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Genetic Diversity in Hawaiian Stream Macroinvertebrates

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Abstract—The Hawaiian archipelago is the world's most geographically isolated island chain, with a high proportion of endemic plant and animal species. We studied DNA sequence variation in all four native species of Hawaiian stream macroinvertebrates. These species all exhibit freshwater amphidromous development, in which eggs are spawned and hatched in freshwater, the resulting larvae disperse in marine waters, and post-larval juveniles return to freshwater streams and grow to sexual maturity. The four species studied here were two caridean shrimps (*Atyoida bisulcata* Randall and *Macrobrachium grandimanus* Randall) and two gastropod molluscs (*Neritina granosa* Sowerby and *Neritina vespertina* Sowerby). Approximately 350 bp of the mitochondrial cytochrome oxidase subunit I (COI) gene was sequenced for 7 – 28 individuals of each species sampled from two islands (Hawai'i and Kaua'i) located at the extreme ends of the high islands. Estimates of nucleotide site diversity among the four species (range, 0.30% – 0.58%) and estimates of F_{ST} (range, -0.028 to 0.172) were comparable to previous studies of Hawaiian freshwater amphidromous fish and macroinvertebrate species, and also comparable to nucleotide site diversity among non-amphidromous shrimp of the infraorder Caridea (mean, 0.62%, N=40 species). The most common sequence haplotype in each species was shared between the two islands. These results suggest that population sizes in these amphidromous species are large enough to maintain genetic diversity, and that larval dispersal is sufficient to prevent the development of island-specific clades.

Introduction

Archipelagos have several potential advantages over continental ecosystems for studies of geographic variation in molecular polymorphisms, because islands (1) set discrete physical boundaries to populations and (2) often have known geological histories, allowing phylogeographic patterns to be correlated with island age and providing a calibration of sequence divergence rates (Zink et al. 1996, Fleischer et al. 1998). The present study takes advantage of the fact that all native freshwater Hawaiian fishes and stream macroinvertebrates are amphidromous. That is, the eggs are spawned and hatched in fresh water, and the resulting larvae are swept into the ocean to become part of the zooplankton. After several months in the plankton (the exact time period is unknown), the juveniles settle out, recruit back into the freshwater streams, and continue to feed and grow for months or years prior to sexual maturity (McDowall 1992). The life cycle of the native freshwater Hawaiian fishes and stream macroinvertebrates raises the question of how freshwater amphidromy affects population genetic structure. It might be assumed that a planktonic larval phase would enhance the chances for dispersal, and thus result in genetic homogeneity among islands. Such a result was previously obtained for native Hawaiian freshwater fishes (Zink et al. 1996, Chubb et al. 1998) and for one Hawaiian stream macroinvertebrate (Hodges & Allendorf 1998). Similar patterns have also been seen in the amphidromous shrimp *Atya innocous* (Fièvet & Eppe 2002) and for other genetic comparisons of diadromous and non-diadromous fishes (Allibone & Wallis 1993, Ward et al. 1994). However, without any data on larval behavior or on the duration in the wild of the larval period, the assumption of genetic uniformity of each of these stream macroinvertebrate species may be unwarranted. For example, despite a pelagic larval duration of three weeks, reef fish in the genus *Elacatinus* show pronounced genetic differences among islands in the Bahamas and Caribbean Sea, with few sequence haplotypes being shared between islands (Taylor & Hellberg 2003).

The Hawaiian archipelago consists of eight major islands and more than 100 smaller islands, stretching for approximately 6000 km from the Central to the Northern Pacific and located more than 3000 km from the nearest continent or similarly sized island group. The primary source of fresh water for the high islands is the year round orographic rain derived from moisture lifted from the sea by tradewinds and deposited on mountain slopes (Fitzsimons et al. 1997). Consequently, perennial streams are limited to the windward side of the main Hawaiian islands (Nishimoto & Kuamoʻo 1997). These streams are depauperate in the numbers of species of native fishes and aquatic macroinvertebrates, as compared to their mainland counterparts. Only five species of fishes, representing two families and five genera, and four species of stream macroinvertebrates, representing two phyla and three genera, are found within the Hawaiian streams.

