Comparative phylogeography to test for predictions of marine larval dispersal in three amphidromous shrimps

Junta Fujita1,4,*, Kei Zenimoto2, Akira Iguchi3, Yoshiaki Kai1, Masahiro Ueno1, Yoh Yamashita1

1Maizuru Fisheries Research Station, Field Science Education and Research Center, Kyoto University, Nagahama, Maizuru, Kyoto 625-0086, Japan
2Atmosphere and Ocean Research Institute/Graduate School of Frontier Sciences, University of Tokyo, 5-1-5, Kashiwanoha, Kashiwa, Chiba 277-8564, Japan
3Department of Bioresources Engineering, National Institute of Technology, Okinawa College, 905, Henoko, Nago, Okinawa 905-2192, Japan
4Present address: Kyoto Prefectural Ayabe High School, 18, Osada, Okacho, Ayabe, Kyoto 623-0042, Japan

ABSTRACT: The ecology of the freshwater life stages of the amphidromous shrimps Caridina leucosticta, C. typus and C. multidentata from southwestern Japan has been well studied. However, the ecology of their marine larval stages remains unclear. Therefore, to compare their genetic population structures and to predict their marine larval dispersal patterns, we collected 504 individuals of these 3 shrimps from throughout their distribution range in Japan and sequenced the mitochondrial DNA cytochrome c oxidase subunit I gene and control region. Comparative phylogeography showed that C. leucosticta had a genetically heterogeneous population structure, suggesting that the zoeal larvae remain near the river mouth, whereas C. typus and C. multidentata displayed homogeneous population structures, indicating high levels of larval dispersal. Consideration of these results alongside ecological data suggests a paradigm for amphidromous shrimps, whereby species with shorter larval stages require lower salinities for larval development and so cannot move with the ocean current, giving a narrower geographic distribution, whereas species with longer larval stages show the opposite pattern. We also detected species-specific demographic processes and local-scale population structures in relation to seascape features, which may have been shaped by the ecological features of each Caridina shrimp species.

KEY WORDS: Molecular ecology · Comparative phylogeography · Amphidromous shrimps · Caridina · Pelagic larval duration · Kuroshio Current

INTRODUCTION

Population connectivity or the exchange of individuals between populations is a central concept for conservation, ecological, and evolutionary studies in the marine environment (e.g. van der Meer et al. 2015). For most coastal marine species, the larval phase is the dominant dispersal stage; therefore, this stage and the processes that influence it have received considerable attention when considering population connectivity in marine systems (Cowen & Sponaugle 2009). However, the small size of the pelagic larvae combined with the vast and complex fluid environment they occupy hamper the tracking of their behavior and movements. Molecular ecology uses genetic markers that are not affected by natural selection to help solve some of our most interesting ecological questions (Beebee & Rowe 2008), includ-
Shrimps belonging to the family Atyidae exhibit high diversity in suborder Caridea and are abundant in nearly all tropical and most temperate regions of the world (De Grave et al. 2008). Most atyids have an amphidromous life history (Fig. 1), whereby the adults live and reproduce in freshwater environments (Shokita 1975), but high salinities are required for the successful development of their larvae (Hayashi & Hamano 1984, Nakahara et al. 2005). Thus, the zoeae hatch as a planktonic stage and passively drift to the ocean (Ideguchi et al. 2000, 2007). After metamorphosis and recruitment to river mouths, juveniles migrate upstream to freshwater habitats after sunset (Hamano & Hayashi 1992). The amphidromous atyid shrimps have a high fecundity and relatively small eggs at maturity (Shokita 1981), indicating an ‘r-selection’ life-history strategy, which should facilitate oceanic (among-river) dispersal over a range of spatial scales (Chubb et al. 1998, Cook et al. 2006, 2008, Crandall et al. 2010, Bauer 2013) and colonization of new suitable habitats (McDowall 2007).

Ecological information about these marine life stages is extremely limited, due to the larvae being tiny, vulnerable, and difficult to study in the wild (Ideguchi et al. 2000, Bauer 2013, Yatsuya et al. 2013). However, one such study in an estuary found that the first stage zoeae dispersed rapidly to the ocean surface, following which the developing larvae switched to become bottom dwellers (Ideguchi et al. 2000). It has been estimated that these shrimps spend 2 wk to 2 mo in the larval stages under optimal rearing conditions (Hayashi & Hamano 1984, Nakahara et al. 2005); however, the pelagic larval duration may be shorter in the wild. Thus, the extent to which zoeal larvae can disperse between rivers via the ocean environment is a longstanding question in crustacean biology (Bauer 2013, Yatsuya et al. 2013) (Fig. 1).

Two methods are available for estimating dispersal: direct field observations (i.e. the mark-recapture method) and indirect genetic approaches (i.e. statistics derived from the genetic variation of populations) (Favre et al. 1997, Bohonak 1999). The mark-recapture method has been successfully used in population ecology (Pradel 1996) but is notoriously difficult to apply, especially for crustacean species, which molt at regular intervals and therefore lose external tags. Furthermore, it is difficult to interpret mark-recapture studies due to the frequent detection of dispersal that has no evolutionary significance (‘abortive migration’) (Slatkin 1985). Genetic methods offer an alternative approach for assessing the larval dispersal of decapod crustaceans in natural situations (Slatkin 1987, Bohonak & Jenkins 2003, Hughes 2007), based on the assumption that if dispersal is high, the genetic composition of contiguous populations will be homogeneous (i.e. with shared alleles at similar frequencies), whereas if dispersal is limited, populations will genetically diverged via random genetic drift, leading to different allele frequencies (Slatkin 1985).

