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Genetic population structure of the fiddler crab *Austruca lactea* (De Haan, 1835) based on mitochondrial DNA control region sequences

Takenobu Tokuyama, Jhy-Yun Shy, Hui-Chen Lin, Yasuhisa Henmi, Peter Mather, Jane Hughes, Makoto Tsuchiya, Hideyuki Imai

Abstract.— The fiddler crab, *Austruca lactea* inhabits tidal flat areas and is widely distributed across Japan, the Korean Peninsula, China, Taiwan and northern Vietnam. Fiddler crab habitat is being lost rapidly due to human impacts and this species is currently listed as endangered in Japan. We studied the population genetic structure of *A. lactea* using mitochondrial DNA control region markers to define management units. *A. lactea* individuals were sampled from Osaka, Hiroshima and Kumamoto in Japan, and from Taichung and the Penghu Islands in Taiwan. All local samples exhibited high levels of genetic diversity, and no sampled populations showed evidence for a significant decline in effective population size that can result from population bottleneck effects. Pairwise F_{ST} estimates distinguished three discrete *A. lactea* populations corresponding with, the Seto Inland Sea (Osaka and Hiroshima), Kyushu (Kumamoto) and Taiwan. The three populations showed clear differences in historical population expansion times and their population dynamics after expansion. Results of the study indicate that *A. lactea* dispersal is limited geographically and that high levels of genetic diversity are maintained both within and among populations.

***Key words**: genetic structure, gene flow, mitochondrial control region, fiddler crab*

■ Introduction

The fiddler crab, *Austruca lactea* (De Haan, 1835) has a natural distribution that includes Japan, Taiwan, the Korean Peninsula, China and northern Vietnam (Crane, 1975; Shih *et al*., 2009; Shih, 2013). In Japan, *A. lactea* is found in tidal flats on the islands of Honshu, Shikoku and Kyushu (Sakai, 1976; Yamaguchi, 1978). Natural habitat in estuarine tidal flats has been significantly reduced in recent times as a result of anthropogenic shore protection measures and reclamation work. As a consequence, local fiddler crab population sizes have been declining, and the species has been listed as endangered (II; Vulnerable) in the 'Threatened Wildlife of Japan, Red data book (Takeda, 2006). In the latter half of the 20th century, the rate of loss of tidal areas in Japan reached 40% (Hanawa, 2006), with Osaka experiencing the highest rate of loss (90%). This remarkable reduction in wild habitat potentially threatens persistence of fiddler crab populations. Clarifying *A. lactea* genetic population structure in Japan will assist with understanding and conserving this species over the longer term.

The magnitude of larval dispersal in invertebrates can impact their natural genetic structures significantly (McConaugha, 1992). Adults of most decapods do not disperse over large geographical distances, rather dispersal and gene flow are often determined by planktonic larval stages. As a consequence, their relative dispersal ability is strongly linked to the duration of each species' larval phase (Scheitema, 1975; Grantham *et al*., 2003). Larval stage du-

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ration in fiddler crabs has been hypothesised to last from three to four weeks (Hyman, 1920; Otani, 1993). In decapods where the length of larval duration has been confirmed experimentally, e.g., the swimming crab, *Portunus trituberculatus*, the first stage after hatching was observed to range from 14 (26°C) to 27 (20°C) days in the laboratory (Hamasaki, 1996). Dispersal distances of *P. trituberculatus* and the acorn barnacle *Balanus glandula*, another crustacean species with a similar larval phase duration, were estimated to be 47 km and 85 km, respectively (Shiota & Kitada, 1992; Schwindt, 2007). Based on these estimates, dispersal ability and genetic population structures of fiddler crab has been assumed to be similar to that of *P. trituberculatus* and *B. glandula*.