Many of the Hawaiian marine and freshwater species are endemics (i.e., entirely restricted to the present islands and not naturally found elsewhere). Among the various major invertebrate phyla with Hawaiian representatives, between 20 – 40% of the species are endemic to Hawaiian waters (Kay & Palumbi 1987). Three of the four species in this study are considered endemic to the Hawaiian islands, and the four species jointly represent the entire native freshwater macroinvertebrate fauna. Two are decapod shrimp species: *Macrobrachium grandimanus* and *Atyoida bisulcata*. *M. grandimanus* are medium sized (up to 7.5 cm long) caridean shrimp, most often found near vegetation along the banks of the lower reaches of streams, before the first waterfall. This species is the only one included in the present study that is not a Hawaiian endemic, being also recorded from the Ryukyu, Philippine and Fiji islands (Short & Marquet 1998). *A. bisulcata* are also caridean shrimp, but they only reach 4 cm in length, and are endemic to the Hawaiian islands (Chace 1983). This species is most often found at the higher reaches of streams, where the water runs fast and clear. The two native freshwater gastropod mollusc species are *Neritina vespertina (=Theodoxus vespertinus)* and *Neritina granosa. N. vespertina* are found near the mouths of streams where the water tends to be brackish, and are distinguished by a smooth, thin brownish-green shell. *N. granosa*, found from the middle to upper reaches of streams in clear, fast-flowing water, are characterized by a robust, knobby shell that is dark brown and gold. These two mollusc species are both endemic to the Hawaiian islands, and appear to be the result of separate colonization events by different ancestral lineages (Unabia 1996).

To explore the genetic diversity and phylogeography of the native Hawaiian macroinvertebrates, we sequenced approximately 350 base pairs of the cytochrome oxidase subunit I (COI) mitochondrial gene from individuals collected from streams on Hawai‘i and Kaua‘i. These two islands were selected because they are the most geographically distant (about 400 km) high islands in the archipelago and are presumably the most widely separated in time of origin (5 million years).

Materials and Methods

Streams on each island were chosen based on accessibility and on the availability of animals. Animals were collected by hand from six streams on three trips during March 1996, January 1997, and May 1998 (see Figure 1 and Table 1 for details). Foot muscle (snails) or tail muscle (shrimps) was removed, placed in 1.5 ml microcentrifuge tubes containing as preservative a dimethyl sulfoxide-salt solution (Seutin et al. 1991) and brought to Louisiana State University in Baton Rouge, where genomic DNA was extracted and purified with standard methods (see Bebler 2001 for details). Amplification of a 375 base pair region of the cytochrome oxidase subunit I (COI) gene was carried out with the following primers: 5'CCGGATCCACNACRTARTANGTRTCRTG-3' [H7005] and 5'-CCGGATCCTTYTGRTTYTTYGGNCAYCC-3' [L6625]. After an initial denaturation step of 96ºC for 3 m, PCR was performed with 35 cycles of denaturation at 93ºC for 30 s, annealing at 50ºC for 30 s, and extension at 72ºC for 1 m. PCR products

Table 1. Collecting locality information and sample sizes for four native Hawaiian stream macroinvertebrate species.

Island	Stream	Geographical Coordinates	*Macrobrachium grandimanus*	*Atyoida bisulcata*	*Neritina vespertina*	*Neritina granosa*
Kaua‘i	Limahuli	N22º13.119', W159º34.615'	0	1	0	5
	Wainiha	N22º12.667', W159º32.824'	2	3	2	4
Hawai‘i	Waikaumalo	N19º55.952', W155º9.763'	0	0	0	6
	Hakalau	N19º54.014', W155º7.731'	2	3	4	4
	Honoli‘i	N19º45.376', W155º5.521'	3	3	4	5
	Pukihae	N19º43.910', W155º5.620'	0	0	0	4
Total			7	10	10	28