In the present study, we investigated the amphidromous atyid shrimps Caridina leucosticta, C. typus and C. multidentata, which are common in the southwestern Japan and have previously been intensively studied, particularly their freshwater life stages (e.g. Shokita 1979, Hamano & Hayashi 1992, Ideguchi et al. 2007, Yatsuya et al. 2013). Shokita (1979) compared ecological data of prawns belonging to the family Palaemonidae, which have the same amphidromous life history as shrimps belonging to the family Atyidae. He connected 4 ecological parameters: (1) the number of larval stages, (2) the salinity requirements for larval development, (3) larval dispersal, and (4) the distribution range of each species. He suggested that the former 2 parameters lead to large or small larval dispersal abilities via the ocean environment (Fig. 2), arguing that species with shorter larval stages and requiring lower salinities for larval development exhibit restricted larval dispersal, resulting in narrower geographic distributions (as shown in the linkages between parameters 1 to 4 in Fig. 2), and vice versa. The ecologies of C. leucosticta and C. typus support this hypothesis: C. leucosticta has fewer zoeal instars (7 stages), an optimum salini-
ity of 50% seawater (17.0) for the first zoeal stages (Shokita 1981, Nakahara et al. 2005, 2007), and a distribution that is restricted to the Japanese archipelago (Hayashi 2007), while *C. typus* has more zoeal instars (9 stages), an optimum salinity of 75% seawater (25.5) for the first zoeal stages (Nakahara et al. 2005, 2007), and is distributed across the Indo-West Pacific region (Hayashi 2007). However, the actual dispersal capabilities of these species remain to be evaluated because it is very difficult to trace zoeal larvae in the wild and compare marine dispersals of each species (Yatsuya et al. 2013).

*C. multidentata* has a characteristic ecology in the freshwater adult stage and is morphologically adapted to live in fast-flowing water (wide and short rostrum shape, body and leg robustness) and to actively walk and climb a vertical wall in the juvenile stage. It is found in fast-flowing habitats in uppermost streams under dense forest and lives beneath rocks under waterfalls and in rapids, whilst other *Caridina* species are adapted to riverside weed beds with slow velocity, usually displaying a more gracile habitus (Hamano & Hayashi 1992). Interestingly, a reported optimum salinity of 50% seawater for *C. multidentata* to survive the first zoeal stages (Hayashi & Hamano 1984) is not in accordance with a larger number of zoeal instars (9 stages) (Hayashi & Hamano 1984) and an extensive distribution across the Indo-West Pacific region that is similar to that of *C. typus* (Hayashi 2007).

Therefore, the main goal of our study was to use phylogeographic analyses with molecular markers to predict marine larval dispersals, and test the Shokita’s (1979) hypothesis (Fig. 2). We used a multi-species comparative approach, which enables broader conclusions to be drawn than species-specific case studies, which cannot compare the relative importance of dispersal (Dawson et al. 2002, Page & Hughes 2007, Hickey et al. 2009, Steele et al. 2009).

**MATERIALS AND METHODS**

**Sampling and DNA sequencing**

In total, 504 specimens across all Japanese populations of the 3 *Caridina* species (*C. leucosticta*, *n* = 244; *C. typus*, *n* = 140; *C. multidentata*, *n* = 120) were collected from 12 sites, 9 on the Japanese mainland and 3 on Nansei Islands (see Fig. 3 for locations, Table 1 for site names and the abbreviations used in the text). Samples were collected using a dip net and preserved in 99.5% ethanol prior to DNA extraction. *C. grandirostris* (*n* = 9) was also investigated to increase the robustness of the phylogenetic data for *C. leucosticta*, due to the extremely similar morphology and

---

**Fig. 2.** Hypothesized ecological relationships for amphidromous atyid shrimps. Species with shorter larval stages require lower salinities for larval development and exhibit restricted larval dispersal, resulting in narrower geographic distributions (upper panels), whereas species with longer larval stages exhibit the opposite pattern (lower panels).
close relationships between these species. The subsets of tissue samples from the mainland populations of *C. leucosticta* (all sites except ICT) were the same as were used in a previous study (Fujita et al. 2011), while the other specimens of *C. leucosticta* (at sites ICT, KWU, YON, and OMJ) and all samples of *C. typus*, *C. multidentata*, and *C. grandirostris* were newly collected for this study (Table 1).

Total genomic DNA was extracted from the abdominal muscle tissue using the DNeasy® Tissue Kit (Qiagen) according to the manufacturer’s protocol. We chose to use the mitochondrial DNA (mtDNA) cytochrome c oxidase subunit I (COI) gene for comparison because its rates of molecular evolution are reasonably well constrained (Lefèbure et al. 2006). The mtDNA COI region was amplified via polymerase chain reaction (PCR) with Ex Taq polymerase (Takara) using newly designed forward primers for *C. leucosticta* and *C. grandirostris* (COIF-leugra: 5’-GGA ATA GTA GGW ACA GCY YTA AGT C-3’), *C. typus* (COIF-typus: 5’-GGA ATA GTT GGC ACT GCT CTC AGA C-3’), and *C. multidentata* (COIF-mul: 5’-GGA ATA GTA GGT ACA GCC CTC AGA C-3’), and a previously designed reverse primer for Caridea (COIR-Caridea; Fujita et al. 2012). The 3 species-specific forward primers were designed as the internal primer to the universal forward primer ‘LCO1490’ (Folmer et al. 1994). In addition, the region extending from 12S rRNA to the control region (CR) was also investigated in 86 *C. leucosticta* specimens (OYB: n = 20; ISZ: n = 20; GN: n = 20; NYD: n = 20; and KWU: n = 6) to assess the robustness of the tree topology using newly designed PCR primers CRF-leu (5’-TAA AGA TCA ACT TAA GAA CAA GCG TGC-3’) and CRR-leu: (5’-ACT ATA TCA TGC GAT TTC ACG AAT ACT C-3’) with the primer walking from both the 12S rRNA side and the NADH dehydrogenase subunit 2 (ND2) side. The PCR and sequencing were carried out using the same protocols as previously reported (Fujita et al. 2012). The same primers were used for the sequencing reactions as for the PCR amplifications. The sequences were aligned using the program BioEdit v7.2.5 (Hall 1999) and deposited in the International Nucleotide Sequence Database (INSD) under accession nos. LC071987 to LC072267.

