiobdellidans: insights into evolutionary history and conservation

（日本産ヒルミミズ類の過去・現在・未来：
進化史と保全への洞察）

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Summary

Branchiobdellida, also known as crayfish worms, are a group of Oligochaeta primarily found on the body surface of crayfish. Despite their unique and important biological features, branchiobdellidans are often overlooked due to their small size. For instance, discrepancies between molecular phylogeny and morphological classification and the presence of cryptic species are suggested. Branchiobdellidans are thought to have co-evolved with crayfish superfamily Astacoidea for over 200 million years, distributed discretely across the Northern Hemisphere, yet their evolutionary history remains poorly understood. Additionally, many host crayfish species face extinction risks due to climate change and anthropogenic impacts. In general, symbiotic organisms like branchiobdellidans are known to become extinct before their hosts, making them especially vulnerable. However, few studies have investigated branchiobdellidans from a conservation biology perspective, leaving their current status largely unexplored.

This dissertation aimed to update the foundational knowledge of branchiobdellidans, examine their co-evolutionary history with crayfish, and highlight the need for conservation. In Chapter 2, branchiobdellidans were sampled across the entire distribution range of their host, the Japanese crayfish, and classified morphologically to understand their diversity patterns. Branchiobdellidans were found in 69 out of 98

crayfish populations, with up to six species co existing on a single host individual. A positive correlation was observed between host density and branchiobdellidans diversity, suggesting that declining host populations may contribute to the loss of branchiobdellidans diversity. Moreover, comparing distribution and species composition between current status and data from approximately 100 years ago revealed a decline in diversity. Occurrence rates varied across species, with some experiencing over a 50% decline. Additionally, in regions invaded by non-native crayfish, Japanese branchiobdellidans shifting hosts from native crayfish to non-native species was reported.

In Chapter 3, the phylogenetic classification of Japanese branchiobdellidans was refined using genetic analysis alongside morphological data. Japanese branchiobdellidans formed a monophyletic lineage in a relatively basal position within the global phylogenetic tree of branchiobdellidans. For many species, morphological features corresponded with genetic clades; however, in some cases, multiple clades were identified within a single species, indicating the presence of cryptic species. Some Japanese species displayed dorsoventral heteromorphy of jaws, a unique characteristic in Branchobdellida. Phylogenetic analyses suggested that speciation among Japanese branchiobdellidans preceded the immigration of crayfish in the Japanese archipelago. Furthermore, phylogenetic relationships across the Northern Hemisphere revealed a rough correspondence between branchiobdellidans and their crayfish hosts, supporting a co-evolutionary relationship.

In Chapter 4, the co-evolutionary history between Japanese branchiobdellidans and Japanese crayfish was investigated through a comparative analysis of their intra-specific phylogenies. Phylogenetic analyses of samples from over 10 sites revealed that, in some cases, the divergence dates and intra-specific phylogenetic structures of branchiobdellidans matched those of their hosts, while in some cases, they did not match. Co-phylogeny analysis provided statistical evidence for a co-evolutionary relationship between the *Cirrodrilus cirratus* complex and Japanese crayfish, with a similar trend observed for *C. homodontus*. These findings suggest that the evolutionary histories of Japanese branchiobdellidans vary by species. Some species have preserved co-evolutionary histories spanning several million years, and the continuation of such relationships may drive macroevolution in the future.

A series of studies has shown that the phylogenetic relationships between branchiobdellidans and crayfish in the Northern Hemisphere are broadly consistent, and co-evolutionary histories spanning several million years at the intraspecific lineage level in Japanese species. These findings indicate that branchiobdellidans and crayfish share a co-evolutionary history extending over millions of years and that co-evolution can occur within a host species at the intra-specific lineage. Notably, up to six branchiobdellidan species have been found on a single host individual, and genetically and morphologically closed species have coexisted for an extended period. This is significant from a community ecology perspective. However, it is also evident that the diversity of Japanese branchiobdellidans is now rapidly declining. The results of studies strongly suggest that the diversity of branchiobdellidans, shaped by long-term co-evolution, is disappearing at an alarming rate. In the future, extinction risks, by each branchiobdellid species, should be reassessed, and conservation efforts for host populations must account for the diversity of their branchiobdellidan symbionts.

CHAPTER 1

General Introduction

Branchiobdellidans, also known as crayfish worm, are a group of Oligochaeta in the phylum Annelids that mainly inhabit the body surface of crayfish. Hermaphroditic, with a body length of 1 to 12 mm and 15-17 body segments, are harbored to hundreds of branchiobdellidans, which are common for crayfish individuals. Branchiobdellidans crawl on the host body surface like leeches by using their mouthparts and posteriorly located suckers (Holt, 1965; Kasprzak, 1984; Nesemann, 1994). During feeding, they use the jaws, a pair of hard tissues located in their mouthparts, to scrape or shred diatoms, rotifers and small free-living animals from the body surface of the host and bite off them, and occasionally prey on smaller individuals of the same species (Yamaguchi, 1935; Meike, 1999; Gale and Proctor, 2011). It has also been reported that different species have different feeding habits (Gale and Proctor, 2011). Reproduction is entirely host-dependent; the cocoons, encased in a yellowish-white protective membrane, are attached to the host’s body surface (Creed et al., 2015). Although branchiobdellidans cannot complete their life cycle without a host, they have been recorded to survive for up to eight months in host-free laboratory conditions (Young, 1966). The symbiotic relationship between branchiobdellidans and crayfish has traditionally been viewed as either commensal or parasitic; however, some studies suggest it may also be mutualistic. Jennings and Gelder (1979) described most branchiobdellidan species as commensal symbionts feeding on algae and small organisms on the crayfish's body surface. In contrast, radioisotope analysis has demonstrated that *Branchiobdella hexadonta*, which inhabits the European crayfish *Astacus astacus*, is parasitic, feeding on host tissue (Grabda and Wierzbicka, 1969). Brown et al. (2002) reported mutualism, noting that increased branchiobdellidan densities enhanced crayfish growth and reduced mortality. This was attributed to the cleaning of debris from crayfish gill chambers, improving respiratory efficiency. Brown et al. (2012) reported that the relationship is density-dependent, shifting from commensalism at low densities to mutualism at medium densities, and parasitism at high densities. The host crayfish is a keystone species and significantly influences the abundance of other aquatic species through predation and environmental modification. Therefore, it has been suggested that differences in the presence or absence and number of branchiobdellidans in crayfish populations can affect the local ecosystem through the crayfish (Creed et al., 2021).

The taxonomy of the branchiobdellidans is relatively well-established at the higher taxonomic category, but considerable gaps remain at the subfamily and genus levels. The body structure of branchiobdellidans closely resembles that of Hirudinea and Oligochaeta, and from an anatomical perspective, they were included in Oligochaeta. Subsequently, Holt (1965) established the order Branchiobdellida as an independent order within the Clitellata. This description has been supported by later researchers (e.g., Kasprzak, 1984; Gelder, 1996). Recent molecular phylogenetic studies have also shown that the Branchiobdellida are monophyletic and the most closely related independent group to Hirudinida and Acanthobdellida (Erséus, 2005; Tessler et al., 2018). In Branchiobdellida, approximately 150 species across 21 genera, grouped into one family and four subfamilies, have been described globally (Gelder and Williams, 2015). However, as all species are exosymbiotic, their body structures are very simple with few differences, making identification difficult, and there is a possibility for major revisions regarding phylogeny and taxonomy in the future. The traits generally regarded as taxonomically important are a pair of jaws and female and male reproductive organs, although these are frequently absent from original descriptions. Classification based on morphology has not been supported by molecular phylogeny (Gelder and Siddall, 2001; Williams et al., 2013). Williams et al. (2013) conducted a comprehensive genetic analysis of North American species, including all subfamilies. There are discrepancies between the results of molecular phylogenetic analysis and morphological subfamily classification, as well as morphologically homologous but genetically separate species. Molecular phylogenetic analyses of the European *Branchiobdella* species have also identified many genetically independent lineages within each species, suggesting the existence of many cryptic species that are not morphologically distinct (Füreder et al., 2009; Šarić et al., 2018).

The main hosts of branchiobdellidans are the crayfish of the superfamily Astacoidea, which are distributed in the Northern Hemisphere, and branchiobdellidans also distributed in North America, Euro-Mediterranean, and eastern Asia (Gelder and Williams, 2015). The phylogenetic relationships and history of the host crayfish species have been relatively well studied, diverging from a common ancestor with the superfamily Nephropoidea about 200 million years ago, which is thought to be the only time in evolution when the ancestor of crayfish made a move to freshwater (Crandal, 2000; Wolfe et al., 2019). Subsequent continental drift first led to the divergence of the superfamily Astacoidea, distributed in northern hemisphere, and the superfamily Parastacoidea, distributed in southern hemisphere. Subsequently, superfamily Astacoidea diverged into families Astacoidae, Cambaroidae and Cambaridae with the continental differentiation of Laurasia. From molecular phylogeny, the family Cambaroidae, has been suggested to be the most basal lineage of crayfish in the northern hemisphere (Crandall and De Grave, 2017; Ahn et al. 2006). The fact that branchiobdellidans are monophyletic and symbiotic only with Astacoidea crayfish species suggests that the evolution of symbiosis in crayfish species occurred after the divergence of the Astacoidea and

Parastacoidea, which is more than 100 million years ago. However, the co-evolutionary history of branchiobdellidans and crayfish has only been discussed in Šarić et al. (2018) for *Austropotamobius* crayfish and *Branchiobdella* species, and no knowledge is available on crayfish and branchiobdellidans share evolutionary histories or not. One reason for the lack of studies on the co-evolutionary history of crayfish and branchiobdellidans is the historically widespread anthropogenic movement of crayfish into and out of their original range, which has greatly complicated their distribution (Albrecht, 1983; Barbaresi et al., 2007). In Europe and North America, crayfish species are transported outside their native range as a food resource and fishing bait, and symbiotic branchiobdellidans are also secondarily translocated (e.g. Vedia et al., 2015; Gelder, 2020; Parpet and Gelder, 2020). Movements of branchiobdellidans between native and alien hosts have also been observed (Gelder, Delmastro & Rayburn, 1999; Vogt, 1999; Ďuriš et al., 2006; Bláha et al., 2017; Williams & Weaver, 2021), making it difficult to determine the original distribution and host-symbiont relationships. On the other hand, the flatworm Temnosephala is known to occupy the same niche, although the southern hemisphere is not inhabited by branchiobdellidans (Gelder, 1999; Sibraa et al., 2021). A comparison of the phylogenetic relationships between the Temnocephala and host crayfish has shown that a long co-evolutionary history of about 70 million years is preserved between them (Cuthill et al., 2016). Branchiobdellidans are also highly dependent on their hosts for most of their life history, including reproduction, food, and migration, so the evolution of their distribution range and speciation processes are likely to strongly reflect those of their hosts.

Thought to have long evolutionary histories, branchiobdellidans, like other organisms, are considered to be threatened with extinction due to anthropogenic factors in recent years. The host crayfish is declining populations worldwide due to direct competition with alien species, introduction of pathogens and habitat destruction (e.g. Schulz and Schulz, 2004; Edwards, Jackson & Somers, 2009; Manenti et al., 2019). It has also been suggested that about half of the described crayfish species are threatened with extinction (Holdich, 2002). Because branchiobdellidans depend on crayfish for their life history, they will become extinct at the same time if their host populations become extinct, and they are therefore endangered as well. Furthermore, symbionts are known to go extinct before the extinction of their hosts, suggesting that their potential extinction risk is higher than that of their hosts (Lafferty, 2012; Rózsa and Vas, 2015). Actually, all Japanese branchiobdellid species are listed as endangered species by the Japanese Ministry of the Environment (Ministry of the Environment, 2020), taking into account the recent decline in the Japanese crayfish population. However, there have been few studies from a conservation biology perspective on Japanese branchiobdellid species, which are usually overlooked and even associated with the negative impression of being "parasitic".

The Japanese branchiobdellidans are very important in studying the co-evolution and conservation of branchiobdellidans. Firstly, the host Japanese crayfish, *Cambaroides japonicus*, belongs to the most basal group of crayfish phylogeny in the northern hemisphere, so the symbiont branchiobdellid species may also be in a basal position. By examining the *Cambaroides* crayfish and their symbionts phylogeny, it may be possible to reveal how branchiobdellidans evolved their symbiotic relationship with crayfish. Interestingly, a few species of Japanese branchiobdellidans have different dorsal and ventral jaws (dorsoventral heteromorphy), which are important foraging organs and species-identifying traits. This is a unique character found only in these species of branchiobdellidans in the world (Timm, 1991). 12 valid species of Japanese branchiobdellidans have been found (Yamaguchi, 1935; Ohtaka and Gelder, 2023), all of which are endemic to Japan and only associate with one species, from the Japanese crayfish. Approximately 430 species of crayfish from North America have been described, whereas only 107 branchiobdellid species have been described. This means that in Japan, 12 species of branchiobdellidans coexist with one species of crayfish, and the diversity is considered to be extremely high in this region of the world. In North America, a maximum of three to four species of branchiobdellidans have been found in symbiosis with one host crayfish, while in Japan, symbiosis of three to four species is not uncommon and up to seven species have been found in a host population (Gelder and Ohtaka, 2002). It is interesting from a community ecology point of view to see how multiple species of Japanese branchiobdellidans coexist in the limited niche of the crayfish body surface. Japanese crayfish are vulnerable to environmental change and are rapidly disappearing in many areas (Kawai et al., 2002; Nakata et al., 2004). Therefore, it is highly likely that the symbiont is also threatened with extinction. Until today, studies on the Japanese branchiobdellidans have been mainly taxonomy based on morphology. First, *C. sapporensis* and *C. cirratus* were described by Dr. Pierantoni of Italy, together with the genus *Cirrodrilus*, followed by *C. japonicus* (= *Stephanodrilus japonicus*) and *C. digitatus* (= *Brachiobdella digitatus*) are described (Pierantoni, 1905, 1906, 1912). Dr. Hideji Yamaguchi conducted extensive research on Japanese species in the 1930s (e.g., Yamaguchi, 1932, 1934, 1935). By two authors, eleven species were described under a single genus. Since then, only two species, *C. tsugarensis* and *C. iwakiensis*, have been described (Gelder and Ohtaka, 2000; Ohtaka and Gelder, 2015). However, some key characters for identification overlap among some species and may be synonyms. For example, *C. cirratus* in Pierantoni (1905) and *C. uchidai* in Yamaguchi (1932) are described as separate species, but the overlap in the number of dorsal appendages and their jaws are morphologically the same. The taxonomy needs to be reviewed to determine whether they are separate species or not. Some species have been described in the past but are not found recently, and it is possible that these were errors in the original descriptions. Alternatively, some species that have not been identified in recent years may have already become extinct due to the decline of the Japanese crayfish population.

Based on the above, this dissertation aims to reveal the phylogeny, distribution, diversity and evolutionary history of the Japanese branchiobdellidans. In Chapter 2, Japanese branchiobdellidans were sampled throughout the distribution range of their host and identified according to morphology to reveal their current distribution and diversity patterns. Furthermore, comparing the distribution of the Japanese branchiobdellidans with that studied approximately 100 years ago revealed that their diversity has been declining. In addition, it was reported that a Japanese branchiobdellid species has changed its host from a native species to a non-native species in areas where the native and non-native crayfish distribute parapatrically. In Chapter 3, the phylogenetic relationships of Japanese branchiobdellidans are reconstructed using molecular analyses. Here, synonyms and cryptic species were identified, and a more robust phylogeny was established.

Furthermore, the acquisition pattern of the jaws, which is unique to Japanese species, and the phylogenetic position of the Japanese species among the world branchiobdellidans are established. In Chapter 4, the co-evolutionary history of Japanese branchiobdellid species and Japanese crayfish was studied by co-evolutionary analyses and comparing the intraspecific phylogenetic relationships based on these results, the history, present status, and future of Japanese branchiobdellidans are discussed.

CHAPTER 2-1

Current distribution and loss of diversity in Japanese branchiobdellidans

Introduction

Twelve valid species of branchiobdellidans have been described from Japan, all of which are associated only with Japanese crayfish *Cambaroides japonicus*. Research on Japanese branchiobdellidans has so far been mainly taxonomic. First, *Cirrodrilus sapporensis* and *C. cirratus* were described by Dr. Pierantoni of Italy, together with the genus *Cirrodrilus*, followed by *C. japonicus* (as *Stephanodrilus japonicus*) and *C. digitatus* (as *Brachiobdella digitatus*) were described (Pierantoni, 1905, 1906, 1912). Subsequently, in the early 1930s, Dr. Hideji Yamaguchi conducted detailed observations and descriptions of branchiobdellid species from Japan, including five species from Hokkaido (*C. uchidai*, *C. homodontus*, *C. nipponicus*, *C. makinoi*, and *C. megalodentatus*), one species from Aomori Prefecture (*C. aomorensis*) (Yamaguchi, 1932, 1934). There were no taxonomic studies on Branchiobdellidans from Japan for more than a half century after that, but *C. tsugarensis* (as *C. makinoi*, Chapter 3) was discovered and described in Gelder and Ohtaka (2000) and *C. iwakiensis* in Ohtaka and Gelder (2015)was described from Aomori Prefecture, Japan.

There have been few studies on the distribution of Japanese branchiobdellidans, it has been thought that, like many other taxa, they differ in species composition between Honshu and Hokkaido, i.e., across the Blakiston Line. Gelder and Ohtaka (2002) examined the distribution and species composition of Japanese Branchiobdellidans from the 19 sites in Japan where they were collected, along with a notion of the whole mounted specimens made by Dr. Hideji Yamaguchi. According to this report, *C. tsugarensis*, and *C. aomorensis* are distributed only in Aomori Prefecture, and the other ten species are distributed only in Hokkaido. *C. iwakiensis*, which was later described as a species in Aomori Prefecture, is also considered to be distributed only in Aomori Prefecture (Ohtaka and Gelder, 2015). The species composition at the collection sites was highly variable, with a range of one to a maximum of seven species per site. Branchiobdellidans in North America have at most three to four species per host population, which is a remarkable diversity in comparison. In addition, all species from Hokkaido were found throughout their distribution range, and the species composition did not differ from region to region. Thus, Japanese branchiobdellidans are known to differ in species composition between Honshu and Hokkaido and the number of species and species composition by host population.

In Japanese crayfish, the molecular phylogenetic study has shown that there are significant genetic differences between the western and eastern groups geographically separated by the Hidaka Mountains and that the specimen from Honshu is genetically included in the western group and share some haplotypes with southern Hokkaido

(Koizumi et al., 2012). In other words, while the Japanese crayfish inhabit Honshu is close to the Hokkaido lineage, the species composition of the branchiobdellidans is completely different between Hokkaido and Honshu, resulting in discrepancies in host phylogeny and symbionts distribution. However, the distribution of branchiobdellidans cannot be said to be well established in the southern Hokkaido and Erimo regions, as there are no confirmed records of their occurrence in these areas, so a more detailed distribution survey covering their entire range is needed.

Crayfish, the main hosts of branchiobdellidans, have been decreasing worldwide due to habitat destruction caused by recent anthropogenic activities, species movement and associated pathogen risks (Schulz and Schulz, 2004; Edwards et al., 2009; Manenti et al., 2019). The Japanese crayfish is no exception, with a declining population due to direct competition with signal crayfish and the spread of crayfish plague (*Aphanomicis astaci*) from the alien crayfish (Kawai et al., 2002; Nakata et al., 2004; Martín-Torrijos et al., 2018). In response to the decline in host populations, the Japanese Ministry of the Environment listed all Japanese branchiobdellidans as endangered species in 2012 (Ministry of the Environment, 2020). As symbionts are generally known to become extinct faster than their hosts (Lafferty, 2012; Rózsa and Vas, 2015), the potential extinction risk of branchiobdellidans is considered to be higher than that of their host crayfish species. Nevertheless, few studies from a conservation perspective have been conducted on branchiobdellidans, and knowledge of their extinction risk remains scarce.

