

Research Article

Population genetics of the non-native freshwater shrimp *Palaemon sinensis* (Sollaud, 1911) in Japan based on mitochondrial 16S rRNA sequence analysis

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Abstract

The palaemonid shrimp *Palaemon sinensis*, a species native to China, was first discovered as an established non-native species in Japan in 2005, and it has since been reported in 25 of 47 prefectures. To genetically elucidate that *P. sinensis* was introduced to Japan from China via importation as a live fishing bait species, this study analyzed partial nucleotide sequences of the mitochondrial 16S rRNA gene of 165 specimens collected in 7 geographical regions in Japan (Tohoku, Kanto, Chubu, Kinki, Chugoku, Shikoku and Kyushu), and from 18 imported specimens from China purchased at a fishing store. All sequences were classified into 11 haplotypes, which included one widespread haplotype shared by more than half of the *P. sinensis* samples. Almost all of the samples of imported specimens possessed the dominant haplotype. Analysis of molecular variance showed no genetic differentiation between the Japanese local and imported samples or among regions in Japan. The overall results suggest that *P. sinensis* was introduced through human-mediated dispersal; furthermore, the haplotype distributions of *P. sinensis* established in Japan reflected the intensity of the species' importation from central and northern China. This is the first study to document the genetic structure of non-native *P. sinensis* in Japan.

Key words: AMOVA, genetic structure, haplotype distribution, live fishing bait, native species, population structure

Introduction

Aquaculture and shipping are major vectors for the transportation of animal species beyond native distribution ranges worldwide (e.g., Campbell and Hewitt 2008). In Japan, imported live fishing baits are also considered as vectors of unintentional introductions of species (Saito et al. 2011, 2014). The freshwater palaemonid shrimp *Palaemon paucidens* De Haan, 1844 is a native species in Japan and has also been supplied under the trade name “Shirasa ebi” as a bait for blackhead seabream *Acanthopagrus schlegelii* (Bleeker, 1854) and Japanese rockfish *Sebastes inermis* Cuvier,

1829 (Niwa and Ohtaka 2006; Saito et al. 2011). “Shirasa ebi” was imported from Korea between 1969 and 2016, and from China between 1990 and 2016, and *P. paucidens* is collected in Japanese freshwaters, such as Lake Biwa (Niwa 2010; Saito 2017, 2018). *Palaemon sinensis* (Sollaud, 1911) is another freshwater palaemonid shrimp, naturally distributed in China, Myanmar, Siberia, and Sakhalin (Cai and Dai 1999; Cai and Ng 2002; Labay 2011). *Palaemon sinensis* was not reported in Japan before 1990 (Liu et al. 1990), but was discovered first in a pond in Shizuoka Prefecture in 2005 (Oonuki et al. 2010), and has since been reported from 25 of 47 prefectures (Oonuki et al. 2010; Imai and Oonuki 2014, 2017; Saito et al. 2016, 2017, 2019; Saito 2017, 2018; Chow et al. 2018a; Uchida et al. 2018; Imai et al. 2018, 2019, 2020). The two species closely resemble each other morphologically, and bait sellers have thus sold both species as “Shirasa ebi” without distinction (Saito 2018). Consequently, sport fishers could have unknowingly released the non-native *P. sinensis* into neighborhood freshwaters for future use.

In Japan, *P. sinensis* is now found in various lentic environments, such as ponds, lakes, agricultural waterways, streams and riverside pools (Saito et al. 2019). Since the lifecycle of *P. sinensis* is characterized by abbreviated larval development as an adaptation to freshwater life (Shen 1939), it is improbable, unlike the other diadromous shrimps, that this landlocked shrimp could expand its distribution via movements through ocean.

The importation routes of bait shrimp indicate that large amounts of *P. sinensis* originated from central China during fall to spring, and to some degree from northern China in the summer (Niwa 2010). Genetic studies based on mitochondrial DNA (mtDNA) can help to reveal not only the phylogeny and genetic structure of a non-native species, but also the origin of invasion (Miura 2007). In addition, genetic studies can provide important information for managing the spread and negative impact of an invasive species (Geller et al. 2010). However, little is known about the genetic and population structure of *P. sinensis* established in Japan. Hence, this study aimed to 1) reveal the genetic structure of *P. sinensis* populations in Japan by analyzing mitochondrial 16S rRNA sequences, and 2) elucidate the possible source regions and importation routes of *P. sinensis* to Japan by obtaining genetic information on Chinese specimens.