**Summary statistics**

We estimated haplotype diversity (*h*), nucleotide diversity (*π*), and Tajima’s *D* using DnaSP v5 (Librado & Rozas 2009). To determine the level of genetic differentiation between pairs of populations, we calculated both pairwise *Φ*ST (genetic distance) and pairwise *F*ST (using haplotype frequencies only). An analysis of molecular variance (AMOVA) was performed to test for genetic structure within and between populations, and a hierarchical AMOVA was also conducted to evaluate the distribution of genetic polymorphism between groups of populations (Japanese mainland vs. Nansei Islands). Pairwise *F*-statistics and AMOVAs were evaluated using 10 100 permutations in Arlequin v3.5.1.2 (Excoffier & Lischer 2010). We also ran a spatial analysis of molecular variance (SAMOVA) at *K* = 2 to maximum using the software SAMOVA 2.0 (http://cmpg.unibe.ch/software/samova2/; Dupanloup et al. 2002) to confirm the groupings used in the AMOVA, and to reveal more effective population groups that were geographically homogeneous and maximally differentiated from each other without any a priori definition.
To examine the effect of geographic distance on genetic structure, we performed correlations between the pairwise genetic distances, represented by $\Phi_{ST}/(1 - \Phi_{ST})$ estimates, and pairwise geographic distances, calculated according to the latitude and longitude of each site using Vincenty Direct formula (Vincenty 1975), among all populations using the Mantel test with 100,000 permutations in the Isolation By Distance Web Service (IBDW) v3.23 (http://ibdws.sdsu.edu/~ibdws/; Jensen et al. 2005). We omitted the populations along the Sea of Japan (sites OYB, ISZ, and GN) and the Inland Sea of Seto (sites KK and SB) from the C. leucosticta dataset to allow a direct comparison of the data from all 3 species.

The incorporation of oceanographic information into the isolation by distance (IBD) model, through calculation of isolation by oceanographic distance (IBOD), has proven useful for estimating population connectivity in the marine environment (White et al. 2010, Alberto et al. 2011). In the present study, given that the mean pathway of the pelagic larvae investigated coincides with the Kuroshio axis, we estimated the oceanographic distance between 2 locations using the latitude and longitude data for this axis (Marine Information Research Center, Japan Hydrographic Association). Since the Kuroshio Current took a large-meander (LM) path during 2004–2005 and a non-large-meander (NLM) path during 2006–2010 (www.jamstec.go.jp/jcope/cgi-bin/show-archive.cgi), with some daily variation, an average of the typical LM path in 2005 and NLM path in 2000 was used to measure the oceanographic distance.

Larval dispersal estimates

Although $F$-statistics provide information about the level of genetic differentiation between populations, they are easily biased by the degree of genetic polymorphism, making it difficult to compare values between species, particularly where they have different demographic histories. Therefore, we used another approach that evaluated the frequency distribution of phylogenetic groups, which can be used in a shallower
ecological time scale significantly minimizing the impact of historical processes.

We used neighbor joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) approaches for phylogenetic reconstruction. NJ and ML trees were built in MEGA v6.0 (Tamura et al. 2013), selecting models of nucleotide substitution (T92+G+I for C. leucosticta and C. typus, T92+G for C. multidentata) under the Bayesian information criterion (BIC). The optimal substitution models selected were also used to obtain ML trees with the nearest-neighbor-interchange (NNI) algorithm as a heuristic method. The reliability of the nodes was assessed using 1000 bootstrap replicates under NJ and ML, and using the posterior probability of the nodes under the Bayesian approach (see ‘Coalescence’ below). To verify the accuracy of the topology for C. leucosticta, the mtDNA 12S rRNA–CR region was also used to reconstruct the NJ tree with 1000 bootstrap replicates. We also reconstructed a statistical parsimony network to estimate the genealogical relationships of the haplotypes using TCS v1.21 (Clement et al. 2000) and PopART (http://popart.otago.ac.nz). We then summarized the frequency distribution of phylogenetic groups with strong statistical support. To detect significant differences between the observed frequencies, we performed the χ² test (Pearson uncorrected, Yates corrected) and Fisher’s exact test under the null hypothesis of no heterogeneity between populations.