In this chapter, I conducted comprehensive sampling throughout the distribution range to clarify the current distribution of Japanese branchiobdellidans and to assess whether the patterns of species composition across regions align with previous knowledge. Additionally, I compared the diversity of branchiobdellidans among sites, focusing on host densities at each population, to identify potential factors contributing to differences in diversity. Furthermore, I examined whether the current diversity of branchiobdellidans has changed over the past century by comparing it with the distribution and species composition reported approximately 100 years ago by Gelder and Ohtaka (2002).

Material and Methods

Field sampling and survey

Japanese crayfish and branchiobdellidans were surveyed at 98 sites in Hokkaido, Japan, between September 2020 and October 2024 (Fig. 1). Crayfish were captured by turning over stones and logs by hand. Catch per unit effort (CPUE) was used as an indicator of crayfish density, calculated as the number of captured crayfish / (number of people*survey time (min)). Branchiobdellidans were collected by shaking the crayfish in a container with 50 ml of 70% ethanol for 30 seconds, followed by shaking in a container with 50 ml of river water for 30 seconds, and then passing the contents through a 63 μm mesh. This method is non-lethal to Japanese crayfish and experiments have confirmed that more than 90% of branchiobdellidans can be separated (Konno, unpublished data). Japanese crayfish were released back to their original habitat after recording cephalothorax length, sex, loss of pedipalps, and injuries.

Species identification

Branchiobdellidans were dehydrated in 100% ethanol, clarified with methyl salicylate, and the anterior part of the organism was detached and fixed with Canada balsam. The posterior part of the organism was preserved in 100% ethanol as a sample for genetic analysis in Chapters 3 and 4. Species identification was based on Yamaguchi (1934), Gelder and Ohtaka (2000), and Ohtaka and Gelder (2015); *C. cirratus* and *C. uchidai* are described as separate species, but there is no difference in taxonomic characteristics, such as overlap in taxonomic characters and no morphological difference in the jaws, an important identification keys. So, the species is treated as *C. cirratus* complex in this study.

At 20 sites, after species identification, numbers of branchiobdellidans per host individual were recorded in 5-10 crayfish.

Statistical analysis

To evaluate changes in species diversity, both for individual species and overall, past and present occurrence rates and species numbers were compared using the Wilcoxon rank-sum test. Statistical methods were also used to investigate whether there is a relationship between branchiobdellidans diversity and population density in the Japanese crayfish population. A generalized linear model (GLM) was used with branchiobdellidans diversity as the dependent variable and the possible factors of Japanese crayfish density, water temperature, and season as independent variables. The number of species (N_0), the Hill number of order 1 (N_1), and the Hill number of order 2 (N_2) were used as diversity indices (Hill, 1973). As the presence or absence of branchiobdellidans may be related to unknown factors such as water quality in addition to the aforementioned factors, the Wilcoxon rank sum test was used to test for differences in median values between the presence/absence groups. I also wrote a Rarefaction Curve based on the number of branchiobdellidans for the number of species, to ascertain how many branchiobdellidans per crayfish population would be considered sufficient for sampling. Sites considered to be insufficiently sampled were excluded from the GLM analysis. All statistical analyses were performed using R version 4.3.1 (R Core Team, 2023), and the significance level was set at 0.05. Using the "lme4" package (Bates et al., 2015) for the linear model and the "iNEXT" package (Hsieh et al., 2016) for the calculation of the Hill number and Rarefaction curve.

Results

Distribution and species composition of branchiobdellidans

Branchiobdellidans were found in 69 of the 98 Japanese crayfish populations in Hokkaido. Nine species, *C. cirratus* complex, *C. digitatus*, *C. japonicus*, *C. sapporensis*, *C. homodontus*, *C. nipponicus*, *C. makinoi* (as *C. tsugarensis*, Chapter 3), *C. meglodentatus* and *C. iwakiensis* were found in this survey (Table 1). Of these, *C. iwakiensis* had been found only in Honshu, but in this survey, it was also found in the Southern Hokkaido and the Erimo region, indicating that this species is distributed both in Hokkaido and Honshu. *C. makinoi* (as *C. tsugarensis*, Chapter 3) was also found in the Northern Hokkaido region and the Erimo region. The occurrence rates of species varied greatly between species, with the most frequently occurring *C. cirratus* complex and *C. digitatus* accounting for 72.5% (occurring at 50 of the 69 sites where branchiobdellidans were found), and the least frequently occurring *C. megalodentatus*, *C. makinoi*, and *C. iwakiensis* accounting for 2.9% (occurring at 2 of the 69 sites). In terms of species composition, a maximum of six species co-occurred, with an average species count of 2.4 across all sites where branchiobdellidans were observed.

An examination of past and present changes in species diversity, both overall and by region, revealed a significant decline in species diversity overall ($P < 0.001$), as well as in the Central and Eastern Hokkaido (Central Hokkaido, $P < 0.01$; Eastern Hokkaido, $P < 0.05$) (Fig. 2). In contrast, no decline in diversity was observed in the Northern Hokkaido ($P = 0.27$).

Comparisons for each species revealed a decrease in occurrence across all species except *C. digitatus* (Fig. 3). Statistically significant declines were observed in *C. makinoi*, *C. japonicus*, *C. megalodentatus*, *C. nipponicus*, and *C. sapporensis*, with *C. sapporensis* exhibiting a 51.6% reduction in occurrence.

Relationship between branchiobdellidans diversity and Japanese crayfish populations

An abundance-based Rarefaction curve showed that sites where more than about 100 branchiobdellidans were collected were suitable for use in the diversity analysis (Appendix Fig. 1), so we examined the relationship between branchiobdellidans diversity and Japanese crayfish population density and environmental factors at 32 sites that met the conditions. The relationship between the diversity of the branchiobdellidans and the Japanese crayfish population and environmental factors was investigated at 32 sites that fulfilled the conditions. In the analysis using the GLM, the best model was selected by model selection using AIC and BIC, where the dependent variable was species diversity, the explanatory variable was CPUE (crayfish density), the dependent variable was gamma distribution and the link function assumed a log-link function. A strong relationship between the number of branchiobdellidans species and crayfish CPUE was found, indicating that the number of branchiobdellidans species increases with increasing crayfish density ($y = 1.54e^{1.09x}$, Cox & Snell Pseudo $R^2 = 0.428$, $P < 0.001$, Fig. 4A).

At 15 of the 20 sites where branchiobdellidans populations per individual of Japanese crayfish were examined, which fulfilled the mentioned conditions, I analysed the relationship between branchiobdellidans diversity, Hill number of order 1 (N_1) and Hill number of order 2 (N_2), and crayfish CPUE and environmental factors using a GLM. The results showed that the model with the respective diversity index as the dependent variable and CPUE as the explanatory variable for both was selected as the best model. The model with the Hill number of order 1 (N_1) as the objective variable showed a weak correlation with crayfish CPUE ($y = 1.34 + 1.4x$, $R^2 = 0.266$, $P < 0.05$, Fig. 4B). There was a weak but not significant correlation between the Hill number of order 2 (N_2) and

CPUE ($y = 1.28 + 1.18x$, $R^2 = 0.214$, $P = 0.083$, Fig. 4C).

A Wilcoxon rank-sum test between the two groups for the presence or absence of branchiobdellidans and crayfish CPUE showed no significant difference, indicating that crayfish population density was not involved in the presence or absence of branchiobdellidans ($P = 0.31$, Fig. 4D).

Discussion

In this study, sampling was conducted across the entire range of Japanese branchiobdellidans, covering 69 sites in Hokkaido. Nine species of branchiobdellidans were identified throughout the Hokkaido, with two species occurring in both Hokkaido and Honshu. A clear relationship was observed between crayfish density (CPUE) and the number of branchiobdellidan species, suggesting that branchiobdellidan diversity increases with higher crayfish densities in the population. Based on these findings, the current distribution of Japanese branchiobdellidans, factors influencing their diversity, and their conservation status were discussed, incorporating comparisons between habitat data from approximately 100 years ago and the present state of these species.

Relationship between branchiobdellidan diversity and crayfish populations

This study highlights a relationship between branchiobdellidans diversity and Japanese crayfish populations (Fig. 4). Specifically, a strong correlation was found between the number of branchiobdellidans species and crayfish population density (CPUE), with branchiobdellid species diversity increasing alongside higher crayfish densities (Fig. 4A). It is known that symbionts tend to go extinct faster than their hosts (Lafferty, 2012; Rózsa and Vas, 2015), and that endangered hosts often harbored lower symbiont diversity (Altizer et al., 2007). This suggests that the reduction in available resources and breeding opportunities caused by declining host populations may play a significant role in the loss of symbiont diversity. The findings of this study indicate that maintaining the population density and overall health of host populations is likely to enhance the availability of resources and breeding opportunities for symbionts, thereby preserving their species diversity.

Distribution patterns of Japanese branchiobdellidans

This study identified nine species of branchiobdellidans in Hokkaido, with *C. iwakiensis* and *C. makinoi* found in both Honshu and Hokkaido. These species were recorded at multiple sites in geographically distant areas, suggesting their widespread distribution across Hokkaido. In contrast, the other seven species were exclusively distributed in

Hokkaido. It remains an intriguing question why only these two species occur in both regions. It is hypothesised that the host Japanese crayfish dispersed within Hokkaido before migrating to Honshu via the Tsugaru Strait land bridge during the Middle Pleistocene (0.9-1.3 million years ago) and glacial the Riss glaciation (0.14-0.18 million years ago) (Yokoyama and Goto, 2002; Koizumi et al., 2012; Ooyagi et al., 2018). Accordingly, it is reasonable to assume that branchiobdellidans followed a similar dispersal pattern, dispersing within Hokkaido before crossing to Honshu. The bottleneck effect during this migration may have allowed only these two species to survive. Alternatively, it is possible that other species also migrated to Honshu but experienced regional extinctions due to factors such as reduced host population densities, as suggested in this study. Climatic factors may also be affected, as Honshu, located south of Hokkaido, experiences higher average temperatures, potentially limiting the distribution of some species.

Comparison of past and present distributions

Gelder and Ohtaka (2002) compiled preliminary specimens prepared by Dr. Hideji Yamaguchi and reconstructed the distribution of Japanese branchiobdellidans collected during the 1920s and 1930s. Although the condition of Japanese crayfish populations at that time, as well as the extent of branchiobdellidans sampling, is unknown due to the lack of preserved information, a comparison with the present distribution is possible since at least some specimens were collected. However, the historical distribution data may overestimate actual conditions, as there is no information on the absence of branchiobdellidans in crayfish populations. Therefore, comparisons in this study were limited to sites where branchiobdellidans were recorded.

From Gelder and Ohtaka (2002), specimens from 19 sites in Hokkaido, with an average species count of 4.1. In the present study, branchiobdellidans were found at 69 sites in Hokkaido, with an average species count of 2.4. The current mean species count is 1.7 lower than in the past. A comparison of occurrence rates by species revealed declines in all species except *C. digitatus* and *C. iwakiensis*, which were newly discovered in Hokkaido during this study. Notably, *C. japonicus* and *C. megalodentatus* exhibited declines of more than 30%, while *C. sapporensis* showed a reduction of over 50% (Fig. 4).

The decline in crayfish population density observed in this study strongly correlates with a decrease in branchiobdellidan species diversity. This finding underscores the importance of conserving crayfish populations to maintain branchiobdellidan diversity. Given that all Japanese branchiobdellidans are currently listed as Endangered species by the Japanese Ministry of the Environment (Ministry of the Environment, 2020), the results of this study highlight the urgent need for conservation action. Among the 69 surveyed sites, some species were found at only two locations. Additionally, significant declines in species diversity were observed in eastern and central Hokkaido, emphasising the need to prioritise these regions for conservation efforts. Further investigations are necessary to assess the impacts of environmental and land-use differences on branchiobdellidan populations in these areas. The conservation status of certain species should be re-evaluated considering the findings from this study.

Tables

Table 1. Study site and species composition of branchiobdellidans

Site	Date	Crayfish CPUE	Species number	Species composition
Abashiri	20200926	0.952	0	
Akaiwa	20230608	0.117	0	
Apoi	20201002	0.179	0	*C. cirratus, C. digitatus, C. homodontus*
Atsubetsu 1	20210824	0.017	0	*C. digitatus*
Atsubetsu 2	20210824	0.067	0	*C. digitatus*
Atsubetsu 3	20210907	0.033	0	*C. digitatus*
Ebetsu	20220926	0.111	1	*C. digitatus*
Enbetsu	20201015	0.089	0	*C. cirratus, C. digitatus*
Erimo 1	20220727	0.200	0	*C. cirratus, C. digitatus*
Erimo 2	20220727	0.267	0	*C. cirratus, C. digitatus*
Erimo 3	20220727	0.133	0	*C. cirratus, C. digitatus*
Erimo 4	20220727	0.267	0	*C. cirratus, C. digitatus*
Erimo 5	20240829	0.244	2	*C. cirratus, C. digitatus*
Erimo 6	20240829	0.044	0	
Erimo 7	20240829	0.767	6	*C. cirratus, C. digitatus, C. japonicus, C. homodontus, C. nipponicus, C. makinoi*
Erimo 8	20240829	0.356	4	*C. cirratus, C. digitatus, C. homodontus, C. nipponicus*
Erimo cape	20220728	0.400	0	
Esashi 1	20201018	0.643	0	*C. cirratus, C. digitatus, C. japonicus, C. homodontus, C. makinoi*
Esashi 2	20201018	0.773	0	*C. cirratus, C. digitatus, C. japonicus, C. homodontus, C. sapporensis*
Garo	20231002	0.800	2	*C. cirratus, C. digitatus*
Naganuma	20231115	0.033	2	*C. cirratus, C. digitatus*
Hamanaka 1	20200924	0.460	0	*C. japonicus*
Hamanaka 2	20241010	0.467	1	*C. japonicus*
Hariusu 1	20230608	0.275	0	
Hariusu 2	20230929	0.500	0	
Hiraoka ST3-1	20231018	NA	2	*C. cirratus, C. digitatus*

Hiraoka ST3-3	20231018	NA	2	*C. cirratus, C. digitatus*
Hiraoka ST8	20240725	NA	1	*C. cirratus*
Hiraoka ST8	20240725	NA	1	*C. cirratus*
Hiraoka ST9	20240725	NA	1	*C. digitatus*
Hiraoka ST9	20240725	NA	1	*C. digitatus*
Horoman	20210514	0.467	0	
Itanki 1	20221123	0.200	0	*C. cirratus*
Itanki 2	20221123	0.422	0	*C. cirratus, C. japonicus*
Itanki Biotope	20221123	0.489	0	*C. cirratus, C. japonicus, C. homodontus*
Iwanai	20240709	0.167	2	*C. cirratus, C. digitatus*
Jozankei	20220715	0.585	0	*C. cirratus, C. digitatus, C. japonicus, C. homodontus*
Kaminokuni	20231004	0.167	1	*C. cirratus*
Kennichi	20240621	0.889	2	*C. cirratus, C. digitatus*
kimobetsu	20240709	0.400	2	*C. digitatus, C. homodontus*
Kitami 2	20211007	0.050	0	
Kitami 1	20211007	0.410	0	*C. cirratus, C. digitatus, C. sapporensis*
Kiyosato	20200926	0.241	0	*C. cirratus*
Kumanosawa	20221016	0.352	0	*C. cirratus, C. digitatus*
Kurisawa	20241025	0.333	1	*C. digitatus*
Kushiro 1	20241008	0.089	0	
Kushiro 2	20241008	0.444	2	*C. cirratus, C. japonicus*
Kyowa	20240709	0.100	2	*C. digitatus, C. japonicus*
Maruyama	20230714	0.417	0	*C. cirratus, C. digitatus, C. japonicus*
Matsumae	20231004	0.175	2	*C. cirratus, C. iwakiensis*
Minamifurano	20240822	0.308	4	*C. cirratus, C. digitatus, C. japonicus, C. sapporensis*
Motohukusima	20231004	0.267	3	*C. cirratus, C. digitatus, C. iwakiensis*
Muroran Aq.	20241107	0.440	0	
Nakayama	20230728	0.150	0	
Nopporo 38	20230817	0.100	0	*C. digitatus*
Notsuporo	20230927	0.044	0	
Nukabira	20200928	0.130	0	*C. cirratus*
Ohnuma	20220708	0.200	0	*C. digitatus, C. japonicus*
Oketo	20241012	0.089	0	
Onenai	20210521	0.400	0	*C. cirratus, C. digitatus*

Onneto	20211006	0.581	0	
Osatsube	20231002	0.078	2	*C. cirratus, C. digitatus*
Oshamanbe	20220707	0.767	0	*C. cirratus, C. digitatus, C. japonicus, C. sapporensis, C. megalodentatus*
Otaru Aq.	20210904	0.633	4	*C. cirratus, C. digitatus, C. homodontus, C. sapporensis*
Rausu	20220806	0.013	0	
Samani	20230728	0.258	0	*C. digitatus*
Sarufutu	20240905	0.667	0	
Saruru 536	20240828	0.311	1	*C. cirratus*
Saruru 542	20240828	0.022	0	
Setana 1	20220705	0.317	0	*C. cirratus, C. digitatus, C. sapporensis*
Setana 2	20231004	0.857	2	*C. cirratus, C. digitatus, C. makinoi*
Shari	20200925	0.154	0	*C. cirratus*
Shihoro	20211006	0.100	0	
Shikaoi	20211005	0.048	0	
Shimamaki	20220705	0.567	0	*C. cirratus, C. digitatus, C. japonicus*
Shimamaki	20240712	0.356	0	
Shimokawa	20201019	0.862	0	*C. cirratus, C. digitatus, C. japonicus, C. homodontus, C. sapporensis, C. nipponicus*
Shiodomari	20230927	0.300	2	*C. cirratus, C. digitatus*
Soranuma	20220902	0.075	0	
Soya	20201017	0.167	0	*C. cirratus, C. digitatus, C. homodontus*
Soya 1	20240905	1.000	5	*C. cirratus, C. digitatus, C. japonicus, C. homodontus, C. nipponicus*
Soya 3	20240905	0.467	3	*C. cirratus, C. digitatus, C. homodontus*
Sunagawa	20241025	0.033	0	
Suttu	20240711	0.311	0	
Shakotan A	20240718	0.067	0	
Shakotan C	20240718	0.500	0	
Tenjin	20230906	0.378	2	*C. cirratus, C. digitatus*
Teshio	20201016	0.270	0	*C. cirratus, C. digitatus, C. homodontus*
Todohokke	20230929	0.600	3	*C. cirratus, C. digitatus, C. nipponicus*
Touya	20240711	0.267	3	*C. cirratus, C. digitatus, C. nipponicus*
Toyokoro	20220803	0.150	0	*C. digitatus, C. homodontus*
Toyoni	20201001	1.815	0	*C. cirratus, C. digitatus*

Toyoura	20240711	0.089	0	
Usujiri	20231003	1.067	6	*C. cirratus, C. digitatus, C. japonicus, C. homodontus, C. sapporensis, C. megalodentatus*
Wakkanai 1	20201017	1.058	0	*C. cirratus, C. digitatus, C. homodontus*
Wakkanai 2	20240905	0.167	0	
Yamanaka	20230614	0.433	0	

Figures

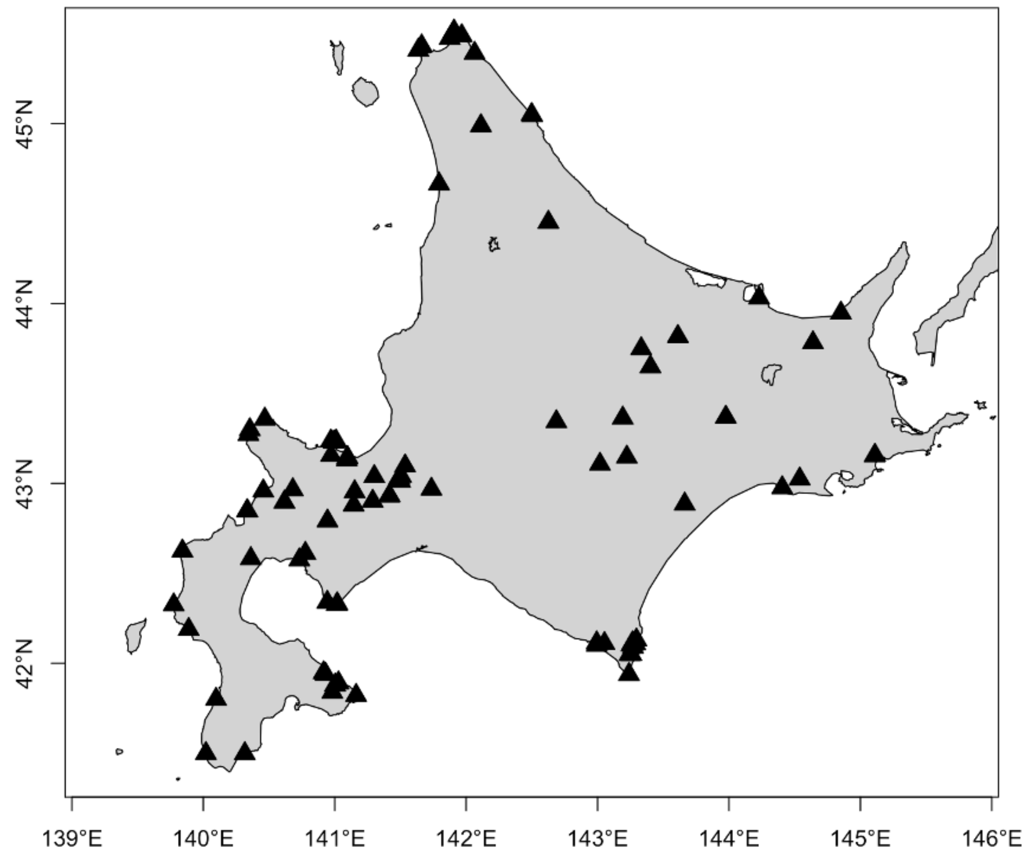


Figure 1. Sampling sites of Japanese crayfish in Hokkaido, Japan, indicated by triangles on the map.