Materials and methods

Sampling

A detailed description of the *P. sinensis* samples used in this study is presented in Supplementary material Table S1. A total of 165 specimens of *P. sinensis* was collected using D-frame sweep nets, from 39 populations across 7 geographical regions in Japan: Tohoku, Kanto, Chubu, Kinki, Chugoku, Shikoku and Kyushu (Figure 1). The species identity of each specimen was verified based on the carapace color pattern (Imai and Oonuki

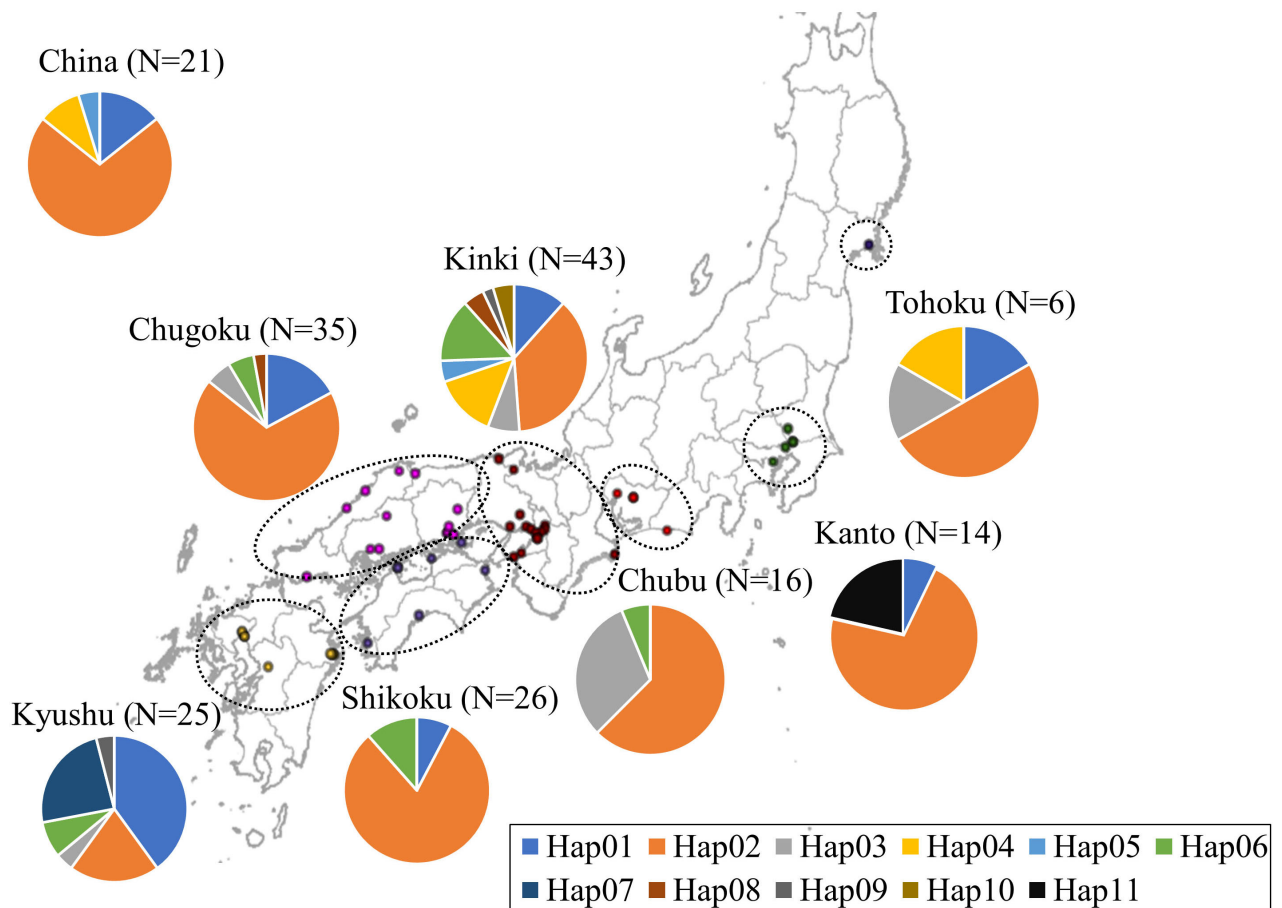


Figure 1. Sampling localities of *Palaemon sinensis*, representing 39 populations in seven regions in Japan, and distribution of the 11 detected haplotypes. Haplotype frequency for the Chinese samples (i.e., 18 specimens purchased at fishing stores in Japan, and three recorded in GenBank) is also presented.