Coalescence

A Bayesian coalescent analysis of the COI datasets was conducted using the Markov chain Monte Carlo (MCMC) method in BEAST v1.8.1 (Drummond et al. 2012). The analysis was run once with 250 × 10⁶ steps for C. leucosticta, 100 × 10⁶ steps for C. typus and 50 × 10⁶ steps for C. multidentata with a random starting tree, sampling every 1000 steps, and discarding the first 10% as burn-in, and the 1st, 2nd, and 3rd codon positions were analyzed independently. We monitored convergence of the sampling process using Tracer v1.5 and v1.6, ensuring that the effective sample size (ESS) values were above 200 and the posterior distributions of the parameters were unimodal. Due to limitations in the BEAST program, all coalescent analyses were performed using the TN93+G model, which was determined to be the best alternative by the BIC criteria in MEGA v6.0. We applied a universal mutation rate for the mtDNA COI gene in Caridea shrimps of 1.4% sequence divergence per million years (Myr) (Knowlton & Weigt 1998). It has been estimated that the species have longevities of 15 to 25 mo for C. leucosticta (Yatsuya et al. 2013) and 12 to 24 mo for C. multidentata (Hamano & Hayashi 1992). However, we used a 1 yr generation time for all species because females reach maturity more rapidly resulting in a more rapid generation turnover.

We generated maximum clade credibility (MCC) trees for each species under the strict clock and constant size models. Bayesian skyline plots (BSP) were used to estimate the demographic history through time for each species under a piecewise constant and a strict clock model using BEAST v1.8.1. We also performed separate BEAST runs using 4 simpler demographic models (constant size, exponential growth, logistic growth, and expansion growth) to objectively assess the historical demography using Bayes factor (BF) tests with 1000 bootstrap replicates in Tracer v1.5. The time to the most recent common ancestor (TMRCA) for each species and distinct lineages with statistical support were estimated using Bayesian inference in BEAST v1.8.1.

RESULTS

Genetic diversity

MtDNA COI sequencing yielded 571 base pairs (bp) of unambiguous sequence corresponding to positions 131 to 701 of the complete mitochondrial genome of the atyid shrimp Halocaridina rubra (INSD accession no. NC008413) and equal in length to that of deep-sea Argois shrimps (Fujita et al. 2012). In total, we identified 65 segregating sites (11.3%) and 25 parsimony informative sites (4.4%) distributed among 63 haplotypes for Caridina leucosticta, 58 segregating sites (10.1%) and 30 parsimony informative sites (5.3%) among 51 haplotypes for C. typus, and 84 segregating sites (14.7%) and 52 parsimony informative sites (9.1%) among 81 haplotypes for C. multidentata (Table 1). Haplotype diversity (h) and nucleotide diversity (π) were slightly lower for C. leucosticta (h = 0.537–0.905, π = 0.182–0.743%) than for C. typus (h = 0.821–0.905, π = 0.848–1.812%). By contrast, the genetic diversity of C. multidentata was extremely high (h = 0.821–0.905, π = 0.848–1.812%), with over 80% of the individuals having unique haplotypes. In C. leucosticta, we further sequenced 87 bp for the posterior part of the 12S rRNA coding region, which corresponded to positions 13475 to 13576 of the H. rubra mitochondrial genome (INSD: NC008413) with 6 conservative domain positions (>3 bp) and a subsequent
524 bp non-coding region, which we considered represented the putative CR of the mitochondrial genome due to its non-coding nature, positional homology with other atyid shrimps (Ivey & Santos 2007), and high A+T content (78.8%) compared with the COI coding region (57.9%) (Diniz et al. 2005). The posterior 12S rRNA and anterior part of CR were merged (611 bp) to define 78 distinct haplotypes among 86 individuals, giving a total of 143 polymorphic sites (23.4%) with six 1 bp indels and overall genetic diversities of \( h = 0.997 \), and \( \pi = 1.832\% \).

**\( F_{ST}\)-based dispersal estimates**

The global AMOVA for *C. leucosticta* indicated that 33.3% of the genetic variation was due to differences between populations, with the remaining 66.7% due to differences within populations, resulting in significant genetic divergence (\( \Phi_{ST} = 0.333, p < 0.001 \)) (see Table S1 in the Supplement at www.intres.com/articles/suppl/m560p105_supp.pdf). By contrast, the global AMOVA for the other shrimps revealed no significant genetic differences between populations (*C. typus*: \( \Phi_{ST} = 0.019, p = 0.181 \); *C. multidentata*: \( \Phi_{ST} = 0.003, p = 0.352 \)). The IBD and IBOD also revealed a significant correlation between genetic distance and geographic distance in *C. leucosticta* (IBD: \( R^2 = 0.5441, p = 0.0088 < 0.01 \); IBOD: \( R^2 = 0.6367, p = 0.0039 < 0.01 \)), while little or no significant correlations were detected in *C. typus* (IBD: \( R^2 = 0.1518, p = 0.1461 > 0.05 \); IBOD: \( R^2 = 0.3817, p = 0.0807 > 0.05 \)) or *C. multidentata* (IBD: \( R^2 = 0.00051, p = 0.2590 > 0.05 \); IBOD: \( R^2 = 0.0191, p = 0.2590 > 0.05 \)) (Fig. 4). Interestingly, the IBOD plots were more effective at explaining population genetic structure than the IBD plots.

We detected unique features of the pairwise genetic distances (\( F_{ST} \) and \( \Phi_{ST} \)) for each species (Table 2). Both *C. leucosticta* and *C. typus* showed a moderate \( F_{ST} \) and a weak \( \Phi_{ST} \), while *C. multidentata* had a weak \( F_{ST} \) and \( \Phi_{ST} \). Based on the pairwise \( \Phi_{ST} \) for *C. leucosticta*, we observed a weak population structure in sites OYB, KWU, and YON, and significant genetic isolation of site OMJ in both *C. leucosticta* and *C. typus*.