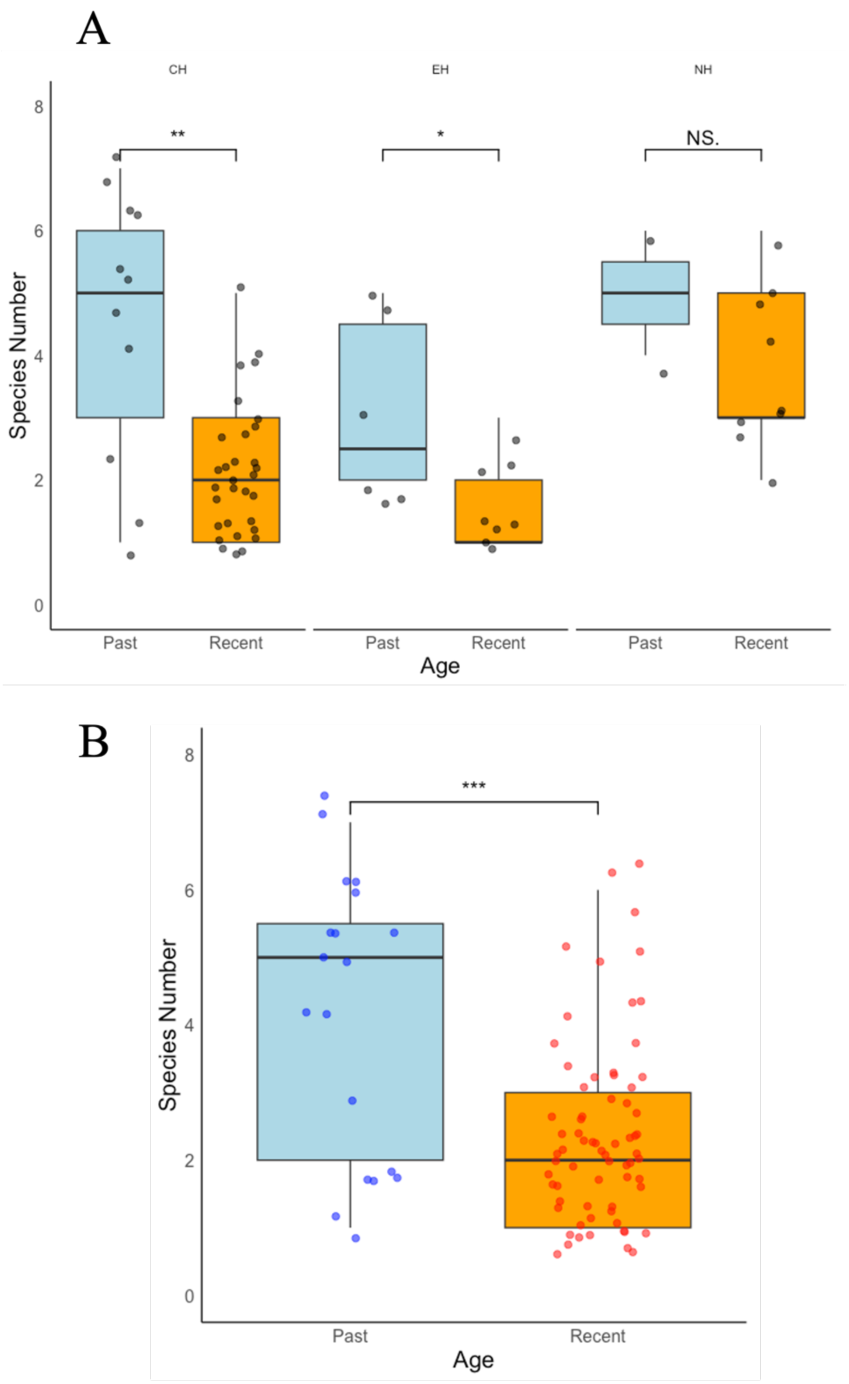


Figure 2. Comparison of historical and current counts of branchiobdellidans species. A: Regional comparison for Central Hokkaido (CH), Eastern Hokkaido (EH), and Northern Hokkaido (NH); B: Overall regions comparison. The plots show species counts per

location, with statistical significance indicated as follows: NS (P > 0.05), * (P < 0.05), ** (P < 0.01), *** (P < 0.001).

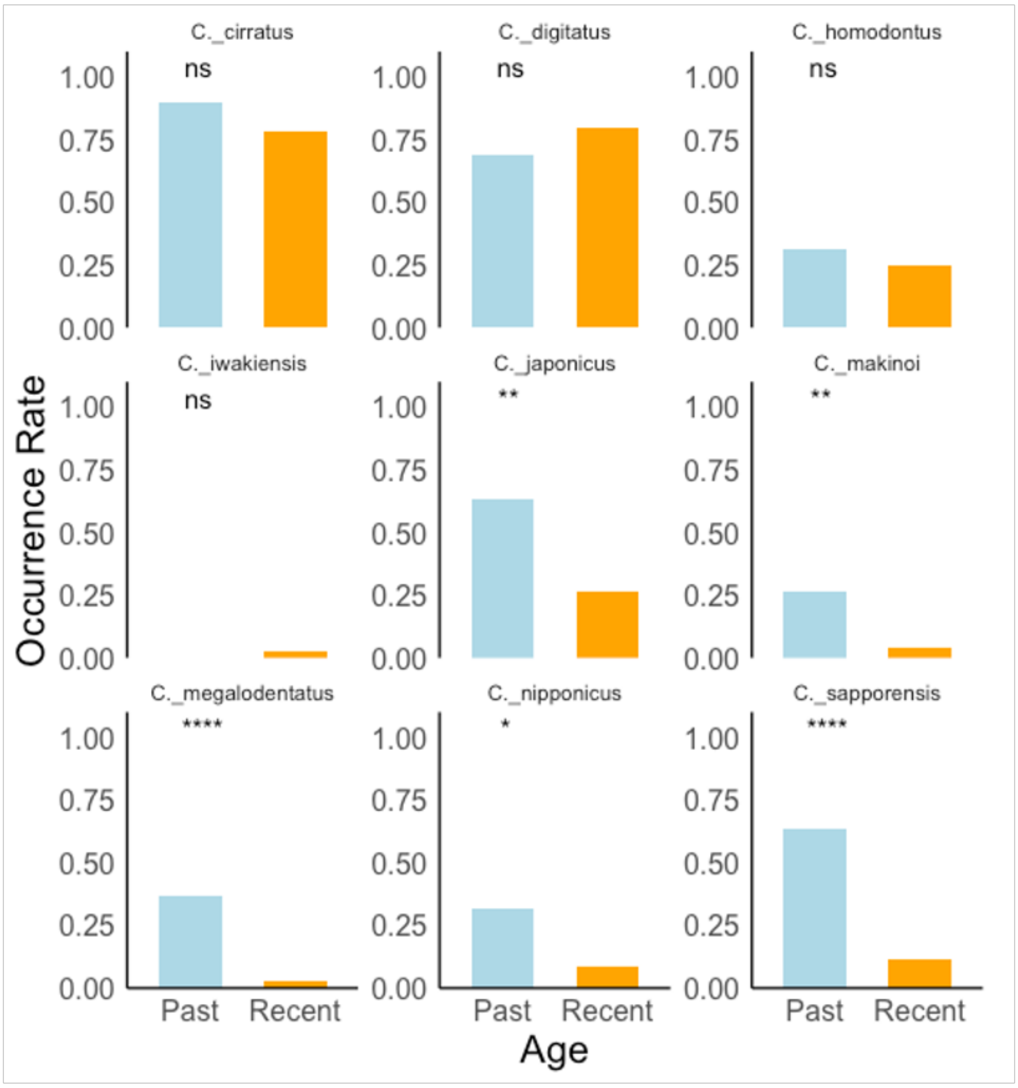


Figure 3. Past and current comparison of occurrence rates of branchiobdellidans species, with statistical significance indicated as follows: ns ($P > 0.05$), * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).

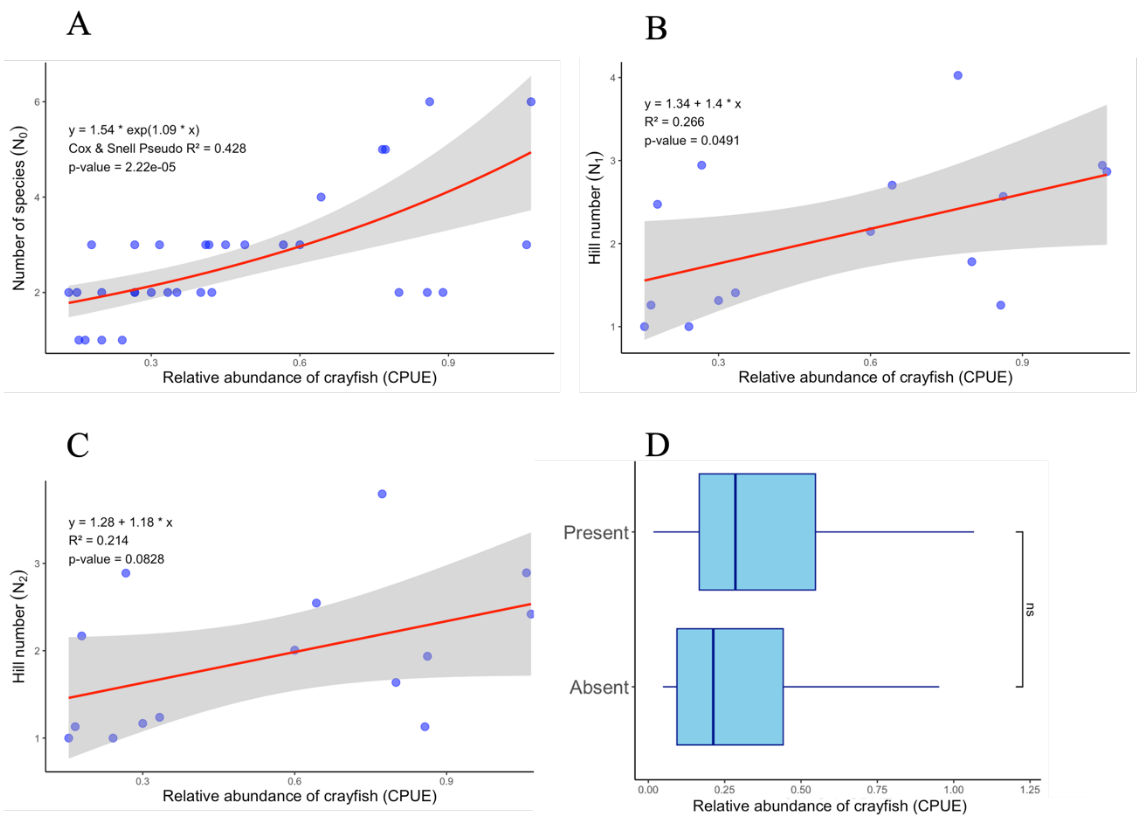
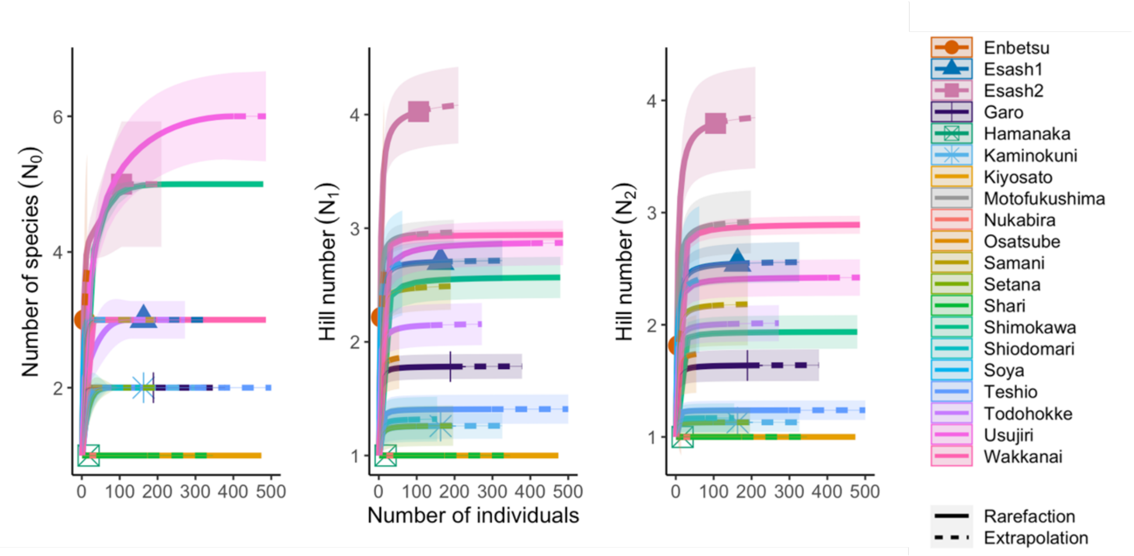


Figure 4. Relationship between branchiobdellidans diversity (Hill number) and relative abundance of crayfish (CPUE). The solid red line represents the fitted regression model, and the gray-shaded area indicates the 95% confidence interval. A: Number of branchiobdellidans species (N_0) vs. crayfish density; B: The Hill number of order 1 (N_1) vs. relative abundance of crayfish (CPUE); C: The Hill number of order 2 (N_2) vs. relative abundance of crayfish (CPUE); D: Presence/absence of branchiobdellidans vs. relative abundance of crayfish (CPUE).

Appendix



Appendix Figure 1. Rarefaction curves for branchiobdellidans diversity and number of observed individuals.

Appendix Table 1. Comparison of species occurrence frequency between past and current periods.

Species	1920s-1930s (19 sites)	%	2020-2023(69 sites)	%	Occurrence Frequency Change (%)
C. cirratus complex	17	89.5	50	72.5	-17.0
C. digitatus	13	68.4	50	72.5	4.1
C. japonicus	12	63.2	18	26.1	-37.1
C. sapporensis	12	63.2	8	11.6	-51.6
C. megarodentatus	7	36.8	2	2.9	-33.9
C. homodontus	6	31.6	17	24.6	-7.0
C. nipponicus	6	31.6	6	8.7	-22.9
C. makinoi	5	31.6	3	4.3	-27.3
C. iwakiensis	0	0	3	4.3	4.3

CHAPTER 2-2

Host shift to non-native species or ecological dead end? Endangered branchiobdellidans found on introduced signal crayfish

Introduction

Recently, occurrences of native symbionts (including parasites) in introduced species (Kelly et al., 2009) or non-native symbionts in native species (Lymbery et al., 2014) have been reported. Because the presence or absence of symbionts often affects host behaviour and survival (Adamo, 2002; Prenter et al., 2004; Poulin, 2010; James et al., 2015), identifying secondary, often overlooked invasions and host shifts is important for the conservation and management of native ecosystems.

Some crayfish species are some of the most successful invaders due to anthropogenic translocations, and their range continues to expand globally (Lodge et al., 2012; Twardochleb, Olden & Larson, 2013). Crayfish in the Northern Hemisphere often carry branchiobdellidans (Annelida: Clitellata: Branchiobdellida) (Gelder and Williams, 2015), and new symbiotic relationships have been created as a result of translocations of the host crayfish and/or secondary invasions of the symbionts (Gelder, 2020). In Europe, several studies have documented the occurrence of native branchiobdellidans on invasive crayfish (Gelder et al., 1999; Vogt, 1999; Ďuriš et al., 2006; Bláha et al., 2017). In North America, three species of native branchiobdelidans were found on non-native crayfish, where the native signal crayfish, *Pasifastacus leniusculus* (Dana 1852), and the non-native snake river pilose crayfish, *Pacifastacus connections* (Faxon, 1914), are sympatric

(Williams and Weaver, 2021). On the other hand, there is only one published report on the transfer of branchiobdellidans from non-native crayfish to native crayfish (Parpet and Gelder, 2020). Host transfer of branchiobdellidans typically occurs during aggressive or mating behaviour among host individuals (Hunt et al., 2018) or by indirect transmission via substrates (Gelder and Williams, 2015).

Pacfastacus leniusculus has been extensively introduced across Japan (Nakata et al., 2010; Usio et al., 2016), and secondary introductions of its branchiobdellidans have also been observed (Kawai et al., 2004; Ohtaka et al., 2005; Nakata et al., 2010). *Pacifastacus leniusculus* is a cold-water species and is widely established in Hokkaido, one of the cooler regions of Japan (Usio et al., 2016). The native and endangered Japanese crayfish, *Cambaroides japonicus* (De Haan, 1841), is present in Hokkaido, but *P. leniusculus* is more competitive and has displaced *C. japonicus* in some sites (Kawai et al., 2002; Nakata et al., 2004). While direct interactions between the native and non-native crayfish have been suggested (Usio et al., 2001; Nakata and Goshima, 2003), no evidence has been reported regarding the transfer of native and non-native crayfish worms to each crayfish species.

In Japan, 12 valid species of branchiobdellidans have been found on *C. japonicus* (Ohtaka et al., 2023). The Red List published by the Japanese Ministry of the Environment lists all these species as being at a higher risk of extinction than their host, *C. japonicus* (Ministry of the Environment, 2020). Biological invasions have a serious impact not only on *C. japonicus* but also on its branchiobdellidans. In this study, I investigated whether a transfer of the symbionts occurred between the two host species at a parapatric area (i.e. not live in the same sites but next to each other) of *P. leniusculus* and *C. japonicus*. And, if it did occur, I investigate where is source population of transmitted branchiobdellidans.

Materials and Methods

Samplings of signal crayfish

Preliminary sampling of *P. leniusculus* is conducted in the Atsubetsu River (mainstem, Site 1) in Sapporo, Japan on August 5, 2021(Fig. 1), using hand net. Two crayfish individuals were fixed together in the same container with 80% ethanol. Later *Pacifastacus leniusculs* were then collected again at the same site on August 30, 2022 (Fig. 1, Table 1), using a cage trap, hand net and backpack electrofisher (model 20B; Smith-Root Inc., Vancouver, WA, USA). Sardine heads and entrails were used as bait in the cage trap (50 cm [L] × 25 cm [W] × 25 cm [H], 2 mm mesh), and 100 V was used for electric fishing. For the crayfish captured on August 30, 2022, the post-orbital carapace length, wet weight and sex of 14 captured *P. leniusculus* were recorded (Table 2), and each individual was fixed in 80% ethanol. Later, the fixative ethanol from each sample container was filtered through a 63 μm mesh, and the number of branchiobdellidans was counted. The species of all obtained branchiobdellidans during this survey were identified according to Yamaguchi (1934) and Holt (1981). Although *Cirrodrilus cirratus* Pierantoni, 1905 and *Cirrodrilus uchidai* (Yamaguchi, 1932) were described as separate species (Pierantoni, 1905; Yamaguchi, 1932; Gelder, 1987), recent studies have argued that the overlap in taxonomic traits between the two species makes it doubtful whether they are valid species or species complex (Ohtaka et al., 2023). Therefore, in this study, I did not distinguish between the two species and considered *C. cirratus* complex.