2014). In addition, 18 specimens were purchased at a fishing store in Hiroshima Prefecture; these specimens were imported from China but the exact locality of their origin was not determined. All specimens were preserved in absolute ethanol immediately after sampling. Three DNA sequences of *P. sinensis* were referenced from GenBank: two of the sequences originated from Liaoning, China (accession number: MH880828) (Li et al. 2019), and the other from Jiangxi, China (accession number: DQ194970) (Liu et al. 2007).

DNA extraction and amplification

Genomic DNA was extracted from tissue samples using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) following the extraction protocols. The quality and concentration of the eluted DNA were evaluated using a spectrophotometer (NanoDrop 2000/200c, Thermo Fisher Scientific Inc., USA). The DNA fragments of mitochondrial 16S rRNA gene were amplified by polymerase chain reaction (PCR) using the primer pair 1471 (5'-CCTGTTTANCAAAAACAT-3') and 1472 (5'-AGATA GAAACCAACCTGG-3') (Crandall and Fitzpatrick 1996). The PCR mixture was set to a volume of 25 μ l, containing 12.5 μ l of 2 \times PCR buffer for KOD

FX Neo, 5.0 μ l of 2 mM dNTPs, 4.0 μ l of sterilized water, 0.5 μ l of 10 pmol/ μ l of each primer, 0.5 μ l of KOD FX Neo polymerase (Toyobo Co. Ltd., Japan), and 20 ng of template DNA. The cycling conditions consisted of initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, extension at 68 °C for 1 min, and a final extension at 68 °C for 1 min. Samples were then cooled and kept at 4 °C until use. PCR products were examined for yield and size by electrophoresis using 2.0% (w/v) agarose gel. Successful amplicons with an expected band size were purified using a FastGene Gel/PCR Extraction Kit (Nippon Genetics Co. Ltd., Japan) and sequenced at Eurofins Genomics (Tokyo, Japan).

Data analyses

Sequence alignments and trimming were performed with MEGA X software (Kumar et al. 2018), and haplotypes were calculated using DnaSP version 6 (Rozas et al. 2017). The degree of genetic variation was estimated by the estimates of haplotype diversity (H_d) and nucleotide diversity (N_d), and the number of segregating sites (S), for all investigated populations and regions, using DnaSP version 6. The genetic relationships among haplotypes was inferred by the molecular-variance parsimony technique, set at 95% probability, to reveal the number of mutations between haplotypes and treating each gap as a single evolutionary event, using PopART version 1.7 (Leigh and Bryant 2015). The partitioning of genetic diversity within and among populations was estimated by analysis of molecular variance (AMOVA), based on 1,023 permutations, implemented in Arlequin 3.5 (Excoffier and Lischer 2010). The AMOVA was accomplished by: (1) grouping all populations as a single group to determine the degree of genetic diversity among and within the entire populations; (2) grouping the Japanese populations according to regions (i.e., Tohoku, Kanto, Chubu, Kinki, Chugoku, Shikoku and Kyushu); and (3) dividing the *P. sinensis* samples collected in Japanese freshwater systems and those imported from China (sampled from fishing stores).

Results

Genetic structure

The length of mitochondrial 16S rRNA sequences determined in 186 individuals was 452 bp, and 11 distinct haplotypes were classified with 9 variable sites (Table 1). All specimens were identified as *P. sinensis* by BLAST searching. The sequences of the 11 haplotypes have been entered in GenBank (accession numbers: LC582784–LC582794). Hap02 was the dominant haplotype, appearing in all geographical regions, with a total membership of 104 sequences representing 55.9% of the entire data set (Figure 1; Table S2). It dominated in $\geq 50\%$ of the specimens in several

Table 1. Sites of sequence variation from 186 sequences of the mitochondrial 16S rRNA region, and positions of the sequence alignment, for the 11 detected haplotypes of *Palaemon sinensis*. Numbers in parentheses are the sample numbers for each haplotype.