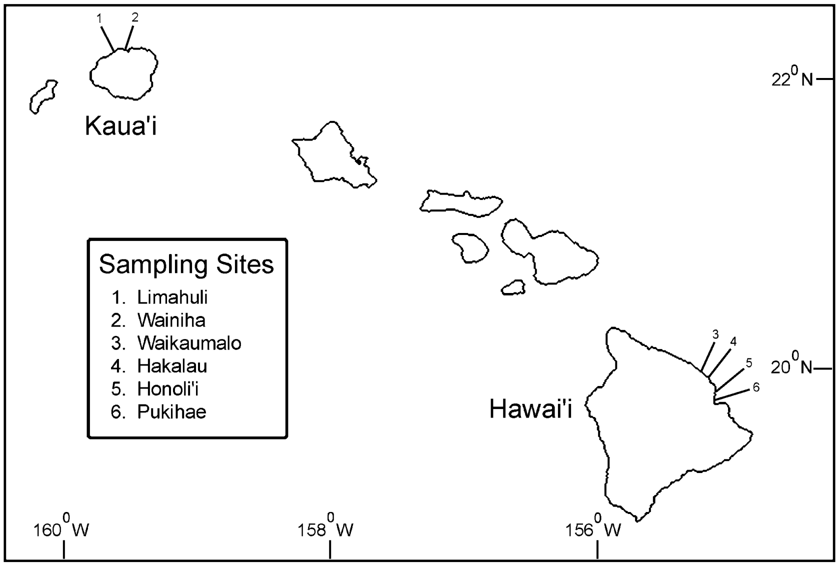

Figure 1. Location of sample streams.

were visualized on 1% agarose gels, excised, purified with the BIO 101 GeneClean® kit (BIO 101, Carlsbad, CA), and stored in water at 4ºC. Cycle sequencing of each mtDNA strand was performed with the ABI PRISM™ Big Dye™ kit (PE Applied Biosystems, Foster City, CA) as directed by the manufacturer, with the following exceptions. Each reaction tube contained 2 µl reaction premix, 2 µl PCR product, 3.2 µl 1 µM primer (either H7005 or L6625) and 2.8 µl 2.5X sequencing buffer. The cycle sequencing protocol consisted of 25 cycles of 96ºC for 10 s, 50ºC for 5 s, and 60ºC for 4 m. Purified, precipitated product was stored at 4ºC until resuspended and electrophoresed on an ABI model 377-XL Genetic Analyzer. The COI gene was chosen because it has previously been used successfully to discriminate sibling species of caridean shrimp (e.g., Mathews et al. 2002), and also because an extensive database of COI sequences in the related genus *Alpheus* is available for comparative analysis (Williams et al. 2001).

Complementary nucleotide sequences were aligned and compared for accurate nucleotide assignment in Sequencher 3.1 (Gene Codes Corporation). Sequence translations were determined in MEGA 2.0 (Kumar et al. 2001), with the invertebrate mitochondrial genetic code. The number of different haplotypes (unique sequence types) within each species was determined by inspection, and the most abundant or cosmopolitan sequence haplotype for each species was deposited with GenBank (Table 2). Arlequin 2.0 (Schneider et al. 2000) was used for construction of minimum-spanning networks, calculation of diversity measures and estimation of F_{ST} values. F_{ST} is the correlation of random haplotypes within streams relative to random pairs of haplotypes drawn from the whole sample, and is calculated as the ratio of the among stream variance component to the total variance, weighted by the observed number of nucleotide site differences

Table 2. Accession and voucher numbers and genetic diversity measures for four native Hawaiian stream macroinvertebrate species.

Species	GenBank accession no.	Sequence length (bp)	Frozen tissue voucher no.[a]	Haplotype diversity[b]	Nucleotide site diversity[b]	F_{ST}
Atyoida bisulcata	AF364063	374	I-310	91.1 ± 7.7	0.42 ± 0.31	0.172
Macrobrachium grandimanus	AF364064	375	I-311	57.1 ± 11.9	0.30 ± 0.26	-0.028
Neritina granosa	AF364065	373	I-312	67.7 ± 9.9	0.58 ± 0.37	-0.012
Neritina vespertina	AF364066	346	I-313	84.4 ± 10.3	0.49 ± 0.36	0.019

[a] Louisiana State University Museum of Natural Science, Baton Rouge, LA 70803

[b] shown as percentages, with standard errors

between haplotypes. Haplotype diversity values (H) and nucleotide diversity values (p) were calculated as in Schneider et al. (2000) and were reported as percentages, with standard errors.