Field survey of Japanese crayfish populations

From August 2021 to July 2022, I surveyed the population of *C. japonicus* at four sites (tributaries, Site 2-5) near the site where *P. leniusculus* was found (Fig.1; Table 1). Hand collecting was conducted for 30-100 minutes per site, with 2-3 people searching under stones and trees. Captured *C. japonicus* were temporarily submerged in 70% ethanol for a short time (30 seconds) to remove branchiobdellidans. This method can remove more than 90% of the attached branchiobdellidans without the death or growth reduction of the host crayfish (T. Konno, in preparation). The collected branchiobdellidans were preserved in 70% ethanol and brought back to the laboratory, and the species were identified according to Yamaguchi (1934). Host *C. japonicus* was rereleased at the site where they were captured.

Results

From preliminary sampling of *P. leniusculus*, in addition to *Sathodrilus tetrodonta* (Pierantoni, 1906) – which is ectosymbiotic with *P. leniusculus* in its native North America – *C. cirratus* complex, which is ectosymbiotic only with *C. japonicus*, was found on *P. leniusculus*. From the survey on August 30, 2022, total of 75 individuals of *P. leniusculus* were captured in the mainstem of the Atsubetsu River (Site 1), but no *C. japonicus* was found in the mainstem (Fig. 1, Table 1). And *C. cirratus* complex was also found on *P. leniusculus* in this survey. Individual information on 14 captured *P. leniusculus* and the number of branchiobdellidans from each host are summarised in Table 2. Two crayfish supported more *C. cirratus* complex than *S. tetrodonta*. The maximum number of *C. cirratus* complex on the host was 232, which was close to the number of *S. tetrodonta* on the same host individual.

C. japonicus was found in four small tributaries (Site 2-5) near the site of the mainstem (Site 1) where *P. leniusculus* was captured (Fig. 1, Table 1). The density of *C.*

japonicus was very low in every site 1 surveyed. No sympatric sites were observed between the native and non-native crayfish. Japanese branchiobdellidans were found in three populations of *C. japonicus* (Site 2-4), and all were identified as *Cirrodrilus digitatus* (Pierantoni, 1906) based on the morphology of the jaws.

Discussion

This is the first record of a native branchiobdellid species being found on a non-native host in Japan. The symbiotic relationships of branchiobdellidans and *P. leniusculus* have been studied at 21 sites in Japan, but only historically associated ectosymbionts, *S. tetrodonta* and *Xironogiton victoriensis* Gelder and Hall, 1990, were detected (Kawai et al., 2004; Ohtaka et al., 2005; Nakata et al., 2010). One of the reasons why Japanese branchiobdellidans have not been found on *P. leniusculus* in past studies could be that it has been a long time since crayfish was introduced (e.g. 70-80 years after initial introduction (Usio et al., 2007)). For example, Ďuriš et al. (2006) reported that four species of native branchiobdellidans – *Branchiobdella italica* Canegallo, 1923, *Branchiobdella pentadonta* Whitman, 1882, *Branchiobdella parasita* (Braun, 1805) and *Branchiobdella hexadonta* Grube, 1883 – were found on the non-native crayfish *Faxonius limosus* (Rafnesque, 1817) in 2001. However, only *B. italica* was observed in 2003, and no branchiobdellidans were detected in subsequent surveys. This suggests that novel symbiotic relationships between crayfish and branchiobdellidans may not last long. The population of *P. leniusculus* was established in the Atsubetsu River system relatively recently (less than 10 years ago, K. Tanaka, unpublished data). Not much time has passed since the first interactions between non-native and native crayfish, and the branchiobdellidans population may be transiently supported by *P. leniusculus*.

No individuals of *C. cirratus* complex were found on *C. japonicus* during this study. I investigated only four populations of *C. japonicus*, but exploring more sites in this river system may lead to identifying the source population of the branchiobdellidans transmitting to *P. leniusculus* from *C. japonicus*. However, the very low densities of *C. japonicus* in the studied area suggest that the host population from which the source of *C. cirratus* complex is already extinct, or that *C. cirratu*s complex became extinct before the *C. digitatus* due to the declining host density at the population. Hinosawa et al. (2023) estimated that *C. japonicus* in northern Honshu Island in Japan will be largely extinct by 2050, suggesting that *C. japonicus* is at a greater risk of extinction than previously thought. In Hokkaido, population extinction has already been confirmed at several sites (Kawai et al., 2002; Nakata et al., 2004), and the estimation in Hinosawa et al. (2023) could apply to Hokkaido populations as well. Since symbionts are known to go extinct earlier than their hosts in some cases (Lafferty, 2012; Rózsa and Vas, 2015), it is highly possible that the extinction of *C. cirratus* complex occurred earlier than that of *C. japonicus* in the Atsubetsu River system.

Japanese branchiobdellidans are specifically ectosymbiotic with *C. japonicus* and could only live in lakes or small streams near water sources inhabited by this host. On the other hand, *P. leniusculus* can inhabit mainstems, and *C. cirratus* complex could permanently use *P. leniusculus* as a host; they have expanded their habitat range to mainstem reaches, where it was originally difficult for them to inhabit. This finding is a unique example of a native symbiont that would normally be lost along with its native host as a result of biological invasions avoiding extinction by shifting hosts to the invasive species as well as expanding the living area of the river due to the high dispersal ability of the new host.

When native endangered symbiotic species transfer to non-native hosts, the removal of non-native species means driving the endangered species to extinction at the hands of humans. This finding suggests that there is a dilemma between the extermination of non-native species and the protection of symbionts caused by an overlooked host shift. Nevertheless, the main goal of the management of introduced species is to eradicate or reduce their population sizes, which leads to the restoration of native biodiversity (Hulme, 2006; Prior et al., 2018). A proper understanding of complex ecosystems includes symbiotic organisms and clarifying on what to conserve and what to eliminate, sometimes at the expense of native species, is important for ecosystem management.

Tables

Table 1. Number of crayfish captured at each site and the species of branchiobdellidans in the Atsubetsu River system

				Host crayfish		Branchiobdellidans
Site	Date	Latitude	Longitude	Species	Number	Species
Site 1	30 Aug. 2022	42.93534 N	141.42446 E	*P. leniusculus*	14	*S. tetrodonta* *C. cirratus*
Site 2	24 Aug. 2021	42.93079 N	141.41415 E	*C. japonicus*	2	*C. digitatus*
Site 3	24 Aug. 2021	42.93111 N	141.41741 E	*C. japonicus*	4	*C. digitatus*
Site 4	7 Sep. 2021	42.92880 N	141.40734 E	*C. japonicus*	4	*C. digitatus*
Site 5	15 Jul. 2022	42.94113 N	141.42438 E	*C. japonicus*	4	-

Table 2. Number of branchiobdellidans on individuals of *P. leniusculus* from Site 1in the Atsubetsu River system

					Number of branchiobdellidans		
Crayfish ID	MC	POCL (mm)	WW (g)	Sex	*S. tetrodonta*	*C. cirratus* complex	Total
1	BT	47.7	71.0	M	250	232	482
2	HN	43.7	48.5	F	151	82	233
3	EF	40.7	46.8	M	61	66	127
4	EF	43.0	43.3	F	39	21	60
5	HN	39.2	39.2	F	90	43	133
6	HN	39.9	38.4	F	16	19	35
7	HN	39.3	36.3	M	44	7	51
8	HN	38.4	33.9	F	25	1	26
9	BT	39.4	33.6	F	77	28	105
10	HN	36.8	28.3	F	38	8	46
11	BT	34.7	27.3	M	15	2	17
12	HN	33.9	22.7	F	16	6	22
13	HN	30.4	16.2	F	4	0	4
14	BT	26.5	10.3	F	9	7	16

MC, method of capture for crayfish (BT, box trap; HN, hand net; EF, electric fisher); POCL, postorbital carapace length; WW, wet weight.

Figures

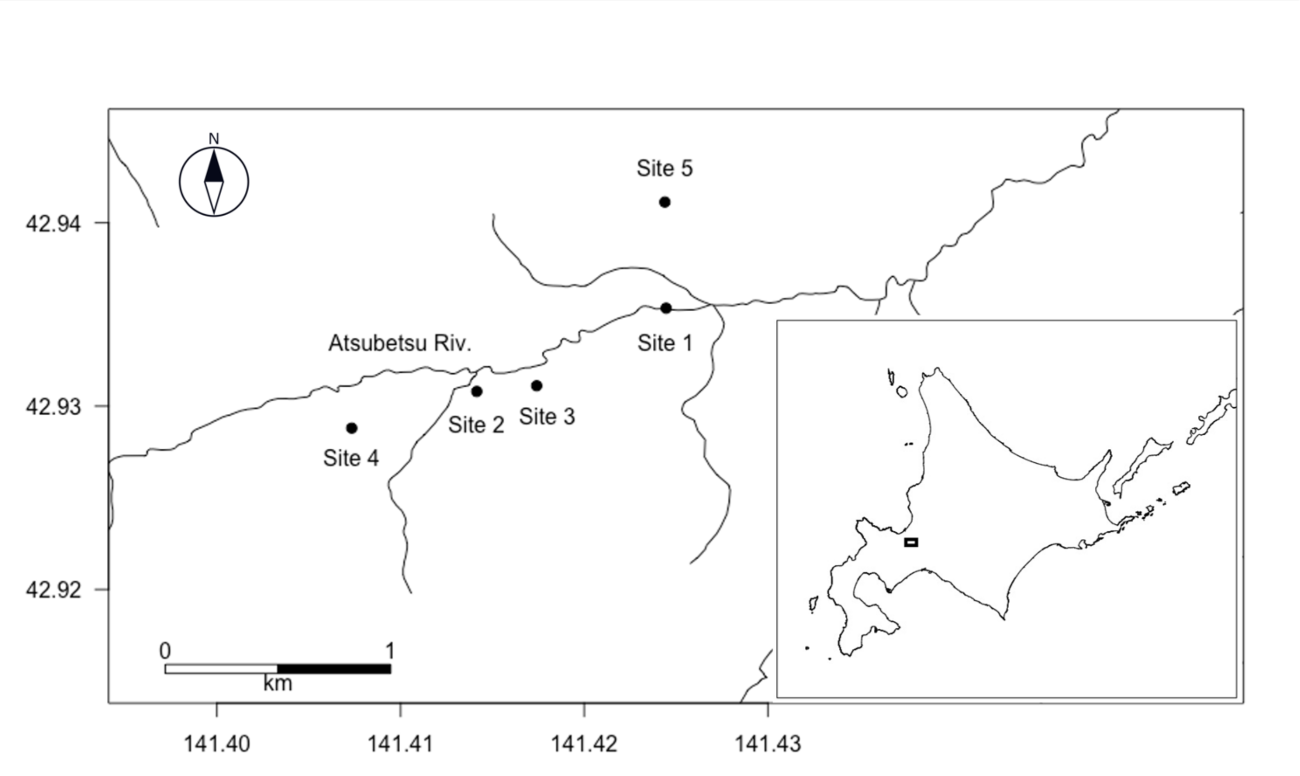


Figure 1. Crayfish were captured in the Atsubetsu River system at the numbered sites. The collection area is indicated by a rectangle on the map of Hokkaido Island; insert bottom right.

CHAPTER 3

Molecular phylogeny of Japanese branchiobdellidans and their phylogenic position within world species: with insight into co-evolution between Astacoidea crayfish and branchiobdellidans

Introduction

Branchiobdellidans are a group belonging to the phylum Annelida and are ubiquitous on crayfish in the northern hemisphere. They are relatively small (1-12 mm in length), hermaphroditic organisms that live symbiotically on the body surface of crayfish. Their life history is host-dependent, as they cannot reproduce in the absence of a host (Creed et al., 2015). Approximately 150 species across 21 genera, within 1 family and 4 subfamilies, have been described worldwide (Gelder and Williams, 2015). Their distribution is discrete, with populations in North America, Europe, and East Asia (Gelder, 1999). The relationships between crayfish and branchiobdellidans have been suggested to vary between parasitism, commensalism, and mutualism, depending on the number of branchiobdellidans per host individual (Brown et al., 2012; Skelton et al., 2013). As crayfish influence other organisms in their habitat through environmental modification and consumption, it has been suggested that branchiobdellidans may indirectly impact the environment through their host crayfish (Creed et al., 2021). Despite their widespread distribution and ecological importance, much of the ecology and evolutionary history of branchiobdellidans remains unknown.

The position of Branchiobdellida within annelids has been debated for many years, but both morphological and molecular phylogenetic study now supports their classification within annelids, forming a sister group together with Acanthobdellida and Hirudinida (Gelder and Siddall, 2001; Martin, 2001; Erseus and Kallersjo, 2004; Tessler et al., 2018). However, few studies have examined phylogenetic relationships within branchiobdellidans, and phylogenetic knowledge remains limited. In Gelder and Siddall (2001), phylogenetic relationships within branchiobdellidans were examined for the first time using molecular biological techniques, specifically 18S rDNA and COI region molecular markers. However, the phylogenetic tree obtained showed significant differences in the relative position of taxa compared to relationships suggested by some non-molecular methods (Holt, 1986; Gelder and Brinkhurst, 1990), and the taxonomical classification of subfamily was not supported. Williams et al. (2013) and Šarić et al. (2018) examined the phylogenetic relationships of North American and European species, but both studies established phylogenetic relationships only within their respective regions. Tessler et al. (2018) analysed the phylogenetic relationships of annelids and included results on the phylogenetic relationships within branchiobdellidans. These results supported those of Williams et al. (2013), but the phylogenetic relationships within branchiobdellidans remain controversial. For example, only one sequence of *Cirrodrilus sapporensis* is available from East Asia, while sequences of European and North American species are relatively well-documented. The phylogenetic relationships within

Branchiobdellida remain a matter of debate. Although there are many taxonomic studies on Far East Asian branchiobdellidans (e.g., Yamaguchi, 1935; Gelder, 1987; Timm, 1991; Gelder and Ohtaka, 2002; Ohtaka and Gelder, 2015), comprehensive molecular data are lacking. The only available sequence in databases such as GenBank is for the C. *sapporensis*. Consequently, comprehensive phylogenetic analyses across continents have not yet been conducted.

Phylogenetic relationships have been well studied in crayfish, the primary hosts of branchiobdellidans, and the Asian crayfish family Cambaroides is considered to represent the most ancestral lineage of crayfish in the Northern Hemisphere (Ahn et al., 2006; Crandall et al., 2017). Since branchiobdellidans are monophyletic and exclusively symbiotic with Astacoidea crayfish, the evolution of symbiosis in crayfish likely occurred after the divergence of the Southern Hemisphere crayfish and Northern Hemisphere crayfish superfamilies, which is thought to have diverged over 200 million years ago (Crandall et al., 2000; Wolfe et al., 2019). The Far East Asian branchiobdellidans that host members of the Cambaroidea crayfish are therefore considered evolutionarily significant, but no studies have yet examined their phylogenetic relationships.

Twenty-five species of branchiobdellidans have been described from Far East Asia, including 12 valid species from Japan (Yamaguchi et al., 1935; Timm, 1991; Ohtaka et al., 2023; Ohtaka and Gelder, 2023). All Japanese branchiobdellidans are endemic to Japan and exclusively hosted by a single species, the Japanese crayfish (*Cambaroides japonicus*). Interestingly, six of the Japanese species possess dorsoventral heteromorphic jaws, a unique feature found only in these branchiobdellidans worldwide (Timm, 1991). The host Japanese crayfish belongs to the phylogenetically most basal crayfish family, suggesting that its symbionts, the branchiobdellidans, may also represent the most basal branchiobdellidan lineage. Although Japanese branchiobdellidans are likely evolutionarily significant, comprehensive phylogenetic analyses within Japanese species and across global branchiobdellidan species have yet to be conducted.

In this study, I focused on Japanese branchiobdellidans and analysed the gene sequences of all 12 species to elucidate their phylogenetic relationships. I also reconstruct these phylogenetic relationships with branchiobdellid species from Europe and North America, aiming to construct a more comprehensive phylogenetic tree that includes a greater number of species than previous studies. Furthermore, by comparing the phylogenetic relationships of branchiobdellidans with the previously established phylogenetic relationships of crayfish, I discussed the co-evolutionary history of branchiobdellidans and crayfish in Northern Hemisphere.

Materials and Methods

Sampling and species identification of branchiobdellidans

Host *C. japonicus* was captured by searching under stones and trees. Captured crayfish were temporarily submerged in 70% ethanol for a short time (30 seconds) to remove branchiobdellidans. This method can remove more than 90% of the attached branchiobdellidans without the death or growth reduction of the host crayfish (T. Konno, in preparation). The collected branchiobdellidans were preserved in 70% ethanol and brought to the laboratory.

For species identification based on morphology, the anterior part of the body was sealed with Canada balsam to prepare preparations. Species identification of branchiobdellidans was mainly based on the morphology of the jaws reference to Yamaguchi (1934). The posterior part of the organism was preserved in 99% ethanol for DNA extraction. In some cases, the whole body was used as a sample for DNA extraction after the morphological observation of specimens under a microscope for species identification. Obtained specimens and some details are shown in Table 1.

C. cirratus and *C. uchidai* have been described as separate species (Pierantoni 1905; Yamaguchi, 1932). However, since the morphology of their jaws, the key identification characteristics, and body shape were very similar, Ohtaka et al. (2023)

mentioned two species from morphology is very difficult. So, they are not distinguished and treated as *C. cirratus* complex in this study.

DNA extraction, amplification, and sequencing

DNA was extracted with a QIAamp® DNA Stool Micro Kit (QIAGEN) following the manufacturer's protocol. I amplified and sequenced regions of two mitochondrial genes, cytochrome c oxidase subunit I (COI) and 16S rDNA, and two nuclear genes, 28S rDNA and internal transcribed spacer 1 (ITS1) using primers listed in Table 2. PCR amplifications were performed in a total volume of 10 µL, and the reaction mixtures contained 0.5 U Master Mix (GoTaq, Promega), 0.2 µM of each primer, 0.2 mM dNTP, 50 Mm KCl, 15 mM TrisHCl (pH 8.0), 1.5 mM MgCl2, and 0.5 µL of extracted DNA as a template. The PCR thermocycling protocol for the mtDNA analysis followed Šarić et al. (2018): initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s, extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min. The PCR products were purified by PEG precipitation. Purified products were cycle sequenced using BigDye Terminator v1.1(Applied BioSystems). Cycle sequenced products were purified by ethanol precipitation and run on the ABI 3130 (Applied BioSystems).

Sequence alignment and download from GenBank

The sequences were analyzed with the software MEGA11 (v11.0.10; Tamura et al., 2021). Fragment sequences were aligned using MUSCLE (Edgar, 2004) with defaults. Alignments of sequences were performed using MEGA11 and Finch TV 1.4.0 (Geospiza Inc.).

All sequences of European and North American species used here were downloaded from GenBank. As the outgroup taxa, sequences of lumbriculid species *Lumbriculus variegatus* were also downloaded and used for analysis (Appendix Table 1).

Phylogenetic reconstruction

Phylogenetic reconstruction of fragments and combined sequences was performed using the maximum likelihood (ML) methods using RAxML-NG v.1.2.2 (Kozlov et al., 2019) and Basian analysis (BA) using MrBayes v3.2.7 (Ronquist and Huelsenbeck, 2003). Prior to running the analyses, the optimal models of nucleotide evolution for each gene were determined based on the collect Akaike information criterion (AICc; Anderson and Burnham, 2004) using Modeltest-NG v.0.1.7 (Darriba et al., 2020). The selected models for each gene are listed in Supplementary Table 2. Phylogenetic trees were analyzed with 2000 bootstrap replicates using RAxML-NG. In

BA, priors were set according to the suggested model. Four Monte Carlo Markov chains were performed for 3,000,000 generations, and trees were sampled every 1000 generations, eliminating the first 25% of trees as burn-in. Nodes with posterior probabilities (PP) ≥0.90 in BA and bootstrap support values (BS) ≥70 in ML analyses were considered well-supported.