It is also important to recognise that planktonic larvae do not necessarily disperse over the entire larval period (Todd, 1998) so the extent of natural genetic differentiation among discrete populations of marine organisms can be significantly impacted by the relative philopatry of their larvae (Shanks, 2009). In particular, many estuarine organisms, including fiddler crabs, employ a larval retention strategy (Bilton *et al*., 2002). Wild capture of fiddler crab larvae (*A. lactea* and *Tubuca arcuata*) and simulation studies of their dispersal has demonstrated that larvae remain close to their local estuaries after hatching (Uno & Nakano, 2002). Based on these observations, it is quite possible that *A. lactea* could show significant population structuring even over fine spatial scales across the natural distribution.

In earlier studies of genetic population structure of *A. lactea* that employed a mtDNA control region marker (Kitamura *et al*., 2005) and in *P. trituberculatus* using RFLP analysis of the whole mtDNA molecule (Imai *et al*., 1999), micro-scale genetic heterogeneity was observed between local samples in Seto Inland Sea populations. In contrast, a study of *T. arcuata* using RFLP analysis of mtDNA control region showed no significant genetic differentiation among localities in mainland Japan and Taiwan apart from Okinawa-jima Island (Aoki *et al*., 2008a). In a study that examined mtDNA CO1 region sequence variation in the mudflat crab, *Chiromantes dehaani* and the horned ghost crab *Ocypode ceratophthalma*, both of which live in the supratidal zone, no significant population structure was evident between Seto Inland Sea and Kyushu Island populations (Kawane *et al.*, 2008). Thus, the extent of wild population structure can vary significantly among inter-tidal decapod species.

Imai & Numachi (2002) and Chu *et al*. (2003) showed that mitochondrial DNA control region of crustacea is useful for detecting and evaluating intraspecific diversity. Here we conducted a genetic analysis of the fiddler crab, *A. lactea*, after designing new primers to amplify a sequence of the mtDNA control region. The study aims to document natural population genetic structure in this species in Japan and Taiwan and to identify wild management units (MUs), where they are present. In addition, we estimated population dynamics following population expansion and clarified whether population bottlenecks may have occurred due to modern declines in available habitat due to human impacts.

■ Materials and Methods

Sampling and DNA extraction

Two hundred and fifty-nine specimens of *A. lactea* were collected from three localities in Japan and two localities in Taiwan (Table 1; Fig. 1). Sampling month and year for each sampled locality, respectively were as follows; Osaka in Sep. 2015, Hiroshima in Oct. 2015, Kumamoto in Dec. 2014, Taichung in Mar. 2014, Penghu in Nov. 2014. Captured adult individuals were preserved in 70% ethanol and stored at 4℃. Muscle tissue was later excised from either the abdomen or legs and samples stored in 0.5 mL of TNES-8M urea buffer (Asahida *et al*., 1996). DNA was extracted us-

Table 1. Sampling localities, numbers of samples and genetic diversity indices for the fiddler crab *Austruca lactea*. *n*: number of individuals. N_h: number of haplotypes. Number in parentheses mean number of unique haplotypes in each locality. *h*: haplotype diversity; π: nucleotide diversity.

Localities	*n*	N_h (unique)	$h \pm$ SD	$\pi \pm$ SD (%)
Sennan, Osaka, Japan	52	48 (45)	0.995 ± 0.006	1.46 ± 0.75
Matsunaga Bay, Hiroshima, Japan	53	50 (46)	0.998 ± 0.004	1.33 ± 0.68
Nagaura Island, Kumamoto, Japan	50	50 (49)	1.000 ± 0.004	1.69 ± 0.86
Taichung, Taiwan	54	54 (53)	1.000 ± 0.004	2.46 ± 1.23
Penghu Island, Taiwan	50	50 (49)	1.000 ± 0.004	2.71 ± 1.34
Total	259	247 (242)		

