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

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**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% (Hana-wa, 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-
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 differentia-

tion 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°C. 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-
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’-ACCCCTTTAAATCAG 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°C for 2 minutes, followed by 35 cycles (denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 1 minute), and a final extension at 72°C 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).

**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* ± SD | *π* ± 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)   |          |             |
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 \((\pi;\) 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 estimated based on the formulae \(\theta = 2N_{ef}\nu\) (Watterson, 1975), \(\nu = m\mu\) (\(m\): the sequence length, \(\mu\): 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 \(N_{em} = [1/F_{ST} - 1]/4\) (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 \(\tau\) and population size \(\theta\) 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 \((\pi)\) 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.](image-url)
$F_s$ 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 FST values and all were significant (P < 0.05). The P-value for the FST estimate between the Osaka and Hiroshima localities was the largest detected (0.9369 ± 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-

| Localities | Osaka | Hiroshima | Kumamoto | Taichung | Penghu |
|------------|-------|-----------|----------|----------|--------|
| Osaka      | 0.9369 ± 0.0310 | 0.0090 ± 0.0091* | 0.0000 ± 0.0000* | 0.0000 ± 0.0000* | 0.0000 ± 0.0000* |
| Hiroshima  | -0.0081 | 0.0000 ± 0.0000* | 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* | 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.

| Localities | Osaka | Hiroshima | Kumamoto | Taichung | Penghu |
|------------|-------|-----------|----------|----------|--------|
| Osaka      | 180   | 490       | 1820     | 1930     | 1930   |
| Hiroshima  | —     | 340       | 1680     | 1790     | 1790   |
| Kumamoto   | 6.87  | 5.59      | 1350     | 1450     | 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.
ghu samples as the Taiwanese population, respectively. \( \tau \) values decreased in the order Taiwan > western Kyushu > Seto Inland Sea (Table 5). \( \theta_1 \) values were higher than the \( \theta_0 \) 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 semiterrestrial 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.

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

| Localities | \( \tau \) | T    | \( \theta_0 \) | \( \theta_1 \) | 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|

\( \tau \): Time since expansion. T: absolute time (years) since expansion. \( \theta_0 \): population size before expansion. \( \theta_1 \): population size after expansion. SSD: sum of square deviations from the sudden expansion model.

* Means significant value (\( P<0.05 \)).
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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