**Phylogroup frequency dispersal estimates**

Phylogenetic inferences based on NJ, ML, and BI analyses for each of the 3 study species plus *C. grandirostris* (6 haplotypes) resulted in consistent tree topologies that were well supported by the boot-strap method and the Bayesian posterior probability. *C. leucosticta* comprised 2 well-supported groups: Group A contained haplotypes that were present in all populations, while Group B was restricted to the Nansei Islands populations (Fig. 5). The COI phylogeny of this species resolved Group A as paraphyletic with respect to Group B (also supported by a haplotype network using the maximum parsimony
Table 2. Genetic differentiation between pairs of populations, calculated using pairwise $\Phi_{ST}$ (genetic distance; below diagonal) and $F_{ST}$ (using haplotype frequencies only; above diagonal) values based on 10100 replicates. See Fig. 1 for site numbers and locations and Table 1 for abbreviations of site names. (*)p < 0.05, (**)p < 0.01 without Bonferroni correction, ***p < 0.001 with Bonferroni correction.

### Caridina leucosticta

<table>
<thead>
<tr>
<th></th>
<th>Japanese mainland</th>
<th>Nansei Islands</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mainland</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. OYB</td>
<td></td>
<td>0.345 ***</td>
</tr>
<tr>
<td>2. ISZ</td>
<td>0.024</td>
<td>0.382 ***</td>
</tr>
<tr>
<td>3. GN</td>
<td>0.084</td>
<td>−0.010</td>
</tr>
<tr>
<td>4. YU</td>
<td>0.023</td>
<td>−0.022</td>
</tr>
<tr>
<td>5. KK</td>
<td>0.119 (*)</td>
<td>−0.004</td>
</tr>
<tr>
<td>6. SB</td>
<td>−0.028</td>
<td>0.053</td>
</tr>
<tr>
<td>7. KZ</td>
<td>0.140 (*)</td>
<td>0.009</td>
</tr>
<tr>
<td>8. NYD</td>
<td>−0.017</td>
<td>−0.016</td>
</tr>
<tr>
<td>9. ICT</td>
<td>−0.031</td>
<td>0.009</td>
</tr>
<tr>
<td><strong>Islands</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. KWU</td>
<td>0.208 (**)</td>
<td>0.221 (**)</td>
</tr>
<tr>
<td>11. YON</td>
<td>0.128 (*)</td>
<td>0.071</td>
</tr>
<tr>
<td>12. OMJ</td>
<td>0.716 ***</td>
<td>0.757 ***</td>
</tr>
</tbody>
</table>

### Caridina typus

<table>
<thead>
<tr>
<th></th>
<th>Japanese mainland</th>
<th>Nansei Islands</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mainland</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. YU</td>
<td></td>
<td>0.111 ***</td>
</tr>
<tr>
<td>7. KZ</td>
<td>−0.009</td>
<td>0.150 ***</td>
</tr>
<tr>
<td>8. NYD</td>
<td>−0.028</td>
<td>−0.027</td>
</tr>
<tr>
<td>9. ICT</td>
<td>−0.004</td>
<td>−0.004</td>
</tr>
<tr>
<td><strong>Islands</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. KWU</td>
<td>−0.021</td>
<td>−0.038</td>
</tr>
<tr>
<td>11. YON</td>
<td>−0.008</td>
<td>−0.037</td>
</tr>
<tr>
<td>12. OMJ</td>
<td>0.090</td>
<td>0.105 (*)</td>
</tr>
</tbody>
</table>

### Caridina multidentata

<table>
<thead>
<tr>
<th></th>
<th>Japanese mainland</th>
<th>Nansei Islands</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mainland</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. YU</td>
<td></td>
<td>0.011</td>
</tr>
<tr>
<td>7. KZ</td>
<td>−0.0002</td>
<td>0.013 (*)</td>
</tr>
<tr>
<td>8. NYD</td>
<td>−0.015</td>
<td>−0.025</td>
</tr>
<tr>
<td>9. ICT</td>
<td>0.007</td>
<td>0.032</td>
</tr>
<tr>
<td><strong>Islands</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. KWU</td>
<td>−0.004</td>
<td>−0.021</td>
</tr>
<tr>
<td>11. YON</td>
<td>0.001</td>
<td>0.033</td>
</tr>
</tbody>
</table>
Caridina leucosticta

Effective population size (Ne) vs. Time before present (kyr)

Caridina typus

Effective population size (Ne) vs. Time before present (kyr)

Caridina multidentata

Effective population size (Ne) vs. Time before present (kyr)

Fig. 5. Geographic distributions of distinct phylogenetic groups with high bootstrap support on the basis of neighbor joining trees. Phylogenetic trees were built from the mitochondrial DNA cytochrome c oxidase subunit I (COI) gene. The phylogenetic trees (lower left) of Caridina leucosticta and C. typus resolved into 2 distinct groups (A and B), whereas C. multidentata COI phylogeny revealed no geographic partitioning. Upper left: Bayesian skyline plots, showing median estimates for population size over time (black lines) and 95% highest posterior intervals for population size estimates (dashed lines). Anomalies in δ¹⁸O on a depth-derived timescale (Huybers 2007) (gray lines) indicate glacial (minima) and interglacial (maxima) periods.
method), and an unrooted molecular phylogeny based on mitochondrial CR sequences also showed high genetic divergence between Groups A and B. The phylogenetic tree of *C. typus* was also resolved into 2 distinct reciprocally monophyletic clades (Groups A and B) with few geographic trends. By contrast, the *C. multidentata* COI phylogeny revealed high sequence divergence between haplotypes in deep population lineages with no geographic partitioning.