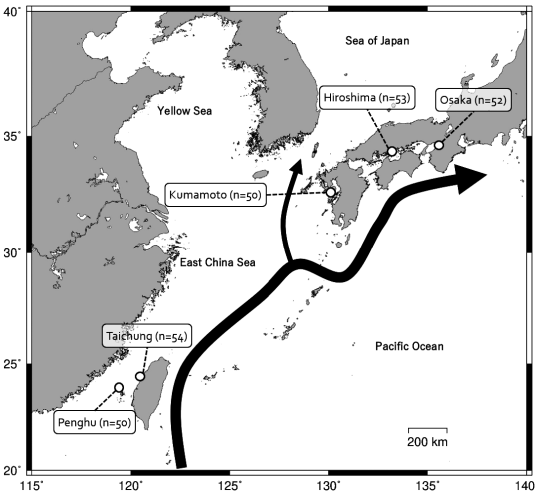

Fig. 1. Sampling sites and sample size (n) for *Austruca lactea.* The black arrow means the route of Kuroshio Current. This map data was designed on GMT (http://gmt.soest.hawaii.edu/projects/gmt).

ing an SDS-phenol-chloroform method with diethyl ether (Imai *et al*., 2004) and DNA samples were stored in Tris-EDTA buffer prior to genetic analysis.

Genetic marker design and PCR amplification

New conserved mtDNA control region primers were designed based on complete mtDNA sequences available for spiny lobster, *Panulirus japonicus* (AB071201; Yamauchi *et al*., 2002), and swimming crab, *P. trituberculatus* (AB093006; Yamauchi *et al*., 2003). The new primer sequences were as follows; Uca-12s-F1 (5′-TTAAGTTTAACCGCAGATGCT-3′) and Uca-tRNA-R1 (5′-ACCCTTTTAAATCAG GCACTAT-3′). PCR amplification was performed with 35 cycles in a 25-μL reaction mixture in an Astec PC-320 (Astec Co., Ltd., Tokyo, Japan) thermal cycler. The reaction mixture contained 12.5 μL of Emerald Amp PCR Master Mix (Takara Bio Inc., Otsu, Japan), 1.0 μL of template DNA, 1.0 μL of each primer, and 9.5 μL of distilled water. Amplification included an initial denaturation step at 94℃ for 2 minutes, followed by 35 cycles (denaturation at 94℃ for 30 seconds, annealing at 59℃ for 30 seconds, and extension at 72℃ for 1 minute), and a final extension at 72℃ for 3 minutes. Electrophoresis was carried out in a 1% Agarose S gel (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After electrophoresis, gels were stained with ethidium bromide and products checked on a transilluminator (Advance Scientific Products Pty Ltd., Queensland, Australia).

Sequencing and population analysis

All PCR products were purified and sequenced at Macrogen Japan Corp. (Kyoto, Japan) using an Applied Biosystems 3730xl DNA analyser (Foster City, CA). Nucleotide sequence data were aligned with MEGA 5.05 (Tamura *et al*., 2011) and unique haplotypes confirmed. A haplotype tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) based on the number of nucleotide substitutions per site. A bootstrap analysis was carried out with 1,000 replicates to determine the robustness of the tree (Felsenstein, 1985).

Evolutionary distances were calculated using a maximum composite likelihood approach (Tamura *et al*., 2004).

Analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) was undertaken in Arlequin ver. 3.5.2.1 (Excoffier & Lischer, 2010) based on the number of samples, the number of haplotypes and the number of nucleotide substitutions per site. Genetic parameters, including haplotype diversity (h; Nei, 1987) and nucleotide diversity (π; Tajima, 1983), were estimated. Statistical tests for neutrality, Tajima's D test (Tajima, 1983) and Fu's F_S test (Fu, 1997), were conducted. Effective population size N_{ef} was stimated based on the formulae $\theta = 2N_{ef}\nu$ (Watterson, 1975), $\nu = m\mu$ (m: the sequence length, μ: the mutation rate) and $\mu =$ 19% (per Myr) from a previous study of the penaeid shrimp, *Farfantepenaeus aztecus* (McMillen-Jackson & Bert, 2003).

Pairwise F_{ST} estimates (Reynolds *et al*., 1983) were calculated to estimate genetic differentiation among populations. Estimates of number of migrants between localities per generation (N_em) were calculated based on the formula $N_e m = [1/F_{ST} - 1]/4$ (Hudson *et al*., 1992). Population dynamics at each sampled locality were generated via mismatch distribution analysis based on pairwise differences between sequences. To estimate population expansions, time since population expansion τ and population size θ were calculated before and after expansion. Absolute time T (years) since population expansion was calculated based on the formula $T = \tau/2u$ and $u = 2\ \mu k$ (k: sequence length) (Rogers & Harpending, 1992; Harpending, 1994). A haplotype network was constructed using PopART (Leigh and Bryant, 2015) applying a median-joining method.