Haplotype		Position								
		76	183	220	279	291	308	316	321	430
Hap01	(28)	C	T	A	A	A	C	A	A	C
Hap02	(104)	.	.	G	G
Hap03	(12)	.	.	G
Hap04	(9)	G	.
Hap05	(3)	.	C
Hap06	(14)	.	.	G	G	G
Hap07	(6)	.	.	G	G	.	.	G	.	.
Hap08	(3)	T	.	G	G
Hap09	(2)	T
Hap10	(2)	.	C	.	.	.	T	.	.	.
Hap11	(3)	G

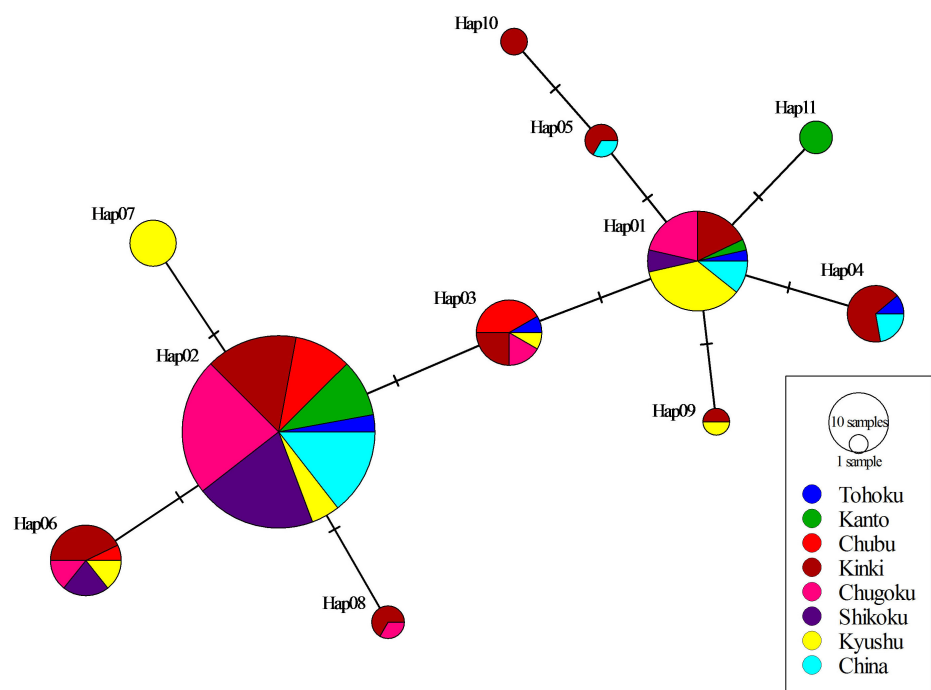


Figure 2. Haplotype network showing the genetic relationship among 11 mitochondrial 16S rRNA haplotypes of *Palaemon sinensis* collected in seven regions of Japan and those derived from specimens from China. The size of the circles is proportional to the haplotype frequency.

regions (Tohoku, Kanto, Chubu, Chugoku, Shikoku and China), but in < 50% of the specimens from Kinki and Kyushu. Hap01 contained 28 sequences representing 15.1% of the total data set, and it appeared in all regions, except for Chubu. Eight haplotypes were shared by at least two regions and at most 8 regions, representing 94.1%, and the remaining three haplotypes, representing 5.9% of the entire haplotype composition, were singletons. The haplotypes Hap07, Hap10 and Hap11 were singularly found in Kyushu, Kinki and Kanto, respectively. Hap07 was detected only in the Saiki population in Kyushu, and Hap10 only in the Shima population in Kinki (Figure 2; Table S2). The predominant haplotype, Hap02, was shared in 15 of the 18 specimens imported from China. Hap01 was detected from one sample from Jiangxi, China, and Hap04 from two samples from Liaoning, China.

Table 2. Results of the analysis of molecular variance (AMOVA) of *Palaemon sinensis* based on the mitochondrial 16S rRNA region.