Phylogenetic reconstructions and divergence time estimation

BEAST v. 2.6.6 (Bouckaert et al., 2014) was employed to estimate divergence times with the coalescent consensus population model under the relaxed clock using the log-normal distribution. BEAUti v.2.6.6 was used to create input files for running BEAST. Here, the molecular clock that is 2.4% per site per million years in earthworms (Chang and James, 2011), the only taxon in Oligochaeta for which a molecular clock rate has been estimated, was used as a rough estimate. The nucleotide substitution model (GTR + I + G) was calculated by Modeltest-NG (Darriba et al., 2019). Four independent MCMC chains were run for 50,000,000 generations (burnin 10%) and were sampled every 1,000 generations. The combined tree was annotated with TreeAnnotator v. 1.10.4 after visualizing the results with Tracer v.1.7(Rambaut et al., 2018). The resulting tree was visualized using FigTree v.1.4.0 with the mean and 95% highest posterior density (HPD) interval for each node age.

Results

Obtained sequences and alignment

Of the 12 valid species described in Japan, 11 species were identified. At least two individuals from each species were sequenced. For the *C. cirratus* complex and *C. digitatus*, preliminary analyses suggested the presence of multiple clades within a single species, so specimens from multiple sites were sequenced.

Sequences of four DNA regions—COI (658 bp), 16S rDNA (509 bp, including gaps), 28S rDNA (330 bp, including gaps), and ITS1 (1552 bp, including gaps)—were successfully obtained from 44 individuals of branchiobdellidans (Table 1). The total alignment length of the combined sequences was 3049 base pairs. The mitochondrial COI sequences were obtained in a total of 350 individuals and were used to estimate divergence ages in inter-specific phylogeny.

Phylogeny of Japanese branchiobdellid species

The results of the phylogenetic analysis showed no significant differences between the Bayesian and ML phylogenetic trees, so only the Bayesian tree is presented (Fig. 1).

Japanese branchiobdellidans demonstrated monophyly, supported by high bootstrap values and Bayesian posterior probabilities in seven species. These results corresponded well with species identification based on jaw morphology (Fig. 1). The *C. cirratus* complex, *C. digitatus*, and *C. japonicus* each contained two, five, and two internal clades, respectively. *C. japonicus* included *C. megalodentatus*, and *C. digitatus* included *C. aomorensis*. The C1 clade of *C. cirratus* was positioned as the most basal position among the Japanese species. *C. tsugarensis* was found to be genetically encompassed within *C. makinoi* and is therefore considered a junior synonym of *C. makinoi*.

Species with dorsoventral heteromorphic jaws (*C. megalodentatus*, *C. japonicus*, *C. nipponicus*, *C. iwakiensis*, *C. digitatus*, and *C. aomorensis*) formed a single clade and were positioned derivatively. Except for several clades of *C. digitatus* (including *C. aomorensis*), which appeared in the most derived position, the Bayesian and ML phylogenetic trees were congruent.

The results of divergence age estimation are shown in Fig. 2. Speciation occurred in all but two clades (*C. aomorensis* included in clades of *C. digitatus*, and *C. megalodentatus* with *C. japonicus*) at a time earlier than the estimated east-west phylogenetic group divergence age of the Japanese crayfish (3-5 million years ago).

Phylogeny of Branchiobdellida

The results of the phylogenetic analysis of the Japanese species suggested that each morphologically identified species was monophyletic. Therefore, a single representative sequence from each species was used for the phylogenetic analysis of the entire branchiobdellidans assemblage. For species with multiple internal clades, one representative sequence from each clade was selected for analysis.

The Bayesian phylogenetic tree constructed from the concatenated sequences of COI, 16S rDNA, 28S rDNA, and ITS1 regions is shown in Figure 3. In the Bayesian phylogenetic tree, the clade consisting of species from eastern North America was monophyletic, supported by a posterior probability (PP) value of 0.96. In contrast, species from northwestern North America (mainly associated with *Pacifastacus* crayfish) occupied the most derived position in the ML phylogenetic tree, with the three clades of species from eastern North America taking a lateral phylogenetic position relative to it. There were other differences in the positions of *Branchiobdella* cf. *kazarovi* and *Triannulata magna*, but overall, the Bayesian phylogenetic tree was better supported, with higher PP values.

The Japanese branchiobdellidans (*Cirrodrilus*) were found to be monophyletic, supported by a bootstrap (BS) value of 100 and a PP value of 1. In this phylogenetic tree, the North American species *Bdellodrilus illuminatus* appeared as the most basal lineage, suggesting that it may represent the most ancestor lineage in Branchiobdellida. Among the western North American species, the genus *Xironogiton* formed a clade positioned next to the Japanese species. In the Bayesian phylogenetic tree, the other western North American species and the European species *Branchiobdella* cf. *kazarovi* did not necessarily form a monophyletic clade but occupied an immediate phylogenetic position relative to the clade consisting of species from eastern North America. However, the phylogenetic tree obtained in this analysis does not strongly support the overall phylogenetic relationships, as some branches had low BS and PP values.

Discussion

This study provides the first molecular phylogenetic analysis of Japanese branchiobdellidans. By examining the genes of Japanese *Cirrodrilus* species, which had previously only been studied in only one species, this study provides a comprehensive analysis of the phylogenetic relationships of Branchiobdellida. The findings contribute to understanding the phylogenetic divergence of Japanese branchiobdellidans, the presence of cryptic species, their phylogenetic position among the Branchiobdellida, and the coevolutionary history of branchiobdellid species and crayfish worldwide.

Inter-species phylogeny of Japanese branchiobdellidans

This study clarified the phylogenetic relationships of 11 Japanese species. Phylogenetic trees were constructed using Maximum Likelihood (ML) and Bayesian methods, and both approaches yielded almost identical results, producing highly supported phylogenetic trees with very high posterior probability (PP) values, although bootstrap (BS) values were occasionally low.

The feature of dorsoventral heteromorphic jaws is a highly specific trait found only in certain Japanese species (*C. nipponicus*, *C. japonicus*, *C. megalodentatus*, *C. iwakiensis*, *C. digitatus*, and *C. aomorensis*) among branchiobdellidans worldwide (Timm, 1991). The phylogenetic position of these species is of particular interest. In this study, it was shown that the group of species with dorsoventral heteromorphic jaws morphology, a monophyletic lineage, is located in the most derived phylogenetic position among Japanese species. The branching with the clade comprising *C. homodontus* and *C. makinoi*—the immediate ancestral lineage of the dorsoventral heteromorphic group—was strongly supported by a BS value of 95 and a PP value of 1, indicating high confidence. These findings suggest that dorsoventral heteromorphic of the jaws is a derived trait in Japanese branchiobdellidans, evolving only once during the divergence from the common ancestor of *C. homodontus* and *C. makinoi*, even in the extensive evolutionary history of branchiobdellidans as a whole.

The estimated divergence age of these species predates the east-west divergence of Japanese crayfish, strongly suggesting that speciation had already occurred before the dispersal of Japanese crayfish in Hokkaido. This indicates that branchiobdellidans already exhibited high species diversity when the ancestors of Japanese crayfish or other Asian crayfish species diverged and migrated to Japan. Therefore, it is unlikely that co-evolution with Japanese crayfish within Japan significantly contributed to the diversification of branchiobdellidans. Over a timescale of millions of years, environmental changes and dispersal events probably led to species extinctions. Consequently, branchiobdellidans may have exhibited greater species diversity in the past than is observed today.

Existence of cryptic species

This study suggests the presence of several cryptic species among Japanese branchiobdellidans that cannot be distinguished based on morphology.

First, two clades within the *C. cirratus* complex are discussed. Both Bayesian and ML phylogenetic trees support the existence of two genetically distinct clades within the *C. cirratus* complex. In this study, *C. cirratus* and *C. uchidai* were grouped together as the *C. cirratus* complex, as their overlapping morphological features made them indistinguishable based on morphological characteristics. Gelder (1987) conducted detailed morphological observations of *C. cirratus* and *C. uchidai*, identifying the main differences as the presence or absence of an oral process and minor variations in male genitalia. However, the supra-oral process may be lost depending on the method of fixation, and male genitalia can vary significantly depending on maturity and individual characteristics. To determine whether the two clades observed in this study correspond to *C. cirratus* and *C. uchidai* or represent entirely different species, more detailed morphological observations of specimens from each genetic lineage are needed. Furthermore, as the type localities of both *C. cirratus* and *C. uchidai* were not noted in the original descriptions, a thorough taxonomic re-assessment will require estimating their type localities from literature and research records. Comparing specimens from these localities and identifying their genotypes (topotypes) would help resolve these taxonomic uncertainties.

Next, two clades of *C. japonicus* encompassing *C. megalodentatus* are examined. These clades are supported by both Bayesian and ML methods. Morphologically, *C. megalodentatus* has significantly larger jaws than *C. japonicus*, suggesting its status as a distinct species. On the other hand, the two clades of *C. japonicus* do not show notable differences in jaw size, making morphological distinction between them as separate species impossible. Three lineages, including *C. megalodentatus*, were found at a single site in Oshamanbe, where they coexist but are genetically isolated. This reproductive isolation fulfills the criteria for biological species, indicating that each is an independent species. However, more detailed morphological observations of specimens from each genetic lineage are needed before describing them as distinct species. Careful consideration is also required to determine which lineage corresponds to the true *C. japonicus*, necessitating comparisons with type specimens.

Finally, six clades, including *C. aomorensis*, were identified within *C. digitatus*. Although some BS and PP values were low and statistically unsupported, and divergence patterns between Bayesian and ML phylogenetic trees differed, the occurrence of multiple genetically independent clades across both analyses suggests that the clades are polyphyletic. Phylogenies within *C. digitatus* were not geographically coherent, as demonstrated by the presence of two phylogenies at the Jozankei locality. Since *C. aomorensis* is nested within the *C. digitatus* clade, it is inferred that *C. aomorensis* arose via allopatric speciation following the immigration of a portion of *C. digitatus* into Honshu. As with the other species, more detailed morphological observations and comparisons with type specimens are needed to clarify these relationships.

Phylogenetic relationship within Branchiobdellida and co-evolution with crayfish

In this study, a phylogenetic analysis of the world's branchiobdellidans was conducted using four regions of mitochondrial and nuclear DNA. The analysis included 11 Japanese species sequenced in this study and 38 additional species from GenBank, covering a wide range of phylogenetic and geographical diversity.

The analysis revealed that *Bdellodrilus illuminatus*, a species that mainly associates *Cambarellus* and *Cambarus* in eastern North America, positioned the most basal lineage. This species is known to host up to 12 species and has a wide geographical range (e.g., Gelder et al., 2001; Gelder and Williams, 2011; Williams et al., 2013). The Japanese branchiobdellidans formed the next oldest lineage, together with *Xironogiton* (hosted by *Pacifastacus*) from western North America. Since the host Japanese crayfish belongs to the family Cambaroidae—the most ancestral lineage of crayfish in the Northern Hemisphere (Ahn et al., 2006; Crandall et al., 2017)—it is reasonable that the Japanese branchiobdellidans located a basal phylogenetic position.

The Bayesian phylogenetic tree showed a monophyletic lineage with high posterior probability (PP = 0.96) composed of species inhabiting eastern North America and hosted by crayfish from the family Cambaridae. This result aligns with the monophyletic lineage of American crayfish, which occupies a more derived position among Northern Hemisphere crayfish. Although the Cambaridae locates derived position in crayfish phylogeny, the family includes over 350 species, suggesting relatively recent and rapid diversification (Crandall et al., 2017; Stern et al., 2017). More than 90 species of branchiobdellidans have also been described from eastern North America (Longshaw, 2011), accounting for more than half of all described species, suggesting that their speciation paralleled the diversification of their host crayfish.

In contrast, species primarily associated with crayfish from the family Astacoidae exhibited polyphyly in the current analysis, making it difficult to reconstruct a clear history of co-evolution with their hosts. The Astacoidae have an extensive evolutionary history, with divergence estimates suggesting they may date back as far as 150 million years (Bracken-Grissom et al., 2014). Within the Astacoidae, the divergence between *Pacifastacus* from northwestern North America and European crayfish has been estimated to have occurred around 140 million years ago. It is possible that multiple events of geographic and genetic isolation over this long evolutionary history contributed to the observed polyphyletic phylogenetic relationships.

Tables

Table 1. Specimens of branchiobdellidans used for phylogenetic analyses.

ID	Species	Locality	Geographic region	Locus
03_06	*C. digitatus*	*Mt. Apoi*	*Eastern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
03_08	*C. homodontus*	*Mt. Apoi*	*Eastern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
24_02	*C. sapporensis*	*Omu*	*Northern Hokkkaido*	COI, 16S rDNA, 28S rDNA, ITS1
24_25	*C. nipponicus*	*Omu*	*Northern Hokkkaido*	COI, 16S rDNA, 28S rDNA, ITS1
24_27	*C. nipponicus*	*Omu*	*Northern Hokkkaido*	COI, 16S rDNA, 28S rDNA, ITS1
50_03	*C. cirratus* complex	*Shimamaki*	*Southern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
50_05	*C. cirratus* complex	*Shimamaki*	*Southern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
50_07	*C. cirratus* complex	*Shimamaki*	*Southern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
50_13	*C. cirratus* complex	*Shimamaki*	*Southern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
56_01	*C. cirratus* complex	*Motofukushima*	*Southern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
56_02	*C. iwakiensis*	*Motofukushima*	*Southern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Er4_01	*C. digitatus*	*Erimo 4*	*Northern Hokkkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Er4_02	*C. digitatus*	*Erimo 4*	*Northern Hokkkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Es_12	*C. digitatus*	*Esashi 2*	*Northern Hokkkaido*	COI, 16S rDNA, 28S rDNA, ITS1
IB_05	*C. digitatus*	*Itanki-biotope*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Im_01	*C. makinoi*	*Imabetsu*	*Aomori*	COI, 16S rDNA, 28S rDNA, ITS1
Im_02	*C. makinoi*	*Imabetsu*	*Aomori*	COI, 16S rDNA, 28S rDNA, ITS1
Iw2_04	*C. aomorensis*	*Mt. Iwaki 2*	*Aomori*	COI, 16S rDNA, 28S rDNA, ITS1
Jo_04	*C. cirratus* complex	*Jozankei*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Jo_08	*C. digitatus*	*Jozankei*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Jo_09	*C. digitatus*	*Jozankei*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Jo_15	*C. homodontus*	*Jozankei*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Jo_24	*C. homodontus*	*Jozankei*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Ki_01	*C. cirratus* complex	*Kitami*	*Eastern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Ki_02	*C. cirratus* complex	*Kitami*	*Eastern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Ki_03	*C. digitatus*	*Kitami*	*Eastern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Ki_04	*C. digitatus*	*Kitami*	*Eastern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Ks_06	*C. digitatus*	*Kumanosawa-park*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Ks_08	*C. digitatus*	*Kumanosawa-park*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
My_11	*C. cirratus complex*	*Maruyama*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
OA_01	*C. sapporensis*	Otaru Aqualium	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Os_05	*C. megalodentatus*	*Oshamanbe*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Os_06	*C. japonicus*	*Oshamanbe*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Os_07	*C. japonicus*	*Oshamanbe*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Os_08	*C. megalodentatus*	*Oshamanbe*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Os_23	*C. sapporensis*	*Oshamanbe*	*Central Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Uj_01	*C. sapporensis*	*Usujiri*	*Southern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Uj_10	*C. megalodentatus*	*Usujiri*	*Southern Hokkaido*	COI, 16S rDNA, 28S rDNA, ITS1
Wa_06	*C. digitatus*	*Wakkanai*	*Northern Hokkkaido*	COI, 16S rDNA, 28S rDNA, ITS1

Table 2. Primers, primer references, each locus used in phylogenetic analyses.

Locus Primer	Primer sequence 5'- 3'	Reference
Nuclear		
28S rDNA		
C1'	ACCCGCTGAATTTAAGCAT	Lê et al. (1993)
C2'	TGAACTCTCTCTTCAAAGTTCTTTTC	Lê et al. (1993)
ITS1		
ITS1A	CACACCGCCCGTCGCTACTACCG	Kerans et al. (2006)
ITS1B	GTGCGTTCGAAGTGTCGATGATCAA	Kerans et al. (2006)
Mitochondrial		
COI		
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
16S rDNA		
ArL	CGCCTGTTTATCAAAAACAT	Palumbi et al. (1991)
BrH	CCGGTCTGACTCAGATCACGT	Palumbi et al. (1991)

Figures

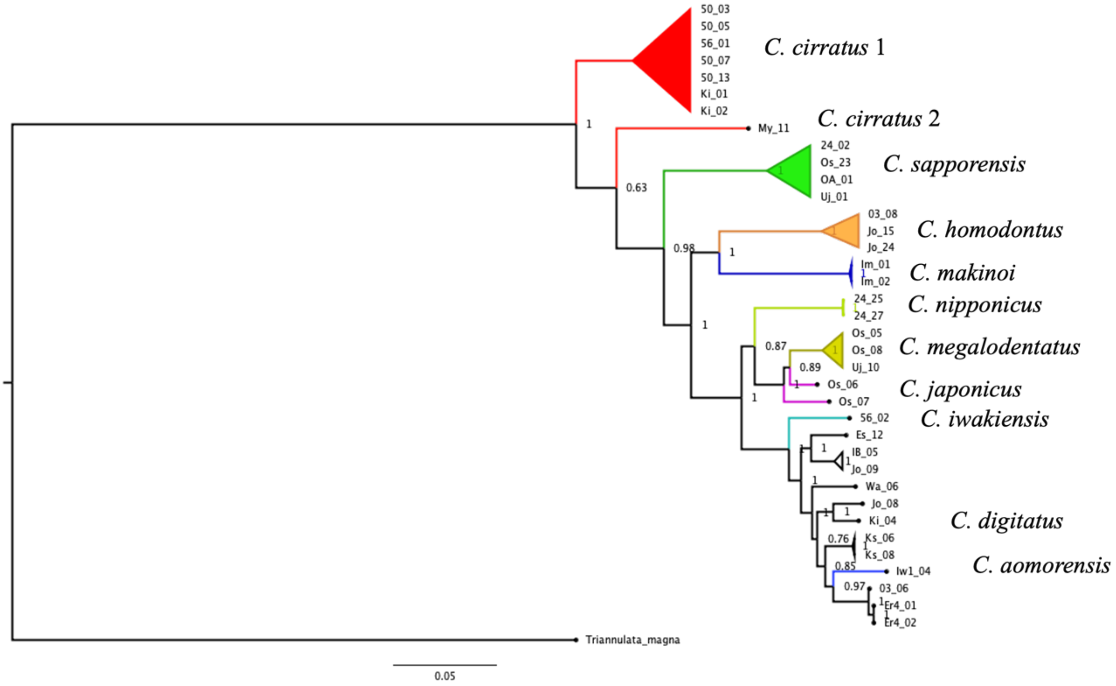


Figure 1. Phylogenetic tree reconstructed using Bayesian methods with concatenated sequences of COI, 16S rDNA, 28S rDNA, and ITS1 regions of Japanese branchiobdellidans. Colour differences indicate species identified through morphological analysis.