Results

Genetic diversity

After alignment and sequence trimming, 816 bp of nucleotide sequence from the mtDNA control region was used in all analyses. 281 variable sites were identified that defined 247 unique haplotypes. Nucleotide sequences of all haplotypes were deposited in the DNA Databank of Japan (DDBJ) under accession numbers LC472550-LC472808. Five haplotypes were shared among multiple locations, while all other haplotypes were unique to individual localities (Table 1). Of the five shared haplotypes, one was found in Penghu and Taichung, one in Kumamoto and Hiroshima, and the others in Osaka and Hiroshima. In the haplotype tree, no population formed an isolated clade (Fig. 2). Results of the haplotype network show that all populations were represented widely across the network. Certain haplotypes identified in the Penghu Islands were more divergent from remaining haplotypes than the average (Fig. 3). Haplotype diversity (h) and nucleotide diversity (π) for each population are presented in Table 1. Haplotype diversity indices were very high at all localities. Nucleotide diversity indices for the Taichung and Penghu sites were higher than for other localities. Results of Tajima's D test and Fu's F_S test were both negative at all sites and P-values for Fu's

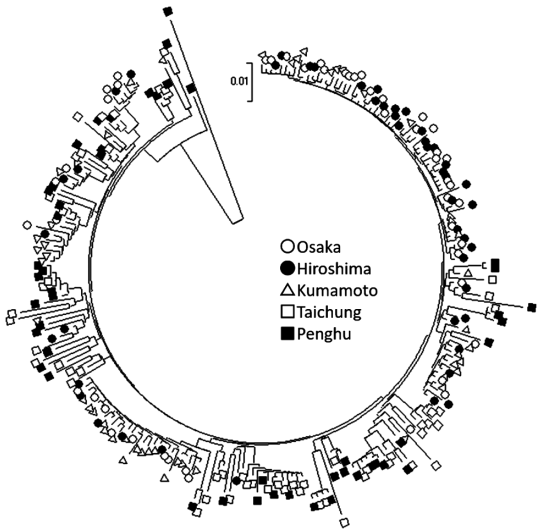

Fig. 2. *A. lactea* haplotype tree constructed using the neighbour-joining method.