Groupings	Source of variation	d.f.	Sum of squares	Variance components	Percentage variation	F - Statistics
All populations	Among populations	41	62.820	0.26421	41.25	$F_{ST} = 0.41253^*$
	Within populations	144	54.180	0.37625	58.75	
	Total	185	117.000	0.64046		
Japanese regions	Among groups	6	9.707	-0.01229	-1.90	$F_{CT} = -0.01898$
	Among populations within groups	32	47.379	0.27710	42.79	$F_{SC} = 0.41989^*$
	Within populations	126	48.236	0.38282	59.11	$F_{ST} = 0.40888^*$
	Total	164	105.321	0.64763		
Local vs imported fishing baits	Among groups	1	0.745	-0.12695	-24.43	$F_{CT} = -0.24435$
	Among populations within groups	38	57.085	0.26759	51.51	$F_{SC} = 0.41392^*$
	Within populations	143	54.180	0.37888	72.93	$F_{ST} = 0.27072^*$
	Total	182	117.000	0.51953		

* $P < 0.001$, derived from 1023 permutations.

The AMOVA indicated that the global F_{ST} value for “All populations” was significantly different from zero ($F_{ST} = 0.413$, $P < 0.001$), indicating a significant genetic structure among populations (Table 2). The AMOVA results also revealed significant genetic differentiation among populations within groups for “Japanese regions” ($F_{ST} = 0.420$, $P < 0.001$) and “Local vs imported fishing baits” ($F_{ST} = 0.414$, $P < 0.001$). Similarly, the values differed significantly within populations for “Japanese regions” ($F_{ST} = 0.409$, $P < 0.001$) and “Local vs imported fishing baits” ($F_{ST} = 0.271$, $P < 0.001$). However, there was no significant genetic differentiation among groups for “Japanese regions” ($F_{ST} = -0.019$, $P > 0.05$) and “Local vs imported fishing baits” ($F_{ST} = -0.244$, $P > 0.05$).

Genetic diversity and test of neutrality

Haplotype diversity (H_d) within the entire population ranged from 0.000 ± 0.000 to 1.000 ± 0.500 , and nucleotide diversity (N_d) from 0.0000 ± 0.0000 to 0.0066 ± 0.0077 , with the number of segregating sites (polymorphisms) ranging from 0 to 4 (Table 3). Nineteen populations (Ishinomaki, Kawasaki, Katsushika, Kobe, Yosano, Shima, Daito, Hannan, Higashiosaka, Osaka, Yao, Miyoshi, Hamada, Oda, Yamaguchi, Kawauchi, Okawa, Saiki, and Saga) recorded relatively higher H_d values (0.600 ± 0.175 to 1.000 ± 0.500) at the intra-population level, whereas the rest of the populations recorded relatively lower H_d values (0.000 ± 0.000 to 0.500 ± 0.265). Intra-regional diversities were relatively higher in Tohoku, Kinki, and Kyushu (0.763 ± 0.054 to 0.816 ± 0.043), whereas Kanto, Chubu, Chugoku, Shikoku, and China showed relatively lower diversities (0.342 ± 0.110 to 0.542 ± 0.099).

Discussion

This study detected 11 haplotypes of *P. sinensis* in Japan. Among these, Hap02 was predominant and the most widely distributed haplotype, shared by more than 100 individuals in the total dataset (Table 1). Furthermore, Hap02 was identified in 15 of the 18 imported specimens

Table 3. Genetic diversity and results of neutrality tests for populations of *Palaemon sinensis* sampled in seven regions of Japan and for imported specimens from China. The data summarize the sample size (N), number of haplotypes (h), estimated haplotype diversity and standard deviation ($H_d \pm SD$), estimated nucleotide diversity and standard deviation ($N_d \pm SD$), and number of segregation sites (S).