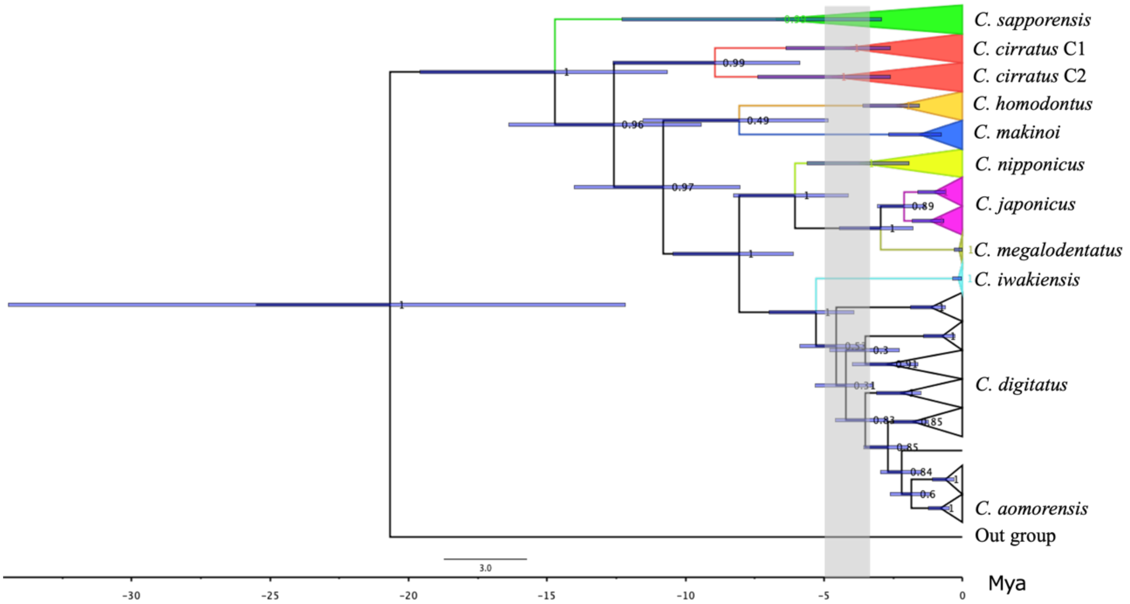


Figure 2. The Bayesian phylogenetic tree is based on COI sequences of Japanese branchiobdellidans, with the horizontal axis representing time in million years. Grey shading shows the estimated divergence time between eastern and western phylogenetic lineages of Japanese crayfish considered in Koizumi et al. (2012). 95% highest posterior density (HPD) range shown as bar lengths on nodes.

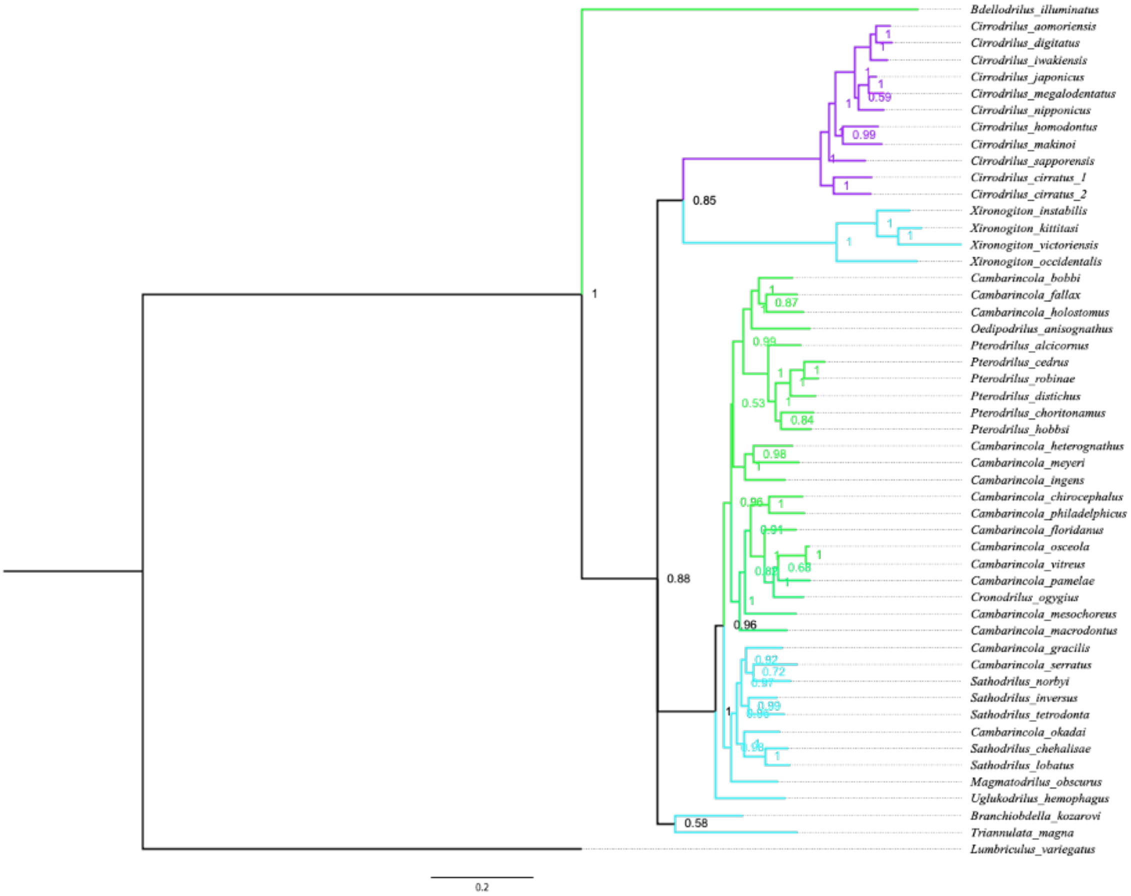


Figure 3. Phylogenetic tree of branchiobdellidans constructed by Bayesian methods using COI, 16S rDNA, 28S rDNA, and ITS1 sequences. Colours of branches represent associated crayfish families of the each branchiobdellid species: purple (Cambaroidae), light blue (Astacoidae), and green (Cambaridae).

Appendix

Appendix Table 1. Sequences used for phylogenetic analyses with GenBank accession numbers listed for each locus.

Species	COI	16S rDNA	28S rDNA	ITS1
Bdellodrilus illuminatus	JQ821634	JQ821459	JQ821577	JQ821683
Branchiobdella cf. *kozarovi*	MH351653	MH351637	MH351644	-
Cambarincola bobbi	JQ821620	JQ821445	JQ821563	JQ821670
Cambarincola chirocephalus	JQ821603	JQ821428	JQ821546	JQ821654
Cambarincola fallax	JQ821621	JQ821446	JQ821564	JQ821671
Cambarincola floridanus	JQ821617	JQ821442	JQ821560	JQ821667
Cambarincola gracilis	JQ821591	JQ821414	JQ821532	JQ821641
Cambarincola heterognathus	JQ821625	JQ821450	JQ821568	JQ821674
Cambarincola holostomus	JQ821639	JQ821465	JQ821583	JQ821686
Cambarincola ingens	JQ821626	JQ821451	JQ821569	JQ821675
Cambarincola macrodontus	JQ821618	JQ821443	JQ821561	JQ821668
Cambarincola mesochoreus	JQ821619	JQ821444	JQ821562	JQ821669
Cambarincola meyeri	JQ821601	JQ821426	JQ821544	JQ821652
Cambarincola okadai	JQ821595	JQ821420	JQ821538	JQ821647
Cambarincola osceola	JQ821616	JQ821441	JQ821559	JQ821666
Cambarincola pamelae	JQ821614	JQ821439	JQ821557	JQ821664
Cambarincola philadelphicus	JQ821602	JQ821427	JQ821545	JQ821653
Cambarincola serratus	JQ821594	JQ821419	JQ821537	JQ821646
Cambarincola vitreus	JQ821615	JQ821440	JQ821558	JQ821665
Cronodrilus ogygius	JQ821613	JQ821438	JQ821556	JQ821663
Magmatodrilus obscurus	JQ821600	JQ821425	JQ821543	JQ821651
Oedipodrilus anisognathus	JQ821624	JQ821449	JQ821567	JQ821673
Pterodrilus alcicornus	JQ821606	JQ821431	JQ821549	JQ821656
Pterodrilus cedrus	JQ821609	JQ821434	JQ821552	JQ821659
Pterodrilus choritonamus	JQ821608	JQ821433	JQ821551	JQ821658
Pterodrilus distichus	JQ821611	JQ821436	JQ821554	JQ821661
Pterodrilus hobbsi	JQ821607	JQ821432	JQ821550	JQ821657
Pterodrilus robinae	JQ821610	JQ821435	JQ821553	JQ821660
Sathodrilus attenuatus	JQ821592	JQ821417	JQ821535	JQ821644
Sathodrilus chehalisae	JQ821596	JQ821421	JQ821539	JQ821648
Sathodrilus inversus	JQ821598	JQ821415	JQ821533	JQ821642
Sathodrilus lobatus	JQ821597	JQ821422	JQ821540	JQ821649
Sathodrilus norbyi	JQ821593	JQ821418	JQ821536	JQ821645
Triannulata magna	JQ821629	JQ821454	JQ821572	JQ821678
Uglukodrilus hemophagus	JQ821628	JQ821453	JQ821571	JQ821677
Xironogiton instabilis	JQ821630	JQ821455	JQ821573	JQ821679
Xironogiton kittitasi	JQ821632	JQ821457	JQ821575	JQ821681
Xironogiton occidentalis	JQ821633	JQ821458	JQ821576	JQ821682
Xironogiton victoriensis	JQ821631	JQ821456	JQ821574	KY982581
Lumbriculus variegatus	JQ821637	JQ821463	JQ821581	JQ821685

CHAPTER 4

Co-evolutionary history of Japanese crayfish and ectosymbiotic branchiobdellidans from a molecular phylogeny perspective

Introduction

Eleven valid species of Japanese branchiobdellidans have been described, all of which are symbionts with the Japanese crayfish (Yamaguchi, 1935; Ohtaka and Gelder, 2023; Chapter 3). Twenty-five species of branchiobdellidans have been described in East Asia, and, like the Japanese crayfish, all are endemic to Japan (Timm, 1991). Notably, there are no other examples globally where as many as 11 species coexist on a single crayfish species. In North America, where branchiobdellidan diversity is relatively high, a maximum of only 3-4 species coexists on a single crayfish host.

The geographical genetic structure of Japanese crayfish is highly distinct, with each river system known to harbour its own haplotypes. This is attributed to their habitat being restricted to river headwaters and their limited mobility, which isolates populations. Two major lineages of Japanese crayfish are distributed east and west of the Hidaka Mountains, with their divergence estimated to have occurred 3-5 million years ago (Koizumi et al., 2012). Although the Tsugaru Strait separates Hokkaido and Aomori, crayfish from Southern Hokkaido and Aomori share some haplotypes, suggesting multiple historical genetic exchanges (Koizumi et al., 2012).

Branchiobdellidans are obligate symbionts that cannot complete their life cycle in the absence of crayfish (Creed et al., 2015). As Japanese branchiobdellidan species exclusively associate with Japanese crayfish, their genetic structure is expected to mirror that of their hosts. If the intraspecific phylogeny of the symbiont reflects that of its host, this would indicate a co-evolutionary relationship. Moreover, it is of great interest to determine whether each of the 11 Japanese branchiobdellidan species is in a coevolutionary relationship with its host.

In this study, I revealed phylogenetic relationships among 60 populations (total of 11 species) of branchiobdellidans on Japanese crayfish from Hokkaido and northern Honshu based on the COI mitochondrial DNA. Furthermore, I reconstructed host phylogeny and conducted cophylogenetic analyses to detect co-evolutional signals between branchiobdellidans and host crayfish. Then, the co-evolutionary history between branchiobdellidans and the Japanese crayfish was discussed.

Material and methods

Sampling and species identification of branchiobdellidans

Japanese crayfish and branchiobdellidans were surveyed at 70 sites in Hokkaido and Aomori prefecture, Japan, between September 2020 and October 2024 (Fig. 1). Host *C. japonicus* was captured by searching under stones and trees. Captured crayfish were temporarily submerged in 70% ethanol for a short time (30 seconds) to remove branchiobdellidans. This method can remove more than 90% of the attached branchiobdellidans without the death or growth reduction of the host crayfish (T. Konno, in preparation). The collected branchiobdellidans were preserved in 70% ethanol and brought to the laboratory.

For species identification based on morphology, the anterior part of the body was sealed with Canada balsam to prepare preparations. Species identification of branchiobdellidans was mainly based on the morphology of the jaw plates reference to Yamaguchi (1934). The posterior part of the organism was preserved in 99% ethanol for DNA extraction. In some cases, the whole body was used as a sample for DNA extraction after the morphological observation of specimens under a microscope for species identification. Obtained specimens and some details are shown in Table 1.

C. cirratus and *C. uchidai* have been described as separate species (Pierantoni 1905; Yamaguchi, 1932). However, since the morphology of their jaws, the key identification characteristics, and body shape were very similar, Ohtaka et al. (2023) mentioned two species from morphology is very difficult. So, they are not distinguished and treated as *C. cirratus* complex in this study.

DNA extraction, amplification, and sequencing

DNA was extracted with a QIAamp® DNA Stool Micro Kit (QIAGEN) following the manufacturer's protocol. I amplified and sequenced the region of mitochondrial genes, cytochrome c oxidase subunit I (COI), using the universal primers LCO1490 and HCO2198 (Folmer et al., 1994). PCR amplifications were performed in a total volume of 10 µL, and the reaction mixtures contained 0.5 U Master Mix (GoTaq, Promega), 0.2 µM of each primer, 0.2 mM dNTP, 50 Mm KCl, 15 mM TrisHCl (pH 8.0), 1.5 mM MgCl2, and 0.5 µL of extracted DNA as a template. The PCR thermocycling protocol for the mtDNA analysis followed Šarić et al. (2018): initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s, extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min. The PCR products were purified by PEG precipitation. Purified products were cycle sequenced using BigDye Terminator v1.1(Applied BioSystems). Cycle sequenced products were purified by ethanol precipitation and run on the ABI 3130 (Applied BioSystems).

Sequence alignment and download from GenBank

The sequences were analyzed with the software MEGA11 (v11.0.10; Tamura et al. 2021).

Fragment sequences were aligned using MUSCLE (Edgar, 2004) with defaults. Alignments of sequences were performed using MEGA11 and Finch TV 1.4.0 (Geospiza Inc.).

As the outgroup taxa, sequences of Asian branchiobdellid species *Cirrodrilus suzukii* and Asian crayfish species *Cambaroides schrekii* were downloaded from Genbank. Additional sequences of Japanese crayfish, analyzed in Hinosawa et al. (2023), are also downloaded from Genbank.

Phylogenetic reconstructions and divergence time estimation

BEAST v. 2.6.6 (Bouckaert et al. 2014) was employed for phylogenic reconstructions and estimate divergence times in a relaxed clock using the log-normal distribution. BEAUti v.2.6.6 was used to create input files for running BEAST. Here, the molecular clock that is 2.4% per site per million years in earthworms (Chang and James 2011), the only taxon in Oligochaeta for which a molecular clock rate has been estimated, was used as a rough estimate. 1.15% per site per million years for crayfish (Brower, 1994). The nucleotide substitution models for each species calculated by Modeltest-NG (Darriba et al., 2019) are listed in Table 2. Four independent MCMC chains were run for 50,000,000 generations (burnin 10%) and were sampled every 1,000 generations. The combined tree was annotated with TreeAnnotator v. 1.10.4 after visualizing the results with Tracer v.1.7(Rambaut et al. 2018). The resulting tree was visualized using FigTree v.1.4.0 with the mean and 95% highest posterior density (HPD) interval for each node age.

Co-phylogenetic analysis

For each species of Japanese crayfish and branchiobdellidans, a phylogenetic tree was constructed using BEAST v. 2.6.6 (Bouckaert et al. 2014), with one representative sequence per locality, or up to two sequences if completely distinct lineages were detected. The phylogenetic trees were analysed in R using the ‘Rtapas’ package (Llaberia-Robledillo et al., 2023) for co-phylogenetic analysis. The MI algorithm was applied to examine the host-symbiont relationship, and the ParaFit Global (PFG) value was calculated using the global fit method (ParaFit) to detect co-evolutionary signals between host and symbiont phylogeny.

Results

Interspecific phylogeny and estimate divergence time of Japanese crayfish

643 bp of COI sequence were obtained from 138 samples of Japanese crayfish in this study, and 18 sequences were downloaded from Genbank (Table 1). Bayesian

phylogenetic tree reconstructed by BEAST is shown in Fig. 2A. The genetic structure of the Japanese crayfish is highly distinct, with clades forming coherent regional groups (Fig. 2A, Fig. 3A). The east-west lineage, separated by the Hidaka Mountains, was the first to diverge, with an estimated median divergence age of approximately 4 million years ago. Within the western group, the Northern Hokkaido clade diverged first, followed by the central and Southern Hokkaido clades, with the Aomori lineage derived from Southern Hokkaido clades. The lineage from Aomori was monophyletic, while Southern Hokkaido lineage was lateral to the Aomori lineage. Specimens collected from the Orumappu River in eastern Hokkaido were included in the Central Hokkaido clade, whereas those from near Lake Onneto were grouped within the Southern Hokkaido clade. The Southern Hokkaido lineage was also detected at sites in Minamifurano and west of Mt. Iwaki.

Interspecific phylogeny and estimate divergence time of Japanese branchiobdellidans

588 bp of COI sequence were obtained from 350 samples, and the phylogenies of the bayesian method are shown in Fig. 2. In this study, I reconstructed phylogenetic trees for three species, a phylogenetic clade of *C. cirratus* complex, *C. sapporensis*, and *C. homodontus*, which sampled from over ten sites around Hokkaido.

For one clade of the *C. cirratus* complex (identified in Chapter 3), 138 sequences were collected from 41 sites in Hokkaido and used for phylogenetic analysis (Fig. 3B). The Bayesian phylogenetic tree generated is shown in Fig. 2B. The lineage formed distinct regional clusters, resembling the intra-specific phylogeny of the host. A significant genetic segregation was observed between the Erimo and Eastern Hokkaido lineages and other lineages composed of individuals from different regions, consistent with the genetic structure of the host Japanese crayfish. The estimated divergence ages for the group comprising Eastern Hokkaido and other regions were 2.06–4.83 million years ago (mean: 3.39 Mya).

For *C. sapporensis*, 20 sequences from 10 sites in Hokkaido were analysed for phylogenetic inference (Fig. 3C). The Bayesian phylogenetic tree generated is shown in Fig. 2C. *C. sapporensis* also exhibited a clear regional pattern within its intra-specific phylogeny. The divergence pattern of the eastern and western lineages across the Hidaka Mountains was consistent with that of the Japanese crayfish. The Central Hokkaido lineage showed greater diversification and deeper divergence within the Eastern Hokkaido and other groups of *C. sapporensis*. The estimated divergence ages for the Eastern Hokkaido and other groups ranged from 1.60 to 4.63 million years ago (mean: 2.94 Mya).

For *C. homodontus*, 30 sequences from 14 sites were used for the analysis (Fig.

3D). Its geographical genetic structure was less distinct compared to that of the Japanese crayfish and other branchiobdellidans. The Northern Hokkaido lineage was not monophyletic and was positioned laterally relative to other lineages. The east-west divergence across the Hidaka Mountains was not the first divergence within the intra-specific lineage, and the estimated divergence age was approximately 2 million years ago.

Co-phylogenetic analyses

Co-phylogenetic analysis of the intra-specific lineages of Japanese crayfish and the three species of branchiobdellidans revealed varying levels of co-evolutionary signals across species.

For *C. cirratus* 1, a statistically significant co-evolutionary signal was observed, with a high ParaFit Global (PFG) value of 411260.9 ($P = 0.001$, Fig. 4A). This indicates a strong co-evolutionary relationship between the host and the symbiont.

In *C. sapporensis*, co-evolutionary signals were detected at specific tips of the phylogenetic tree; however, the overall PFG value was low (458.3), and no significant co-evolutionary signal was found across the entire lineage ($P = 0.853$, Fig. 4B). This suggests a weaker or less consistent co-evolutionary relationship between the host and this symbiont.

For *C. homodontus*, the PFG value was moderate (112212.2), indicating a statistical trend towards co-evolution (P = 0.091, Fig. 4C). While not statistically significant, the result points to a potential co-evolutionary relationship that warrants further investigation.

Discussion

The history of the symbiotic branchiobdellidans was very old and the phylogenies of some symbionts were similar to the host phylogeny. In addition, I found deep intra-specific phylogenies of the branchiobdellidans with strong regional structures, as long as host crayfish. Below, I discuss the dispersal histories of Japanese crayfish and branchiobdellidans and the host-symbiont's co-evolutionary history.