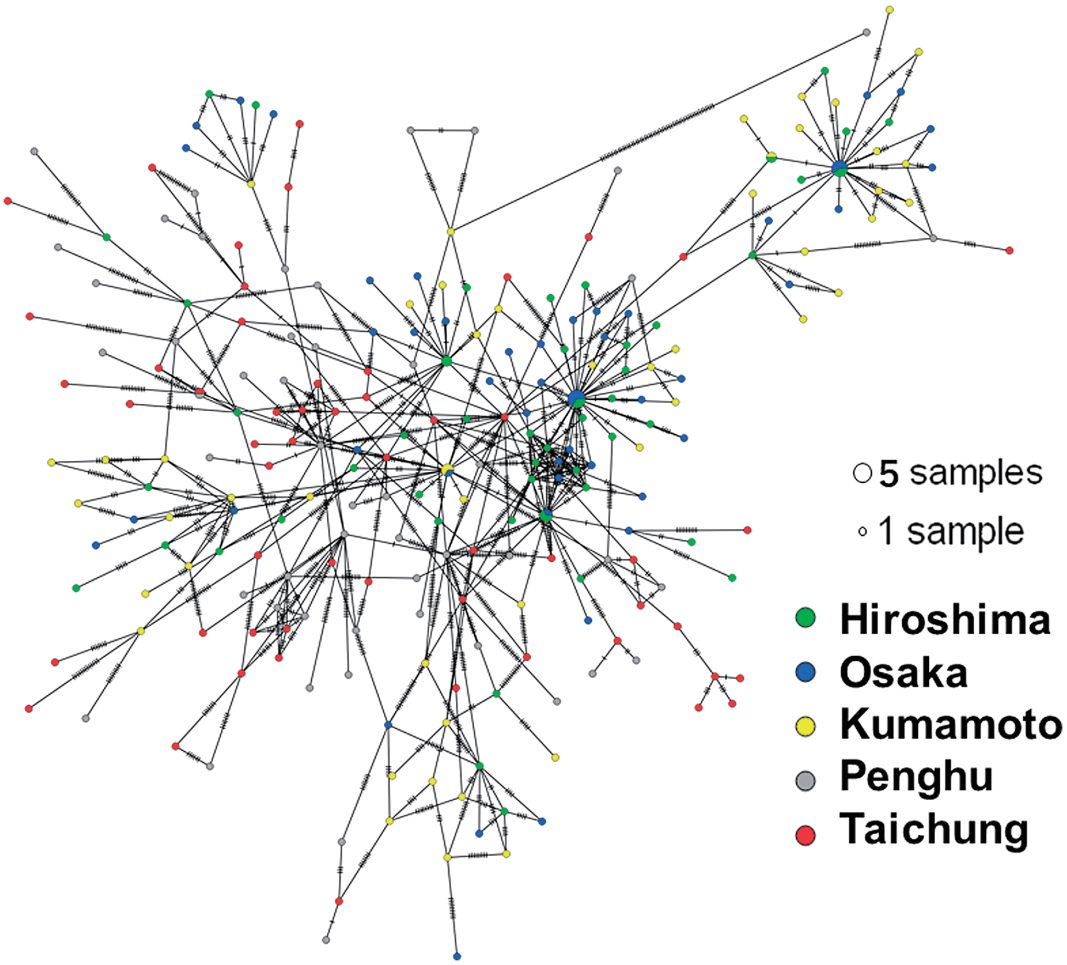

Fig. 3. *A. lactea* haplotype network generated using a median-joining method with PopART software. Sizes of circles are proportional to the frequency of each unique haplotype, while lengths of lines indicate the number of mutations between haplotypes.

Table 2. Effective female population size (N_{ef}) and neutrality test values for the localities of *Austruca lactea* sampling.

Localities	N_{ef}	Tajima's *D*	Tajima's *D* *p*-value	*Fs*	*Fs* *p*-value
Osaka	62803.6	−1.670	0.022	−24.462	0
Hiroshima	60405.5	−1.744	0.016	−24.559	0
Kumamoto	70558.8	−1.567	0.022	−24.353	0
Taichung	115356.8	−1.827	0.010	−24.148	0
Penghu	130317.8	−1.862	0.009	−24.142	0

F_S test were zero at all locations (Table 2). Effective population size estimates, N_{ef}, are shown in Table 2. Effective population sizes at the Taichung and Penghu localities were slightly larger than at the other three localities.

Genetic population structure

Mean F_{ST} estimate among localities obtained from the AMOVA was 0.06298, suggesting presence of integrated population structure ($P<0.05$). Pairwise F_{ST} estimation between Osaka and Hiroshima and between Taichung

and Penghu were negative indicating essential genetic homogeneity (Table 3). Other comparisons of pairs of localities showed positive F_{ST} values and all were significant ($P<0.05$). The P-value for the F_{ST} estimate between the Osaka and Hiroshima localities was the largest detected (0.93694 ± 0.0310). Mismatch distribution plots for the Taichung and Penghu localities showed a single peak while plots for the Osaka and Hiroshima localities showed two peaks (Fig. 4). Osaka and Hiroshima samples will hereafter be referred to as the Seto Inland Sea population, the Kumamoto samples as western Kyushu population, and the Taichung and Pen-

Table 3. Pairwise F_{ST} (below the diagonal) and pairwise F_{ST} P values (above the diagonal) for between-localities of *Austruca lactea*.