Results

The number of unique sequences (molecular haplotypes) identified per species ranged from two to 11 (Figure 2). In none of the four sequence alignments was there any inferred insertion/deletion events. All six inferred substitutions in *Atyoida bisulcata* (line segments in the network in Figure 2) were synonymous at the level of the amino acid sequence. Both substitutions in *Macrobrachium grandimanus* were synonymous, as were all 15 substitutions in *Neritina granosa.* Of the seven inferred substitutions in *Neritina vespertina,* six were synonymous and one was non-synonymous. Nucleotide site diversities were uniform among species, ranging from 0.30% to 0.58%; estimates of F_{ST} were somewhat more variable, ranging from -0.028 to 0.172 (Table 2). Seven sequence haplotypes were identified among 10 *A. bisulcata* (Figure 2). The most cosmopolitan haplotype (N=3 individuals) was found in three streams and on both islands, another haplotype was found in only one stream (N=2), and all other haplotypes were singletons (that is, they occurred only once among all individuals sampled). Two haplotypes were found among seven *M. grandimanus*, each found on both islands. Six haplotypes were identified in 10 *N. vespertina*. The most cosmopolitan hap-

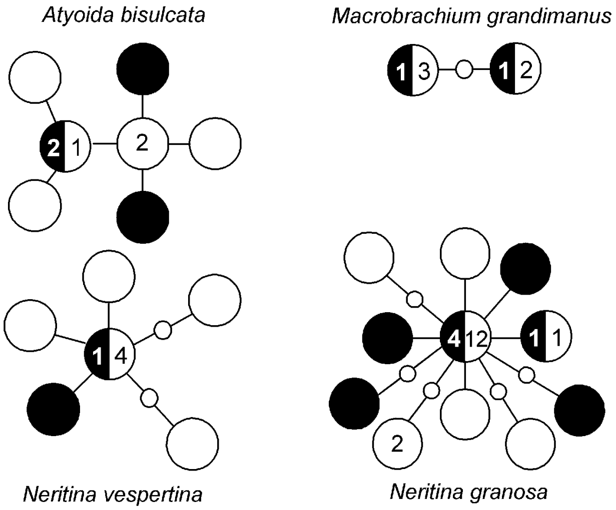

Figure 2. Minimum-spanning networks for four native Hawaiian freshwater macroinvertebrate species. Large circles denote observed sequence haplotypes; small circles denote inferred but unobserved sequence haplotypes. Open, filled and half-filled circles denote haplotypes observed on Hawai‘i only, Kaua‘i only, and on both islands, respectively. Numbers within circles or half-circles denote sample sizes; circles without numbers represent haplotypes observed only once.

lotype (N=5) was found in three streams and on both islands, and the remaining haplotypes were singletons. Eleven haplotypes were found among 28 *N. granosa*. The most cosmopolitan haplotype (N=16) was found in six streams and on both islands, another haplotype was found in two streams, both on Hawaiʻi (N=2), a third haplotype was found in one stream on each island (N=2) and the remaining eight haplotypes were all singletons.

There was no tendency for haplotypes from the same island or stream to form a clade that was monophyletic relative to haplotypes from other islands or streams (Figure 2). For example, in *Atyoida bisulcata,* two singleton haplotypes found on Kauaʻi were connected in the network by a haplotype that was found only on Hawaiʻi. In each species, the most abundant haplotype was found on both islands, and two species (*Macrobrachium grandimanus* and *Neritina granosa*) each had a second, less abundant, haplotype that was also found on both islands. As often happens with intraspecific phylogenies (Crandall & Templeton 1993), more abundant and widespread sequence haplotypes occurred at the interior of the networks in three species, suggesting that the interior sequences are ancestral to those occurring at the tips of the networks. The exception to this pattern is *M. grandimanus*, for which two equally widespread and abundant sequence haplotypes were observed. Finally, the small inter-haplotypic distances observed in Figure 2 (in each species, the maximum sequence divergence was less than 1.2%) suggested that none of the four nominal taxa included two or more undetected sibling species.