Evolutionary history of Japanese crayfish

As shown by Koizumi et al. (2012), the genetic structure of Japanese crayfish species in this study exhibited clear regional differentiation, with a particularly pronounced divergence between the eastern and western lineages, using the Hidaka Mountains as the boundary. The divergence age between the eastern and western lineages, based on molecular clocks, was estimated at approximately 4 million years ago, consistent with the

3–5 million years ago estimate reported by Koizumi et al. (2012). The Aomori lineage placed a relatively derived position, with the median divergence age of the southern Hokkaido lineage estimated at approximately 600,000 years ago. This does not align with the glacial period of the Middle Pleistocene (0.9–1.3 million years ago) and the Riss glaciation (0.14–0.18 million years ago), during which terrestrial and freshwater organisms are believed to have migrated across a land bridge formed in the Tsugaru Strait, connecting Hokkaido and Honshu (Kurose et al., 1999; Yokoyama and Goto, 2002; Sota and Hayashi, 2007; Koizumi et al., 2012; Ooyagi et al., 2018).

Interestingly, the genetic lineages of central Hokkaido and southern Hokkaido were detected near Lake Onneto and the Orumappu River in Eastern Hokkaido, respectively. The samples from near Lake Onneto closely resembled those from Oshamanbe (Central Hokkaido), while those from the Orumappu River closely resembled those from Setana (Southern Hokkaido). This strongly suggests artificial introductions. Historically, Japanese crayfish were distributed as medicine and food, with records of their introduction to Honshu, but no confirmed cases of introduction within Hokkaido. These findings indicate that, as reported in Europe and North America (Albrecht, 1983; Trontelj et al., 2005; Barbaresi et al., 2007), crayfish may have been introduced within their habitat range in Japan.

Individuals with genes similar to the Southern Hokkaido lineage were also collected from Minami-Furano and the western side of Mt. Iwaki. Each represents an endemic lineage, making it notable that the Southern Hokkaido lineages are distributed across the Central Hokkaido and Honshu, across the Tsugaru Strait. One possibility is that the ancestor of the Southern Hokkaido lineage was originally widespread from Central to Southern Hokkaido but experienced habitat contraction and southward migration during the glacial period, resulting in the current lineage distribution. Minami-Furano is located south of the volcanic zone in Central Hokkaido, where the stable spring-fed environment and geothermal heat from volcanic terrain may have enabled the survival of older lineages during the Ice Age. The western Mt. Iwaki population, located at the southwestern edge of the crayfish range, may have retained a lineage from the early period when Japanese crayfish first migrated to Honshu. Further genetic analyses of Japanese crayfish populations in volcanic regions and at the margins of their range in Honshu are necessary to support these hypotheses.

Co-evolutionary history of Japanese crayfish and branchiobdellidans

In the *C. cirratus* complex, the estimated divergence age of the eastern and western lineages and the general pattern of phylogenetic divergence matched those of the host

Japanese crayfish (Fig. 3B). For *C. sapporensis*, the pattern of phylogenetic divergence was consistent with that of the host, but the divergence age of the eastern and western lineages occurred slightly later than that of the host (Fig. 3C). For *C. homodontus*, neither the pattern of phylogenetic divergence nor the age of divergence of the eastern and western lineages matched those of the host (Fig. 3D). The co-evolutionary signals also varied in degree between species (Fig. 4). These results suggest that the evolutionary histories of branchiobdellidans are not always identical, even when they coexist on the same host species.

It is reasonable to assume that species whose phylogenetic relationships align with those of their host and exhibit co-lineage signals form symbiotic relationships with Japanese crayfish, as they disperse and become isolated in a similar manner, thereby maintaining a co-evolutionary relationship with their host. For species where no evidence of co-evolution was found, it is possible that their dispersal processes are independent of the host, or that differing phylogenetic processes are at play due to variations in population size. If different branchiobdellidan species exhibit varying population sizes within individual host crayfish, the potential for individual transfer between populations via crayfish migration may differ, resulting in varying degrees of gene flow. In this study, however, the smaller sample sizes for *C. sapporensis* and *C. homodontus* compared to the

C. cirratus complex may have hindered the construction of accurate phylogenetic trees. The further accumulation of samples and the inclusion of additional genetic regions in future analyses could lead to a more robust and reliable interpretation. A deeper understanding of the fundamental ecology of Japanese branchiobdellidans will contribute to resolving these uncertainties.

Implication for lineage level co-evolution

The phylogenetic relationship between host and symbiont will be mirrored and their distributions match, a phenomenon that is thought to be more common when the relationship between them is closer (Mazé-Guilmo et al., 2016). In this study, I focused on the intra-specific phylogeny between the Japanese crayfish and their ectosymbiotic branchiobdellidans, which is considered to be an obligate symbiosis. Some of the branchiobdellid species showed intra-specific phylogenies that correspond well to the host phylogeny, indicating the host-symbionts co-evolution over long periods (probably over 5 million years). The continuation of this condition in the future could potentially lead to speciation. This study suggests that focusing on the intra-specific phylogeny of both hosts and symbionts can provide deeper insights into their co-evolutionary and distributional patterns and processes.

Tables

Table 1. List of *C. japonicus* specimen used for phylogenetic analysis.

ID	Locality	Geographic region
01_01	*Lake Toyoni*	*Eastern Hokkaido*
01_02	*Lake Toyoni*	*Eastern Hokkaido*
03_01	*Mt. Apoi*	*Eastern Hokkaido*
03_02	*Mt. Apoi*	*Eastern Hokkaido*
11_01	*Hamanaka*	*Eastern Hokkaido*
11_02	*Hamanaka*	*Eastern Hokkaido*
15_01	*Kiyosato*	*Eastern Hokkaido*
15_02	*Kiyosato*	*Eastern Hokkaido*
17_01	*Syari*	*Eastern Hokkaido*
17_02	*Syari*	*Eastern Hokkaido*
22_01	*Esashi 1*	*Northern Hokkkaido*
22_02	*Esashi 1*	*Northern Hokkkaido*
23_01	*Enbetsu*	*Northern Hokkkaido*
24_01	*Omu*	*Northern Hokkkaido*
24_02	*Omu*	*Northern Hokkkaido*
46_01	*Ohnuma*	*Central Hokkaido*
46_02	*Ohnuma*	*Central Hokkaido*
50_01	*Shimamaki*	*Southern Hokkaido*
50_02	*Shimamaki*	*Southern Hokkaido*
50_03	*Shimamaki*	*Southern Hokkaido*
50_04	*Shimamaki*	*Southern Hokkaido*
51_01	*Setana*	*Southern Hokkaido*

51_02	*Setana*	*Southern Hokkaido*
56_01	*Motofukushima*	*Southern Hokkaido*
56_02	*Motofukushima*	*Southern Hokkaido*
Ab_01	*Abashiri*	*Eastern Hokkaido*
Ab_02	*Abashiri*	*Eastern Hokkaido*
As_01	*Asamushi*	*Aomori*
As_02	*Asamushi*	*Aomori*
Er7_01	*Erimo 7*	*Eastern Hokkaido*
Er7_02	*Erimo 8*	*Eastern Hokkaido*
Es_01	*Esashi 2*	*Northern Hokkkaido*
Es_02	*Esashi 2*	*Northern Hokkkaido*
Gr_01	*Garo-river*	*Southern Hokkaido*
Gr_02	*Garo-river*	*Southern Hokkaido*
Gs_01	*Kagehizawa*	*Aomori*
Gs_02	*Kagehizawa*	*Aomori*
Hk_01	*Todohokke*	*Southern Hokkaido*
Hk_02	*Todohokke*	*Southern Hokkaido*
Hn_01	*Hamanaka*	*Eastern Hokkaido*
Hr4_01	*Hiraoka 4*	*Central Hokkaido*
Hr4_02	*Hiraoka 4*	*Central Hokkaido*
Hr8_01	*Hiraoka 8*	*Central Hokkaido*
Hr8_02	*Hiraoka 8*	*Central Hokkaido*
IB_01	*Itanki-biotope*	*Central Hokkaido*
IB_02	*Itanki-biotope*	*Central Hokkaido*
Ik_01	*Ikebana*	*Eastern Hokkaido*
Ik_02	*Ikebana*	*Eastern Hokkaido*

It1_01	*Itanki-beach 1*	*Central Hokkaido*
It1_02	*Itanki-beach 1*	*Central Hokkaido*
It2_01	*Itanki-beach 2*	*Central Hokkaido*
It2_02	*Itanki-beach 2*	*Central Hokkaido*
Iw1_01	*Mt. Iwaki 1*	*Aomori*
Iw1_02	*Mt. Iwaki 1*	*Aomori*
Iw2_01	*Mt. Iwaki 2*	*Aomori*
Iw2_02	*Mt. Iwaki 2*	*Aomori*
Iw3_01	*Mt. Iwaki 3*	*Aomori*
Iw3_02	*Mt. Iwaki 3*	*Aomori*
Iw3_03	*Mt. Iwaki 3*	*Aomori*
Jo_01	*Jozankei*	*Central Hokkaido*
Jo_02	*Jozankei*	*Central Hokkaido*
kg_01	*Komagome*	*Aomori*
kg_02	*Komagome*	*Aomori*
Kh_01	*Kamishihoro*	*Eastern Hokkaido*
Kh_02	*Kamishihoro*	*Eastern Hokkaido*
Kh_03	*Kamishihoro*	*Eastern Hokkaido*
Ki_01	*Kitami*	*Eastern Hokkaido*
Ki_02	*Kitami*	*Eastern Hokkaido*
Km_01	*Kaminokuni*	*Southern Hokkaido*
Km_02	*Kaminokuni*	*Southern Hokkaido*
Ks_01	*Kumanosawa-park*	*Central Hokkaido*
Ks_02	*Kumanosawa-park*	*Central Hokkaido*
Ku_01	*Kushiro*	*Eastern Hokkaido*
Ku_02	*Kushiro*	*Eastern Hokkaido*

Kw_01	*Kurisawa*	*Central Hokkaido*
Kz_01	*Kizukuri*	*Aomori*
Mt_01	*Matsumae*	*Southern Hokkaido*
Mt_02	*Matsumae*	*Southern Hokkaido*
My_01	*Maruyama*	*Central Hokkaido*
My_02	*Maruyama*	*Central Hokkaido*
Nn_01	*Naganuma*	*Central Hokkaido*
Nu_01	*Nukabira*	*Eastern Hokkaido*
Nu_02	*Nukabira*	*Eastern Hokkaido*
OA_01	*Otaru*	*Central Hokkaido*
OA_02	*Otaru*	*Central Hokkaido*
Ok_01	*Oketo*	*Eastern Hokkaido*
Ok_02	*Oketo*	*Eastern Hokkaido*
On_01	Onneto	*Eastern Hokkaido*
On_02	Onneto	*Eastern Hokkaido*
Os_01	*Oshamanbe*	*Central Hokkaido*
Os_02	*Oshamanbe*	*Central Hokkaido*
Ot_01	*Osatsube*	*Southern Hokkaido*
Ow_01	*Oowani*	*Aomori*
Ow_02	*Oowani*	*Aomori*
Ra_01	*Orumappu*	*Eastern Hokkaido*
Sa_01	*Samani*	*Eastern Hokkaido*
Sa_02	*Samani*	*Eastern Hokkaido*
Sd_01	*Shiodomari*	*Southern Hokkaido*
Sd_02	*Shiodomari*	*Southern Hokkaido*
Se_01	*Setana*	*Southern Hokkaido*

Se_02	*Setana*	*Southern Hokkaido*
Se_03	*Setana*	*Southern Hokkaido*
Se_04	*Setana*	*Southern Hokkaido*
Sg_01	*Sunagawa*	*Central Hokkaido*
Sg_02	*Sunagawa*	*Central Hokkaido*
ShA_01	*Sotogahama A*	*Aomori*
ShA_02	*Sotogahama A*	*Aomori*
ShB_01	*Sotogahama B*	*Aomori*
ShB_02	*Sotogahama B*	*Aomori*
Sk_01	*Shikaoi*	*Eastern Hokkaido*
Sn_01	*Soranuma*	*Central Hokkaido*
Sn_02	*Soranuma*	*Central Hokkaido*
So_01	*Soya*	*Northern Hokkkaido*
So_02	*Soya*	*Northern Hokkkaido*
Sr_01	*Saruru*	*Eastern Hokkaido*
Sr_02	*Saruru*	*Eastern Hokkaido*
SS_01	*Minami-Furano*	*Central Hokkaido*
SS_02	*Minami-Furano*	*Central Hokkaido*
SS_03	*Minami-Furano*	*Central Hokkaido*
SS_04	*Minami-Furano*	*Central Hokkaido*
Syo_01	*Onenai*	*Central Hokkaido*
Syo_02	*Onenai*	*Central Hokkaido*
Te_01	*Teshio*	*Northern Hokkkaido*
Te_02	*Teshio*	*Northern Hokkkaido*
Tj_01	*Tejin*	*Central Hokkaido*
Tj_02	*Tejin*	*Central Hokkaido*

To_01	*Toya*	*Central Hokkaido*
To_02	*Toya*	*Central Hokkaido*
Tyk_01	*Toyokoro*	*Eastern Hokkaido*
Tyk_02	*Toyokoro*	*Eastern Hokkaido*
Tyk_03	*Toyokoro*	*Eastern Hokkaido*
Tyk_04	*Toyokoro*	*Eastern Hokkaido*
Uj_01	*Usujiri*	*Eastern Hokkaido*
Uj_02	*Usujiri*	*Eastern Hokkaido*
Up_01	*Usujiri*	*Eastern Hokkaido*
Wa_01	*Wakkanai*	*Northern Hokkkaido*
Wa_02	*Wakkanai*	*Northern Hokkkaido*

Table 2. The nucleotide substitution models for each species.

Species	Model
C. japonicus	*HKY + I + G4*
C. cirratus 1	*HKY + I + G4*
C. sapporensis	*GTR + I*
C. homodontus	*HKY + I + G4*

Figures

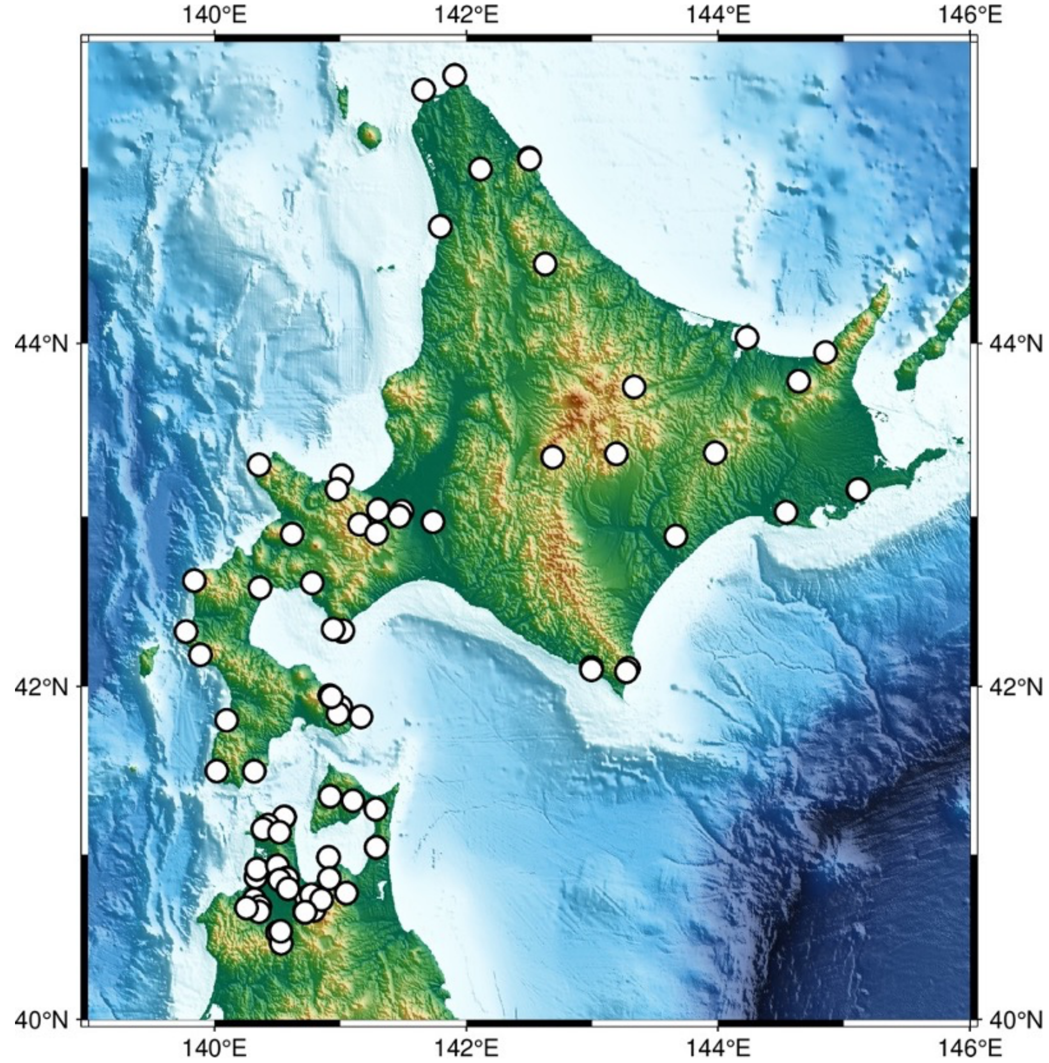


Figure 1. Sampling locality of Japanese crayfish

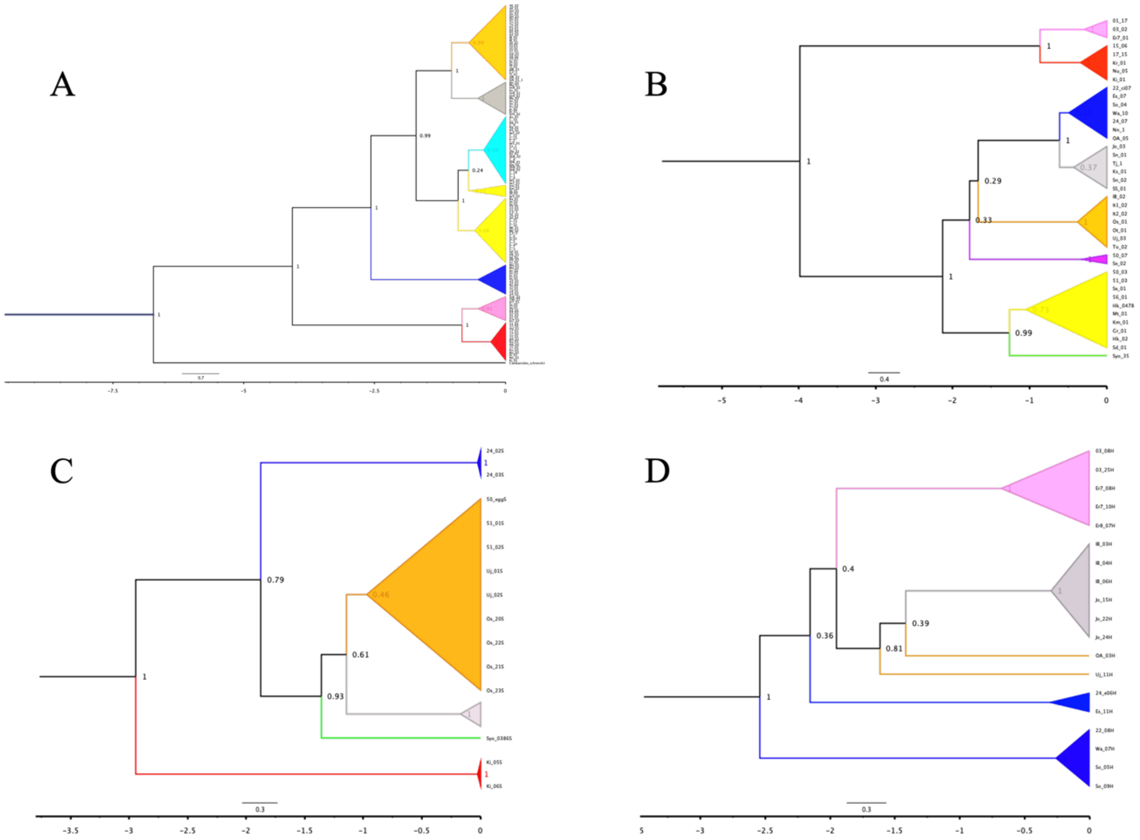


Figure 2. Phylogenetic trees generated using Bayesian methods with COI sequences for each species and estimated divergence ages. A: Bayesian phylogenetic tree of Japanese crayfish. B: Bayesian phylogenetic tree of *C. cirratus* 1. C: Bayesian phylogenetic tree of *C. sapporensis*. D: Bayesian phylogenetic tree of *C. homodontus*. In the phylogenetic tree of Japanese crayfish, the colours of each lineage represent the sampling region. In the phylogenetic tree of branchiobdellidans species, the colours are based on the phylogeny of the associated Japanese crayfish. The horizontal axis represents time, in millions of years.