Localities	Osaka	Hiroshima	Kumamoto	Taichung	Penghu
Osaka		0.9369 ± 0.0310	0.0090 ± 0.0091*	0.0000 ± 0.0000*	0.0000 ± 0.0000*
Hiroshima	−0.0081		0.0000 ± 0.0000*	0.0000 ± 0.0000*	0.0000 ± 0.0000*
Kumamoto	0.0351	0.0428		0.0000 ± 0.0000*	0.0000 ± 0.0000*
Taichung	0.1021	0.1030	0.0645		0.5135 ± 0.0360
Penghu	0.0998	0.1027	0.0572	−0.0001	

*: Significant P-values (Bonferroni correction, $P<0.05$). The error values are shown ± standard deviation.

Table 4. *Nem* values (below the diagonal) between localities of *Austruca lactea* and direct geographical distances (km) between localities measured using Google Maps (https://maps.google.co.jp) (above the diagonal). If the F_{ST} value between localities was negative, *Nem* was not calculated, and is showed with a hyphen.

Localities	Osaka	Hiroshima	Kumamoto	Taichung	Penghu
Osaka		180	490	1820	1930
Hiroshima	—		340	1680	1790
Kumamoto	6.87	5.59		1350	1450
Taichung	2.20	2.18	3.63		110
Penghu	2.26	2.18	4.12	—	

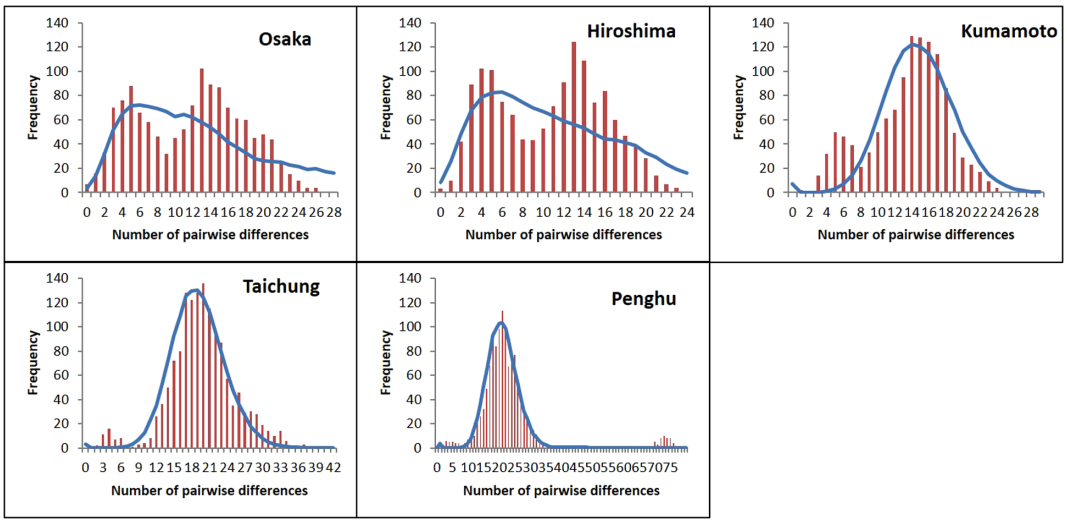

Fig. 4. Mismatch distribution based on *A. lactea* mtDNA control region. Bars represent observed frequency of pairwise differences among unique haplotypes, and the line shows the expected curve predicted for a population that has undergone range expansion in the past.

Table 5. Results of the mismatch distribution analysis for *Austruca lactea*. The absolute time after expansion (T) was calculated based on τ.

Localities	τ	T	θ_0	θ_1	*SSD*
Osaka	3.394	5472.8	13.31	8232.58	0.0084
Hiroshima	2.879	4642.4	11.02	9882.37	0.0104
Kumamoto	15.046	24261.5	0.05	188.62	0.0062*
Taichung	18.757	30245.4	1.01	527.63	0.0015
Penghu	18.617	30019.7	1.95	420.40	0.0013

τ: Time since expansion. T: absolute time (years) since expansion. θ_0: population size before expansion. θ_1: population size after expansion. SSD: sum of square deviations from the sudden expansion model.
* Means significant value ($P<0.05$).

ghu samples as the Taiwanese population, respectively. τ values decreased in the order Taiwan＞western Kyushu＞Seto Inland Sea (Table 5). θ_1 values were higher than the θ_0 values at all localities.