Region/Population	N	h	$H_d \pm SD$	$N_d \pm SD$	S
Tohoku*	6	4	0.800 ± 0.172	0.0032 ± 0.0026	3
Ishinomaki	6	4	0.800 ± 0.172	0.0032 ± 0.0026	3
Kanto*	14	3	0.473 ± 0.136	0.0027 ± 0.0021	3
Kashiwa	5	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Bando	3	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Kawasaki	3	2	0.667 ± 0.314	0.0015 ± 0.0018	1
Katsushika	3	2	0.667 ± 0.314	0.0044 ± 0.0042	3
Chubu*	16	3	0.542 ± 0.099	0.0013 ± 0.0012	2
Nagoya	5	2	0.400 ± 0.237	0.0009 ± 0.0011	1
Toyota	6	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Hamamatsu	5	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Kinki*	43	9	0.816 ± 0.043	0.0042 ± 0.0027	8
Ashiya	3	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Kobe	6	3	0.733 ± 0.155	0.0019 ± 0.0018	2
Toyooka	3	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Yosano	3	2	0.667 ± 0.314	0.0015 ± 0.0018	1
Shima	3	2	0.667 ± 0.314	0.0059 ± 0.0053	4
Daito	5	2	0.600 ± 0.175	0.0040 ± 0.0032	3
Hannan	2	2	1.000 ± 0.500	0.0066 ± 0.0077	3
Higashiosaka	2	2	1.000 ± 0.500	0.0044 ± 0.0054	2
Osaka	6	3	0.600 ± 0.215	0.0030 ± 0.0024	4
Sakai	4	2	0.500 ± 0.265	0.0044 ± 0.0037	4
Yao	3	2	0.667 ± 0.314	0.0015 ± 0.0018	1
Wakayama	3	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Chugoku*	35	5	0.508 ± 0.091	0.0018 ± 0.0015	5
Higashihiroshima	3	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Hiroshima	2	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Miyoshi	3	2	0.667 ± 0.314	0.0030 ± 0.0030	2
Okayama	14	2	0.143 ± 0.119	0.0006 ± 0.0008	2
Hamada	3	2	0.667 ± 0.314	0.0015 ± 0.0018	1
Matsue	1	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Oda	3	2	0.667 ± 0.314	0.0030 ± 0.0030	2
Yonago	3	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Yamaguchi	3	2	0.667 ± 0.314	0.0030 ± 0.0030	2
Shikoku*	26	3	0.342 ± 0.110	0.0011 ± 0.0011	3
Imabari	4	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Uwajima	3	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Mitoyo	4	2	0.500 ± 0.265	0.0011 ± 0.0014	1
Tonosyo	5	2	0.400 ± 0.237	0.0009 ± 0.0011	1
Tosa	5	2	0.400 ± 0.237	0.0009 ± 0.0011	1
Kawauchi	5	2	0.600 ± 0.175	0.0027 ± 0.0024	2
Kyushu*	25	6	0.763 ± 0.054	0.0036 ± 0.0025	5
Kumamoto	5	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Okawa	3	3	1.000 ± 0.272	0.0044 ± 0.0042	3
Saiki	10	4	0.644 ± 0.152	0.0029 ± 0.0022	4
Saga	7	3	0.714 ± 0.127	0.0025 ± 0.0021	2
China*	21	4	0.481 ± 0.121	0.0025 ± 0.0019	4
Imported	18	3	0.307 ± 0.131	0.0015 ± 0.0014	3
Liaoning	2	1	0.000 ± 0.000	0.0000 ± 0.0000	0
Jiangxi	1	1	0.000 ± 0.000	0.0000 ± 0.0000	0

* Region.

(Table S2), supporting the notion that *P. sinensis* was introduced to Japan from China, most probably via importation as a live fishing bait.

Niwa (2010) described the probable supply routes of *P. sinensis* as follows: 1) Collection sites in China have been Jiangsu, Zhejiang, Henan provinces in central China, and Liaoning, Hebei, and Jilin provinces in northern China. 2) Collection in Henan ended by 1998, and in Jilin by 2004, and in the other four provinces the shrimp was collected after 2005. 3) The supply routes varied depending on the season, with most bait shrimp imported from central China from autumn to spring, but with a small amount imported from northern China in summer. In our study, since all imported samples were obtained between November and May, the dominant haplotype Hap02 is presumed to have originated from Jiangsu or Zhejiang in central China.