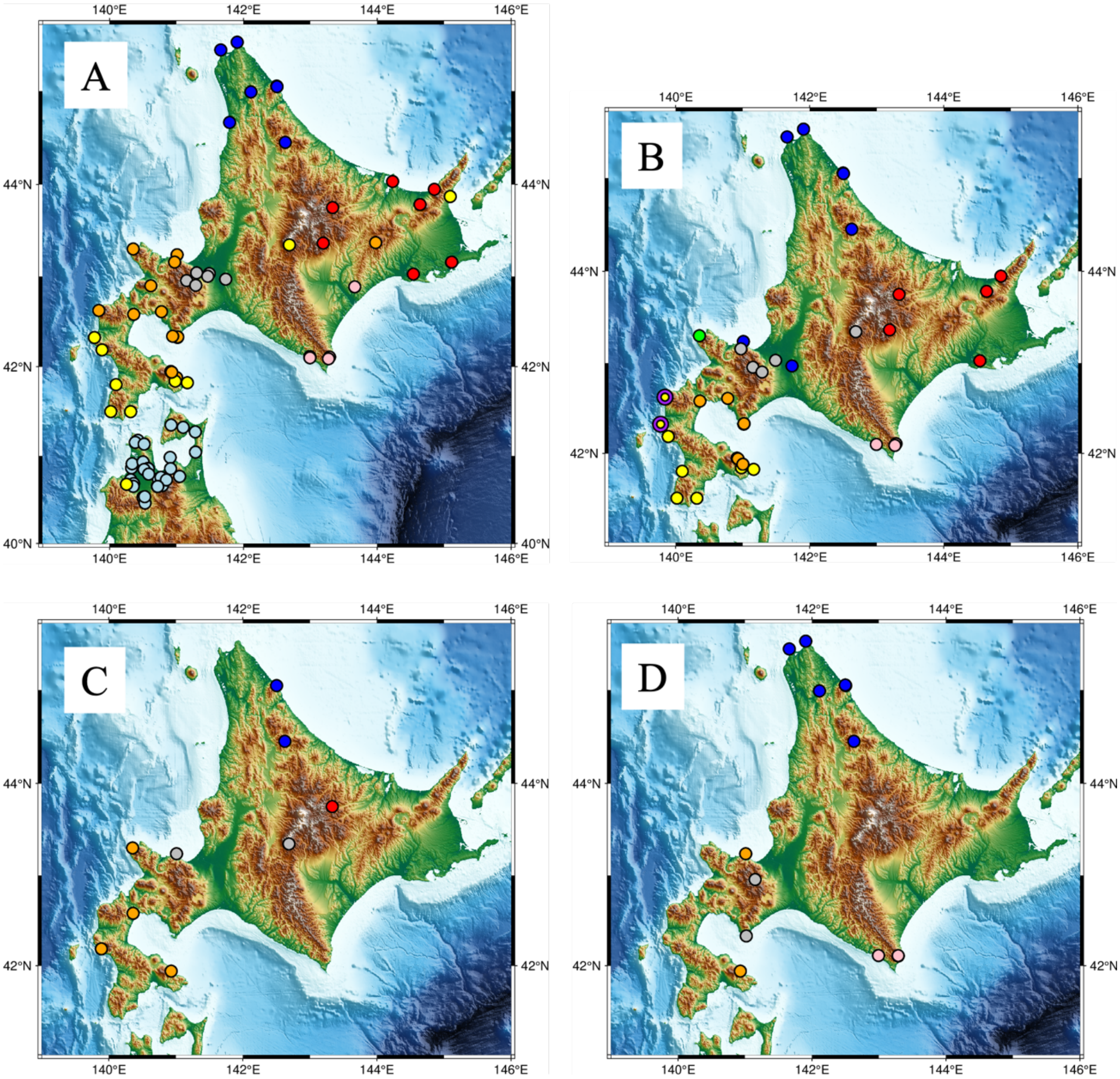
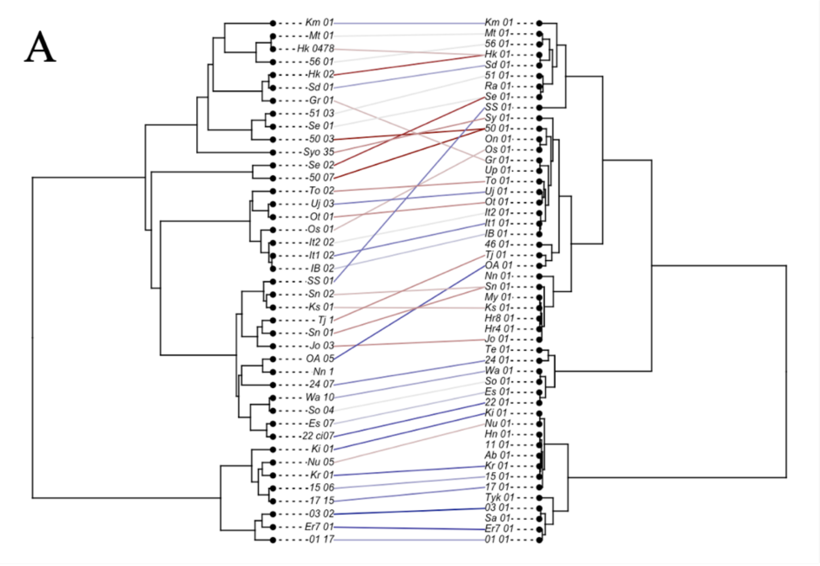


Figure 3. Map showing the geographical distribution of each phylogenetic lineage. Colours correspond to those in Figure 2. A: host crayfish *C. japonicus*; B: *C. cirratus* 1; C: *C. sapporensis*; D: *C. homodontus*.


A
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Mt 01
Hk 0478
56 01
Hk 02
Sd 01
Gr 01
51 03
Se 01
50 03
Syo 35
Se 02
50 07
To 02
Uj 03
Ot 01
Os 01
It2 02
It1 02
IB 02
SS 01
Sn 02
Ks 01
Tj 1
Sn 01
Jo 03
OA 05
Nn 1
24 07
Wa 10
So 04
Es 07
22 ci07
Ki 01
Nu 05
Kr 01
15 06
17 15
03 02
Er7 01
01 17
Km 01
Mt 01
56 01
Hk 01
Sd 01
51 01
Ra 01
Se 01
SS 01
Sy 01
50 01
On 01
Os 01
Gr 01
Up 01
To 01
Uj 01
Ot 01
It2 01
It1 01
IB 01
46 01
Tj 01
OA 01
Nn 01
Sn 01
My 01
Ks 01
Hr8 01
Hr4 01
Jo 01
Te 01
24 01
Wa 01
So 01
Es 01
22 01
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Ab 01
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Tyk 01
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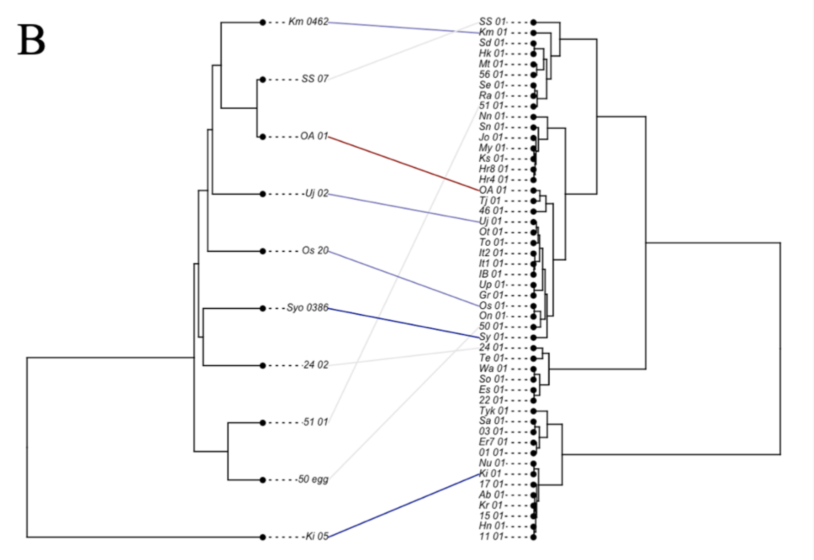
B
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SS 07
OA 01
Uj 02
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Syo 0386
24 02
51 01
50 egg
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Hk 01
Mt 01
56 01
Se 01
Ra 01
51 01
Nn 01
Sn 01
Jo 01
My 01
Ks 01
Hr8 01
Hr4 01
OA 01
Tj 01
46 01
Uj 01
Ot 01
To 01
It2 01
It1 01
IB 01
Up 01
Gr 01
Os 01
On 01
50 01
Sy 01
24 01
Te 01
Wa 01
So 01
Es 01
22 01
Tyk 01
Sa 01
03 01
Er7 01
01 01
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Ki 01
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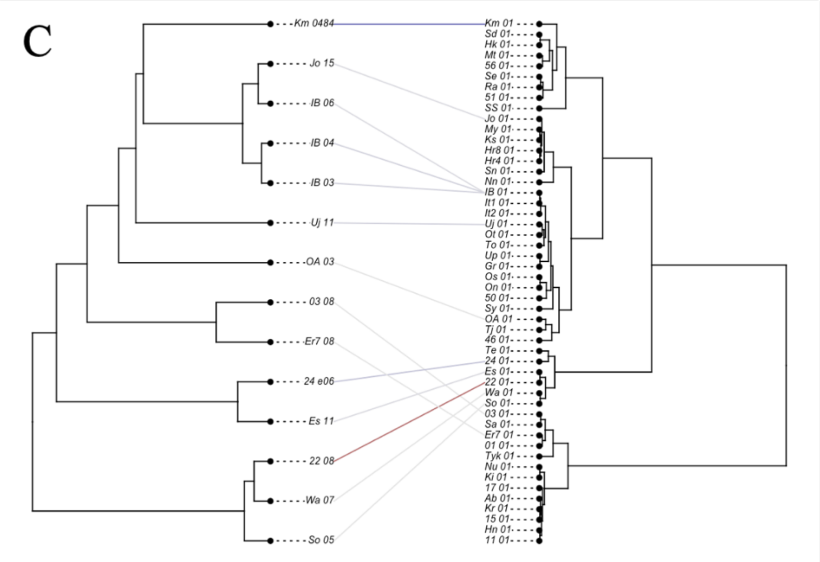
Kr 01
15 01
Hn 01
11 01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Jo 15
IB 06
IB 04
IB 03
Uj 11
OA 03
03 08
Er7 08
24 e06
Es 11
22 08
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Km 01
Sd 01
Hk 01
Mt 01
56 01
Se 01
Ra 01
51 01
SS 01
Jo 01
My 01
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Figure 3. Results of a co-phylogeny analysis comparing the intraspecific phylogeny of Japanese crayfish and three of branchiobdellid species. A: Japanese crayfish and *C. cirratus* 1; B: Japanese crayfish and *C. sapporensis*; D: Japanese crayfish and *C. homodontus*. Lines between tips indicate associations, with darker blue representing a stronger co-evolutionary signal and darker red representing a weaker signal.

CHAPTER 5

General Discussion

The aim of this dissertation was to update the basic knowledge of branchiobdellidans and to investigate their co-evolutionary history with crayfish, as well as the necessity for their conservation.

In Chapter 2, branchiobdellidans were sampled across the entire distribution range of their host, the Japanese crayfish, and classified based on morphology to examine their diversity patterns. Positive relationships were found between host density and branchiobdellidans diversity, suggesting that the decline in host population density may cause a loss of branchiobdellidans diversity. Moreover, a comparison of the distribution and species composition of Japanese branchiobdellidans between the present and approximately 100 years ago revealed a current decline in species diversity. Occurrence rates varied among species, with declines exceeding 50% in some cases. Additionally, it was discovered that Japanese branchiobdellidans have shifted hosts from native to non-native crayfish in areas invaded by non-native species.

In Chapter 3, the phylogeny of Japanese branchiobdellidans was reconstructed using morphological and genetic analyses. Japanese species formed a monophyletic lineage at a basal position in the global phylogenetic tree of branchiobdellidans. While clades corresponding to morphological traits appeared in many species, multiple clades were observed in some species, indicating the presence of cryptic species. The estimated divergence ages of most Japanese species were earlier than those of the eastern and western groups of Japanese crayfish, suggesting that speciation was not driven by co-evolution with Japanese crayfish. However, the overall phylogenetic relationships of branchiobdellidans corresponded roughly to those of their host crayfish, suggesting a broader co-evolutionary relationship.

In Chapter 4, the co-evolutionary history of Japanese branchiobdellidans and Japanese crayfish was investigated by comparing the intraspecific phylogenetic relationships. Phylogenetic analysis of samples from over ten sites revealed that, for some species, the divergence and estimated ages of eastern and western groups aligned with those of their hosts, although some species did not show such a match. The co-phylogenic analysis provided statistical support for a co-evolutionary relationship between the *C. cirratus* complex and Japanese crayfish, and a co-evolutionary trend was observed in *C. homodontus*. These findings suggest that different branchiobdellidans species have distinct evolutionary histories. The co-evolutionary history of some species appears to have been maintained for several million years, indicating that this relationship could continue in the future, potentially driving macroevolution.

Co-evolutionary histories of Japanese branchiobdellidans and Japanese crayfish

The results of Chapter 3 suggest that many Japanese branchiobdellidans species may have undergone speciation before the estimated divergence age of the eastern and western lineages of the Japanese crayfish. This implies that, prior to the dispersal of the Japanese crayfish across Hokkaido, branchiobdellidans were as diverse as, or perhaps even more diverse than, they are today. In Chapter 4, some species of Japanese crayfish and branchiobdellid species showed a rough concordance in the divergence ages of their eastern and western lineages and in the genetic structure of their intraspecific lineages. A comparison of the intraspecific phylogenies of the two species revealed a co-lineage signal, indicating that their phylogenetic coevolutionary history has been preserved at a phylogenetic level over millions of years.

The findings in Chapter 2 indicate that a reduction in host population size leads to a corresponding loss of the branchiobdellidans diversity. Based on these results, possible scenarios for the co-evolution of Japanese crayfish and branchiobdellidans are as follows. First, the ancestors of Japanese crayfish in eastern Eurasia harbored branchiobdellidans diversity that was as rich as or even greater than the current diversity observed in Japan. When the ancestors of the Japanese crayfish immigrated to Japan and dispersed across their present distribution range, they maintained a high diversity of branchiobdellidans species. Subsequent expansions and contractions in host population sizes may have shaped the current diversity and distribution pattern of branchiobdellidans.

Implication to the conservation necessity of branchiobdellid species

The Japanese branchiobdellidans have preserved a co-evolutionary history spanning millions of years with the Japanese crayfish or its ancestral species. However, comparisons made in Chapter 2 between past distributions and species compositions and their current status reveal a decline in the diversity of Japanese branchiobdellidans across their entire range and within each region. Hinosawa et al. (2023) reported that Japanese crayfish in Aomori Prefecture could face extinction by 2050 due to global warming. Local extinctions of Japanese crayfish in Hokkaido have already been documented at locations (Kawai et al., 2002; Nakata et al., 2004). Furthermore, symbionts are known to become extinct before their hosts (Lafferty, 2012; Rózsa and Vas, 2015), suggesting that branchiobdellidans, which are highly dependent on crayfish, may face an even greater extinction risk than previously anticipated. Indeed, species-specific occurrence rates for most branchiobdellidans species have declined significantly compared to approximately 100 years ago, with some species now rarely found. The Japanese branchiobdellidans *C. cirratus* complex was detected in signal crayfish but was absent from nearby Japanese crayfish populations. These findings strongly indicate that branchiobdellidans may experience local extinctions before their crayfish hosts. These results highlight that the extinction risk for branchiobdellidans is considerably higher than that for Japanese crayfish, emphasizing the need for immediate conservation efforts. In particular, a significant diversity loss was observed in Central and Eastern Hokkaido, making it critical to prioritize populations in these regions for conservation initiatives. Further investigations are necessary to assess the impacts of environmental and land-use differences on branchiobdellidan populations in these areas. The conservation status of certain species should be re-evaluated considering the findings from this study.

No branchiobdellidans were found in the crayfish populations that were potentially introduced. This absence could be attributed to the mortality of branchiobdellidans caused by changes in water quality and temperature during the translocation process, or it may indicate a local extinction resulting from the reduction in host population size experienced during the transfer. Indeed, Gelder (2004) reported a bottleneck effect during host translocation, which led to a decrease in symbiont diversity. While the translocation of crayfish populations following road construction or river improvement projects may help sustain crayfish populations, it could simultaneously result in a loss of branchiobdellidans diversity.

Future prospect of study on branchiobdellidans

Recent studies using molecular phylogenetic techniques have suggested the presence of cryptic species in branchiobdellidans that cannot be distinguished by morphology (Füreder et al., 2009; Williams et al., 2013; Šarić et al., 2018). The findings of Chapter 3 indicate that cryptic species are also present within Japanese branchiobdellidans. These species may be reproductively isolated, highlighting the importance of reassessments their taxonomy by detailed morphological observations using specimens tied to their respective genetic lineages in future studies. In Chapter 2, the distribution and diversity of Japanese branchiobdellidans were examined with morphological observations. However, now that the existence of cryptic species has been confirmed, genetic-based analyses of diversity will be essential for a more accurate understanding of their true diversity.

In Chapter 4, co-evolutionary signals at the intraspecific phylogenetic level were detected in some Japanese branchiobdellidans species, suggesting that the relationship with the host Japanese crayfish represents a fundamental process in macroevolution. Although each lineage exhibited distinct regional differentiation, there were no observable differences in jaw morphology between lineages, and speciation did not appear to have occurred. However, as the observed traits were limited to jaw and body shape, more detailed morphological examinations may reveal previously undetected differences between lineages.

The relationship between branchiobdellidans and crayfish is known to shift from parasitism to mutualism depending on the density of branchiobdellidans, influencing the local ecosystem through their crayfish hosts (Brown et al., 2012; Creed et al., 2021). It is not uncommon for a single Japanese crayfish to host more than 100 branchiobdellidans, and if the symbiosis between branchiobdellidans and crayfish has an impact on crayfish, it is highly likely to produce cascading effects on the entire ecosystem. In Chapter 2, it was found that many Japanese crayfish populations lack branchiobdellidans (29 out of 98 sites). These findings provide a foundation for investigating the ecological effects of the presence or absence of branchiobdellidans and pave the way for future studies on their impact via crayfish, such as comparing decomposition rates of aquatic invertebrates and litter among sites.

Up to six species of branchiobdellidans can coexist on a single Japanese crayfish. It is fascinating that organisms with similar morphology, ecology, and phylogeny can coexist in the relatively restricted habitat surface of the crayfish body. Variations in jaw-plate morphology, dietary resources, and preferred locations on the crayfish body surface by each species may facilitate the coexistence of multiple species. This relationship between Japanese crayfish and branchiobdellidans is highly intriguing from a community ecology perspective and holds potential as a model system for future research.

While the ecological significance of branchiobdellidans, this study highlights their risk of extinction. It is not difficult to imagine that, beyond branchiobdellidans, other unique and scientifically valuable organisms are also disappearing unnoticed. It is crucial to turn attention to the wide range of small, often overlooked organisms before they are lost forever.

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