■ Discussion

Significant differentiation between localities

Results of genetic diversity estimates, pairwise F_{ST} estimates and mismatch distribution analysis in combination indicate essential genetic homogeneity between the two Japanese (Osaka–Hiroshima) and between the two Taiwanese (Taichung–Penghu) localities. Aside from these pairs, all other comparisons between localities indicate significant population differentiation. Thus, across the geographical scale of sampling in this study, *A. lactea* can be categorised into three 'populations' that correspond geographically with a Seto Inland Sea population, a western Kyushu population and a Taiwanese population. At the same time in the phylogenetic analysis, the three 'populations' formed a single monophyletic clade (Fig. 2 & Fig. 3), so accordingly, they can best be defined as MUs for short-term conservation purposes (Moritz, 1994).

The pattern of population structuring observed here for *A. lactea* here however, does not conform with patterns reported in earlier phylogeographic studies of a number of semi-terrestrial crabs, *T. arcuata*, *O. ceratophthalma* and *C. dehaani* (Aoki *et al*., 2008b; Kawane *et al*., 2008) across the same region. *T. arcuata* showed essential genetic homogeneity across the largest geographical scale sampled i.e. between Japan and Taiwan, and very little genetic differentiation between the Seto Inland Sea and western Kyushu regions was observed in the latter two species. While these comparisons may appear to provide conflicting patterns, direct comparisons among these species may not be simple. Aoki *et al.* (2008b) used only an RFLP approach which targets only a limited number of mutational sites, while Kawane *et al*. (2008) employed sequencing of a large fragment of the whole mitochondrial DNA COI region. In the current study we also sequenced a large fragment of the complete mitochondrial control region and so were able to examine the extent of genetic differentiation among sites in greater detail.

In the current study, the western Kyushu population was genetically differentiated from the other two Japanese populations sampled. An earlier study of *T. arcuata* by Aoki *et al.* (2008b) also reported genetic differentiation between the Seto Inland Sea and Kyushu regions for this species even though the two regions are geographically close. This pattern potentially results from historical geological conformation of the coastline of western Kyushu. Complex coastal geological structure may have restricted gene flow between *T. arcuata* populations in the two regions.

The Kuroshio Current flows from the east side of Taiwan along the southern coast of Kyushu to the southern coast of Shikoku Island. In addition, a component of the Kuroshio Current flows through the East China Sea facing western Kyushu (Fig. 1). While the current is sufficiently powerful to potentially impact larval gene flow in some marine organisms (Kawabe, 1986; Sugimoto & Kobayashi, 1988; Tomita *et al*., 2016; Abdullah *et al*., 2017; Hamasaki *et al*., 2017), it has also been demonstrated to act as a potential genetic barrier to dispersal in other marine species (Aoki *et al*., 2008a; Iwamoto *et al*., 2012; Imai *et al*., 2013; Yamakawa & Imai, 2014; Iwamoto *et al*., 2015). Results here at least suggest that the Kuroshio Current potentially limits larval dispersal by fiddler crabs between the Seto Inland Sea and Kyushu regions. An earlier experimental study showed that planktonic larvae of the fiddler crab have a relatively short time to achieve pelagic larval dispersal (Otani, 1993). After this period, zoea larvae cannot metamorphose to the megalopa stage and will die. Because of this time limit and the apparent larval retention strategy (Bilton *et al*., 2002), fiddler crabs are unlikely to use the Kuroshio Current effectively to disperse between the Seto Inland Sea and Kyushu regions.

Population dynamics and expansion times

All sampled localities showed significant negative values in Tajima's D and Fu's F_S tests ($P<0.05$) (Table 2). For the Mismatch distribution analysis, all localities also showed relatively low SDD values and a fit to the sudden expansion model (Table 5). These, data suggest that all modern populations are expanding.