The regional occurrence of Hap02 was $\geq 50\%$ in the samples in Tohoku, Kanto, Chubu, Chugoku and Shikoku, but was $< 50\%$ in Kinki and Kyushu (Figure 1; Table S2). These percentages correspond to the observed levels of genetic diversity (Table 3). Previous studies have suggested that multiple introductions of a species might have influenced within-population genetic diversity (e.g., Fatsi et al. 2020). The bait shrimp supply was usually limited to autumn to spring for rockfish in many regions, but there was also demand in summer in Kansai and Kyushu, where fishing for blackhead seabream is popular. Therefore, the high diversity of haplotypes in samples from Kinki and Kyushu may reflect the imported bait shrimp from northern China. In fact, Hap04 was mostly detected in the Kinki samples, and likewise in the sequences of individuals from Liaoning in northern China.

The GenBank sequences originated from Jiangxi and Liaoning provinces in China corresponded to Hap01 and Hap04, respectively (Table S2). Hap01, Hap02, and Hap05 were also detected from fishing bait samples imported from China. However, seven of the haplotypes confirmed in this study (Hap 03 and Hap06–Hap11) were found only in the field samples in Japan. Studies of crustacean species suggest that the mitochondrial 16S rRNA gene does not evolve faster than 1.0% per million years (Schubart et al. 1998, 2000; Groeneveld et al. 2007). *Palaemon sinensis* was not reported from Japan before 1990 (Liu et al. 1990), and only about 30 years have passed since its first importation into the country as a fishing bait (Niwa 2010). Therefore, it is presumed that these seven haplotypes did not differentiate independently in Japan but were introduced with the Chinese imports. Additional haplotype information on this species in China is expected to provide a more detailed estimation of the origin of non-native populations in Japan.

Although there was significant genetic variation among and within populations, our study did not detect genetic variation in *P. sinensis* among geographical regions in Japan (Table 2). In general, the genetic structure of native freshwater organisms is likely to be influenced by geographical separation, such as water systems (Keenan 1994). An earlier study of *P. paucidens* mitochondrial 16S rRNA suggested the existence of regional

haplotype differentiation in Japan (Chow et al. 2018b). Another study found ample genetic divergence between western and southern populations of *P. paucidens* in South Korea (Song et al. 2016). Conversely, an AMOVA between native and non-native clusters of Asian estuarine shrimp *Palaemon macrodactylus* Rathbun, 1902, a successful invader in American and European estuaries, indicated no significant genetic differentiation (Lejeune et al. 2014). Similarly, the *P. sinensis* collected in Japan and the imported specimens from China were not genetically differentiated (Table 2). These indicate that there has been insufficient time for regional populations of *P. sinensis* to genetically differentiate since introduction.

Importation of palaemonid shrimp species, including *P. sinensis* and *P. paucidens*, have been stopped in Japan since 2016, following a revision of the Fisheries Resource Protection Act (Saito 2018; Shichiri et al. 2020). However, *P. sinensis*, which has already become established in Japanese waterbodies, was still collected and sold as fishing bait (Saito 2018); therefore, secondary spread of this species in Japan owing to its sale as a live bait is worrisome. In contrast, it was reported that *P. paucidens* could be introduced to other Japanese freshwaters with the release of ayu *Plecoglossus altivelis* (Temminck & Schlegel, 1846) from Lake Biwa in western Japan (Takamura 2009; Chow et al. 2018a, b). Since the halt of bait shrimp imports, *P. paucidens* collected from Lake Biwa has become a substitute for *P. sinensis* as a bait sold in many fishing stores in Japan (Saito 2018). Fortunately, *P. sinensis* has not yet been found in Lake Biwa, but its invasion into the lake should be prevented, because it will lead to unintentional and artificial secondary spread and consequent habitat expansion of this non-native shrimp.

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Authors' Contribution

CO, HS – research conceptualization; CO, TI, HS – sample design and methodology; CO, TI, HS – investigation and data collection; CO, PSKF, SH, EKA, PAT – data analysis and interpretation; HS – funding provision; CO – original draft; CO, TI, PSKF, SH, EKA, PAT, HS – review and editing.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Details of the 186 samples of *Palaemon sinensis* used in this study.

Table S2. 16S rRNA haplotype frequency in *Palaemon sinensis* samples collected in Japan or imported from China.

This material is available as part of online article from:

http://www.reabic.net/aquaticinvasions/2021/Supplements/AI_2021_Ogasawara_etal_SupplementaryMaterial.xlsx