To compare the genetic heterogeneity of the species, phylogenetic groups with high bootstrap support on the NJ tree were used as 'phylogroup' for each species (Fig. 5). This included 2 phylogroups (Groups A and B) for *C. leucosticta*, 2 phylogroups (Groups A and B) for *C. typus*, and no phylogroup for *C. multidentata*. Genetic heterogeneity was found for the *C. leucosticta* site pairs KWU−OMJ and YON−OMJ (p < 0.001 using uncorrected and Yates corrected χ^2 tests, and Fisher’s exact test), as well as for KWU−YON on a pie chart, although there was no statistical support for the KWU−YON pair. By contrast, there was no statistical heterogeneity between populations of *C. typus*, with the pie chart of *C. typus* phylogroups clearly showing genetic homogeneity among populations, with slight, non-significant geographic isolation of OMJ.

**Phylogeography and demographic history**

The haplotype networks of *C. leucosticta* and *C. typus* had quite similar patterns, with 2 separate groups and star-shaped phylogenies for the main networks, while *C. multidentata* exhibited well-dispersed branches within and between populations (Fig. 6). In *C. leucosticta*, Group A was further divided into 2 subgroups: the major phylogeny contained Japanese mainland and Nansei Islands populations, while the other intermediate phylogeny contained only mainland populations. In Group B of *C. leucosticta*, the difference of a main haplotype frequency, and unique haplotypes from sites KWU and OMJ were detected, suggesting genetic heterogeneity among the Nansei Island populations. In *C. typus*, Group A was also divided into 2 subgroups with no geographical isolation, whereas Group B possessed unique haplotypes from 4 OMJ individuals.

Hierarchical AMOVA for the mainland vs. islands data for *C. leucosticta* partitioned 36.22% of the variation between regions (Φ_{CT} = 0.362, p < 0.01), 11.03% between populations within regions (Φ_{SC} = 0.173, p < 0.001), and 52.74% within populations (Φ_{ST} = 0.473, p < 0.001) (Table S1). By contrast, no genetic divergence between regions was detected for the other shrimps (*C. typus*: Φ_{CT} = −0.013, p = 0.796; *C. multidentata*: Φ_{ST} = −0.008, p = 0.951). This geographic partitioning was supported by the SAMOVA (Fig. S1 in the Supplement). The best partitioning with statistical significance among *C. leucosticta* mainland + YON, KWU and OMJ was obtained with K = 3 (Φ_{CT} = 0.577, p < 0.05), whereas the 3 island populations were separated at K = 4 (Φ_{CT} = 0.509, p < 0.01) and OYB was first distinguished from remainder of the Japanese mainland at K = 5 (Φ_{CT} = 0.456, p < 0.01). The best partitioning of genetic polymorphism was obtained with K = 4 for *C. typus* (YU, ICT, OMJ and other populations; Φ_{CT} = 0.073, p < 0.05) and K = 3 for *C. multidentata* (YU, KZ + NYD + KWU, and ICT + YON; Φ_{CT} = 0.028, p < 0.05), suggesting that there was no population boundary between the mainland and islands for either of these species.

All posterior estimates of the TMRCAs were distinctly unimodal, although with wide 95% highest posterior density intervals (HPD) (Fig. S2). The TMRCAs of *C. leucosticta* were estimated at 1.4114 Myr before present (Myr BP) (95% HPD: 0.3653–17.5286; ESS = 461) for the entire dataset and 0.3869 Myr BP (95% HPD: 0.0374–4.8571; ESS = 556) for Group B, which is endemic to the Nansei Islands. The TMRCAs of Groups A and B for *C. typus* were estimated at 0.8314 Myr BP (95% HPD: 0.1961–7.9286; ESS = 891) and 0.3758 Myr BP (95% HPD: 0.0437–3.6071; ESS = 863), respectively, with these lineages separating 3.0990 Myr BP (95% HPD: 0.8286–29.6571; ESS = 811). The TMRCA of *C. multidentata* was estimated at 2.6659 Myr BP (95% HPD: 0.7571–28.0000; ESS = 894).

The BSPs indicated larger effective population sizes through time for *C. multidentata* (ESS = 243) than for *C. leucosticta* (ESS = 344) and *C. typus* (ESS = 442).
DISCUSSION

Prediction of marine larval dispersal

Our laboratory had investigated the life history of amphidromous *Caridina leucosticta* in Isazu River along the Sea of Japan from 2005 to 2009. Their ovigerous females were observed in early summer, and juveniles were observed in early autumn to winter (Yatsuya et al. 2012, 2013). Few drifting larvae were found in the ocean environment (Yatsuya et al. 2013), whereas massive numbers of larvae were caught in the brackish river estuary (S. Yamato pers. comm.). Our previous study predicted restricted larval dispersals among rivers in *C. leucosticta*, and then compared genetic population structure of *C. leucosticta* with that of *Neocaridina denticulata denticulata*, an obligate freshwater-dwelling species (Fujita et al. 2011). The result showed unexpectedly strong population connectivity in *C. leucosticta*, but whether this species had really panmictic populations remained to be elucidated (Fig. 1). The present study used a molecular ecology approach to assess the relationships between the life histories and dispersal abilities of 3 congeneric amphidromous shrimps over a wide geographical range.

The 3 *Caridina* species displayed species-specific demographies (particularly *C. multidentata*) and biased fixation index scores ($F_{ST}$ and $\Phi_{ST}$), making it difficult to directly compare their larval dispersal using a traditional population genetics approach. Therefore, we proposed a simple method for evaluating the frequency distribution of statistically supported groups on the phylogenetic tree. *C. leucosticta* displayed genetically heterogeneous populations in 2 phylogenetic groups, suggesting that the zoeal larvae remained near the river mouth during their development. By contrast, *C. typus* clearly exhibited homogeneous populations in 2 distinct clades, indicating high levels of larval dispersal via the ocean environment. These findings supported Shokita’s (1979) hypothesis that the number of zoeal stages, salinity requirements for larval development, and distributional ranges of species can predict marine larval dispersal (Fig. 2).