Heterogeneous mismatch distributions were observed among three 'populations' (the Seto Inland Sea, Kyushu and Taiwan) (Fig. 4), indicating that differences between Japan and Taiwan were substantial. The mismatch distribution analysis also provided estimated population expansion times for the five sampled localities. Reconstructions of sea level changes across the late Pleistocene, approximately 20,000 years ago suggest that the islands of Honshu, Shikoku and Kyushu were connected physically and at this time when sea levels were much lower than at present, the Seto Inland Sea did not exist (Maeda, 1976, 1980). Approximately 10,000 years ago after sea levels had risen, seawater began to flow into the area of the modern Seto Inland Sea from the Pacific Ocean and sea levels reached their present status approximately 6,000 to 7,000 years ago. Molecular results in the current study do not conflict with this inferred geological history of the region with T-values for the Osaka 'population' suggesting that this population expanded earlier (estimated at 1,000 years before) before the Hiroshima 'population'. Based on this timing hypothesis, we can infer that *A. lactea* likely colonised and expanded from the south to form populations after the Seto Inland Sea level had stabilised. The mismatch distribution analysis results for the Osaka and Hiroshima population showed two peaks (Fig. 4). A number of hypotheses were considered for this pattern. The first hypothesis is that *A. lactea* may have invaded the Seto Inland Sea two times. Another is that historical population drastically declined in size at certain times in the past. We can evaluate and compare these competing hypotheses with the dynamics of *A. lactea* from eastern Kyushu and Shikoku Island.

Taiwan and Penghu Island are believed to have been connected physically and located on the edge of the Eurasian Continent during the late Pleistocene (estimated at 90,000 to 10,000 years ago) (Ujiie, 1990; Oshiro & Nohara, 2000). Considering the reported geological history for this region and the T-values estimated in this study, the coastal environment (i.e. sea levels and/ seawater temperatures) in the Taiwan region probably stabilised approximately 30,000 years ago, a time during which *A. lactea* populations likely expanded. Following

historical population expansions, sea surface elevation across the Holocene separated Penghu from Taiwan approximately 4,700 years ago (Chen & Liu, 1996). The mismatch distribution analysis results suggest however, that *A. lactea* populations did not decline significantly at this time (Fig. 4). They indicate that *A. lactea* gene flow was ongoing between Taiwan and Penghu after insularisation of the Penghu Islands, so this event probably did not significantly affect *A. lactea* population dynamics.

Conclusions

A. lactea populations in Japan and Taiwan are genetically differentiated into three discrete population groupings as follows; Seto Inland Sea, western Kyushu and Taiwan populations. Results of the current study, suggest that modern *A. lactea* populations do not utilise the Kuroshio Current for effective larval dispersal. This in-shore marine crab species has a relatively low larval dispersal capacity resulting from possessing a relatively short pelagic larval phase. Results here confirm a need to establish MUs for each of the three identified differentiated modern population areas and to develop conservation strategies for each of these populations.

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Addresses

(TT and HI) Laboratory of Marine Biology and Coral Reef Studies, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa

903–0213, Japan
(JYS) Department of Aquaculture, College of Marine Resources and Engineering, National Penghu University of Science and Technology, Magong, Penghu 880, Taiwan
(HCL) Department of Life Science, College of Science, Tunghai University, Taiwan Boulevard, Taichung 40704, Taiwan
(YH) Aitsu Marine Station, Center for Water Cycle, Marine Environment, and Disaster Management, Kumamoto University, Chuo-ku, Kumamoto City, Kumamoto 860–8555, Japan
(PM and JH) Australian Rivers Institute, Griffith University, Nathan, Brisbane, Queensland 4111, Australia
(MT) International Institute of Okinawan studies, University of the Ryukyus, Nishihara, Okinawa 903–0213, Japan
(TT) Present address: Kansai Division, World Intec Co., Ltd., 5F Dai-san Bldg., Osakaekimae, 1–1–3–500 Umeda, Kita-ku, Osaka 530–0001, Japan

E-mail address of corresponding author
(HI) imai@sci.u-ryukyu.ac.jp