Discussion

The only previous study of population structure in Hawaiian stream macroinvertebrates is that of Hodges & Allendorf (1998), who studied variation at 15 allozyme loci in 12 population samples of *Neritina granosa* from Kauaʻi, Molokaʻi, Maui and Hawaiʻi. The mean estimate across loci of F_{ST} (0.034) was significantly different from zero, but most of the departure from zero was attributable to samples from Hanalei (Kauaʻi), Kawainui (Molokaʻi) and Honomanu (Maui) streams. These three streams were not sampled in the present study for any of the four species. Comparison to studies of Hawaiian freshwater amphidromous fishes is also difficult, because of differences in number of islands sampled, and differences in statistical methods. Zink et al. (1996) used mitochondrial DNA restriction site data analyzed by UPGMA clustering to determine if the native freshwater fishes showed island differences in genetic structure. For four of five species of the indigenous fishes collected from Kauaʻi, Oʻahu, Molokaʻi, Maui, and Hawaiʻi, they found considerable genetic variation (nucleotide site diversity values of 0.22% – 0.46%), but no evidence of geographic structure. In the single species *(Sicyopterus hawaiiensis)* that did show some geographic structuring, haplotypes from Maui tended to cluster together and those from the other islands clustered together. Another study on Hawaiian freshwater fishes used DNA sequence data of coding and non-coding gene regions and maximum parsimony analysis to explore further the possibility of geographic structuring (Chubb et al.

1998). Again, there was a lack of geographic structuring of populations, even in *S. hawaiiensis*, for which no island-specific clades were detected in the maximum parsimony analysis.

The presence of one or more shared sequence haplotypes between islands could result from (1) shared ancestral polymorphisms maintained within islands because of large effective population size (N_e), or (2) ongoing gene flow (Avise 1994). These explanations are not mutually exclusive, and it is likely that the presence of shared haplotypes in each species studied here is due to both gene flow resulting from the amphidromous life cycle of these organisms, and to relatively large N_e maintained within streams over long evolutionary time periods. For example, Hodges & Allendorf (1998) reported that population sizes of *Neritina granosa* per stream on Maui ranged from 70,000 to 350,000 individuals. Also, the nucleotide site diversity values in Table 2 for the two caridean shrimp species are not significantly lower ($P > 5\%$ by ANOVA) than the corresponding values for non-amphidromous species in the infraorder Caridea (mean ± standard error, $0.62 \pm 0.07\%$, range 0 – 1.20%, N = 40 species in the genus *Alpheus* [sources: Williams et al. 2001, Mathews et al. 2002]). This result parallels the finding of Ward et al. (1994) that average heterozygosity for nuclear genes is not significantly different among freshwater, marine and amphidromous fish species, and suggests that long-term species-wide effective population sizes are similar between amphidromous and non-amphidromous species. Because of the limited sample sizes, the F_{ST} values in Table 2 are necessarily preliminary estimates. A more precise determination of the amount of gene flow among streams and islands in these four amphidromous species would require larger samples of individuals and genes. However, the available data for each species are sufficient to show the absence of island-specific clades or species, and the occurrence of presumed ancestral haplotypes on both islands is a pattern that is unlikely to be altered by additional sampling. These results differ from those obtained for molecular phylogenetic analyses of some terrestrial animal species in the Hawaiian islands, such as the honeycreeper *Loxops virens* (Fleischer et al. 1998), in which each major clade is restricted in its present distribution to only one island, with more basal clades being found on older islands and derived clades being found on younger islands. Furthermore, some terrestrial animal genera show geographic clustering of sibling species on the same or adjacent islands within the Hawaiian archipelago.

Chubb et al. (1998) suggested that the relatively depauperate native freshwater fish fauna of the Hawaiian islands reflected low rates of speciation and extinction, with the amphidromous life cycle moderating levels of both genetic drift and demographic stochasticity. The native stream macroinvertebrate fauna studied here may have comparable evolutionary dynamics to the freshwater amphidromous fish studied by Zink et al. (1996) and Chubb et al. (1998). Both groups contrast greatly with the marked taxonomic diversification within and among islands that is characteristic of the terrestrial Hawaiian fauna (Kay & Palumbi 1987, Fleischer et al. 1998).

For many years, water has been diverted from Hawaiian freshwater streams for agriculture. It has only been in recent years that the people and the government of Hawaiʻi have shown renewed interest in returning streams to their natural flow. With this idea, there has also been a limited trial of using *Neritina granosa* from undisturbed streams on Molokaʻi to restock a stream on Oʻahu that no longer supports native species (J. M. Fitzsimons, personal communication). The lack of reciprocal monophyly between islands observed in this study supports the idea that inter-island restocking of *N. granosa* would not result in the mixing of otherwise genetically differentiated populations. It can also be inferred that, in time, a "natural" restocking from the planktonic larval population might occur (Fièvet & Eppe 2002). In fact, if recruitment of these species to all islands does occur from a common planktonic pool, a kind of "natural insurance" against extinction is already in place (Chubb et al. 1998).

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