*C. multidentata*, however, displayed unclear phylogenetic groups. Unfortunately, we were unable to collect *C. multidentata* specimens from OMJ, where *C. leucosticta* and *C. typus* exhibited slight geographical isolation, making it difficult to compare the global AMOVA and IBD, especially between *C. typus* and *C. multidentata*. Dawson et al. (2014) previously compared the genetic signatures of 8 marine intertidal species with different pelagic durations: 0 to 1 d (‘low dispersal’), 1 to 2 wk (‘medium dispersal’), and 1 to 2 mo (‘high dispersal’). The haplotype networks of *C. leucosticta* and *C. typus* fitted the medium dispersal pattern, which was characterized by the major haplotypes being evenly distributed across populations and a typically star-like phylogeny, while that of *C. multidentata* fitted the high dispersal pattern, characterized by many haplotypes, each at a low frequency. The high genetic diversity and large population size of *C. multidentata* also matched the high dispersal pattern. A star-shaped phylogeny generally reflects a stochastic distribution of genotypes following a post-bottleneck expansion, while a haplotype-scattered shape reflects a constant population size over a long period (Slatkin & Hudson 1991). Species with lower levels of dispersal are more likely to remain in the same location (Siegel et al. 2003) and so may have been influenced by geological history (e.g. the Pleistocene glacial cycles), resulting in a star-shaped phylogeny with genes coalescing at the same time. By contrast, species with higher levels of dispersal may have escaped historical processes through continuous gene flows, maintaining a constant population size with high genetic diversity. Therefore, these findings indicate that the larval dispersal of *C. multidentata* > *C. typus* > *C. leucosticta*.

The findings for *C. multidentata* linked to ecological data previously published did not support Shokita’s (1979) hypothesis, and the data about salinity requirements seemed inconsistent, i.e. the lower salinity required for larval development was not in concordance with longer zoeal stages, the high larval dispersal predicted by the genetic approach and an extensive disp-
tribution (Fig. 2). Hence, this hypothesis is not always supported for amphidromous species. The fact that there are few ecological parameters to choose from makes it difficult to interpret the data. For instance, it is not clear from the hypothesis why very subtle differences in the number of larval stages (7 vs. 9) should have a very strong effect on larval dispersal capabilities. Furthermore, the optimal salinities during the first larval stages may be less important for explaining dispersal capabilities than the regulatory capacities of the later larval stages. Thus, the later larval stages should be analyzed to explain the reason why \textit{C. multidentata} has a larger distribution area than \textit{C. leucosticta}, even though the 2 species have equally low optimal salinity (50%). The low optimum salinity for surviving the first zoal stages in \textit{C. multidentata} may be explained by this species’ adaptation to a unique adult habitat: the adults live in the uppermost stream, and so the first stage zoal larvae are adapted to a long trip without food in the freshwater environment. Hayashi & Hamano (1984) reported that the larvae of \textit{C. multidentata} begin to take food at the third zoal stage, whereas those of \textit{C. leucosticta} and \textit{C. typus} begin at the first stage, reflecting the ability of \textit{C. multidentata} to rely on internal nutrition for longer (Nakahara et al. 2005). Anger & Hayd (2010) also reported that the greater dependence on larval lecithotrophy in one population of an amphidromous prawn \textit{Macrobrachium amazonicum} is likely an adaptation to the very long drift times in moving river water from upstream hatching sites to coastal estuaries. Thus, \textit{C. multidentata} has evolved several adaptations to its unique adult habitat, but the longer zoal instars and wider geographic distribution suggest high levels of larval dispersal between populations.

\textit{C. multidentata} is likely to be highly isolated in its headwater habitat, and so can be expected to show lower genetic diversity as a result of inbreeding and random drift. However, over 80% of \textit{C. multidentata} samples per sampling station showed unique haplotypes even in the mtDNA coding region (COI), suggesting that it has maintained high genetic diversity by linking to the neighboring rivers via the sea. Therefore, to conserve the genetic diversity of this species, we need to apply integrative management from the mountains to the sea.

**Comparative phylogeography: the Kuroshio Current as barrier and conveyor belt**

\textit{Caridina} shrimps are only found in coastal regions of the Japanese archipelago, which are influenced by the Kuroshio Current (Shokita 1979). The Kuroshio Current, which originates in the westward flowing North Equatorial Current of the central Pacific, is an important carrier of heat, nutrients, and anoxic water from the western Pacific warm pool to the middle latitudes of the northern Pacific Ocean (Wong et al. 1991). The present Kuroshio Current flows between Taiwan and Iriomote Island, northeastward along the Nansei Islands, and then traverses the Tokara Strait.

This current plays 2 roles in marine biogeography: geographic isolation between the Japanese mainland and the Nansei Islands (the ‘Kuroshio barrier’, e.g. Matsumoto et al. 2010, Kuriwa et al. 2014) and the active transport of marine organisms from the Nansei Islands to the Japanese mainland (the ‘Kuroshio conveyor belt’, e.g. Yasuda et al. 2009). In this study, the ‘Kuroshio barrier’ effect was displayed in \textit{C. leucosticta}, while the ‘Kuroshio conveyor belt’ effect was demonstrated in \textit{C. typus} and \textit{C. multidentata}. Usami et al. (2008) found that \textit{C. leucosticta} is not distributed around Hachijo jima Island, which is approximately 280 km from the Japanese mainland (Kawabe 1995), suggesting that this species cannot move with the Kuroshio Current.

A unique phylogenetic relationship was revealed for \textit{C. leucosticta}, albeit in only a single locus: the main phylogeny (Group A) was identified as basal, and the Nansei Islands endemic phylogeny (Group B) branched off this, despite the fact that the Kuroshio Current travels in the opposite direction and no Nansei Islands population exhibits an intermediate phylogeny. Such unique histories are likely to be associated with the Late Pleistocene glacial cycles, when the Kuroshio activity was weaker than at present (Chinzei et al. 1987, Sawada & Handa 1998), and the Okinawa Trough—a back arc basin behind the Nansei Islands — was fully isolated from the open Pacific Ocean due to a land bridge connecting Taiwan and the Nansei Islands. This meant that the Kuroshio Current could not enter the Okinawa Trough area and so shifted to a position east of the Nansei Islands (Ujiie & Ujiie 1999). The Kuroshio then reentered the Okinawa Trough during the last glacial maximum 11.2 kyr BP, and since then, the path of the current has shifted in and out of the Okinawa Trough several times (Jian et al. 2000, Diekmann et al. 2008). Thus, \textit{C. leucosticta} Group B (and the intermediate lineage within Group A) may have been isolated within the Okinawa Trough and later contacted the other phylogenies. The BSP analysis also indicated that \textit{C. leucosticta} has undergone population expansion since the glacial period. The formation of \textit{C. leucosticta} lineages would have been influenced by further
complicated vicariant and dispersal processes during the late Pleistocene. The nuclear markers are necessary to assess the phylogenetic position of *C. leucosticta* groups and their evolutionary histories.

We also detected geographic isolation of the Iriomotejima Island in *C. leucosticta* and *C. typus*, despite both having higher levels of larval dispersal. The biogeographic boundary is not only located between the Japanese mainland and Amamioshima Island (the Tokara Gap), but also between Iriomotejima Island and Okinawajima Island (the Kerama Gap) (Ota 1998), despite the latter only previously having been reported for terrestrial organisms. The endemism on Iriomotejima Island may be due to: (1) the Kuroshio Current turning eastward off Iriomotejima Island (the Kuroshio Counter Current; Qiu & Imasato 1990), which may prevent marine larval dispersal; or (2) the longer distance between sampling locations OMJ and YON compared to the distance between YON and KWU. Therefore, further studies are required to understand the mechanisms that have led to the marine phylogeography in the Japanese archipelago. In particular, it would be useful to investigate whether the Kuroshio Current acts as a geographic barrier or conveyor belt for marine organisms with planktonic stages using a seascape genetics approach (e.g. Selkoe et al. 2010) to incorporate environmental factors such as oceanographic distances into the analysis (White et al. 2010, Alberto et al. 2011).

Cook et al. (2012) compared dispersal and demographic patterns between 2 amphidromous atyid shrimps *Atya scabra* from the Caribbean (an island-dominated landscape) and *Australatya striolata* from eastern Australia (a continental landscape). The islands shrimp *A. scabra* had molecular signatures indicative of a recent population expansion, thus providing the idea that disturbance regimes on islands facilitate periodical extinction, recolonization and population growth cycles. Similar results were found from the other amphidromous atyid shrimps on the Caribbean island (Cook et al. 2008) and amphidromous gastropods in the Pacific islands (Crandall et al. 2010). By contrast, the continental shrimp *A. striolata* contained 2 genetic lineages, neither of which had experienced recent population expansions. Cook et al. (2012) therefore hypothesized that a recent population expansion is attainable for amphidromous species distributed within island-dominated landscapes, but the demographic and drift-mutation equilibrium is for those species on continents. In our study, *C. leucosticta* and *C. typus* matched an ‘islands species’ demographic pattern, and *C. multidentata* matched a ‘continental species’ pattern (Fig. 6, Table 1). *C. leucosticta* is endemic in the Japanese Islands, but the other 2 species have wide geographic distributions across the Indo-West Pacific region (Hayashi 2007) although their southwestern geographic limits are unknown. Under Cook et al.’s (2012) hypothesis, we speculated that the evolutionary origins of *C. typus* is the Pacific islands while that of *C. multidentata* is a continent, and then the 2 species have migrated to the Japanese Islands. This supposition requires validation by future research aimed at revealing phylogeographic patterns throughout their distributions.

**Acknowledgements.** We thank K. Watanabe (Kyoto University), K. Sakai, T. Naruse, (University of the Ryukyus), and Y. Fujita (Okinawa Prefectural University of Arts) for their help with sampling. We are also grateful to M. Yatsuya (Fisheries Research Agency), Y. Usami (Tokyo University of Marine Science and Technology), K. Nakata (Okayama University), K. Mashiko (Teikyo University), O. Tominaga (Fuku Prefectural University), M. Hatsumi (Shimane University), S. Yamato, K. Nakayama, H. Sawada and members of Maizuru Fisheries Research Station (Kyoto University) for their fruitful discussions on an earlier draft of the manuscript. This research was partly supported by the Sasakawa Scientific Research Grant 22-722 from the Japan Science Society, and also funded by Kuroshio Biological Research Foundation.

**LITERATURE CITED**


Librado P, Rozas J (2009) DnaSP v5: a software for compre-


Editorial responsibility: Karen Miller, Hobart, Tasmania, Australia

Proofs received from author(s): November 13, 2016

Submitted: February 24, 2016; Accepted: September 28, 2016