Patterns of mtDNA Variation in Hawaiian Freshwater Fishes: The Phylogeographic Consequences of Amphidromy

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MtDNA sequencing was used to assess the phylogeographic structure of four species of Hawaiian freshwater fishes: *Lentipes concolor, Stenogobius hawaiiensis, Sicyopterus stimpsoni,* and *Awaous guamensis.* Samples of each species were collected from streams on the northeast side of Kauai, Maui, Molokai, Oahu, and Hawaii. We sequenced segments from both coding and noncoding regions (638–1391 bp) in each species. Sequence analysis uncovered genetic variability in these fishes but no evidence of strong geographic structure among island populations. This result is most readily explained by the fishes' larval marine life stage (amphidromy), which likely facilitates gene flow among island populations. By constraining genetic differentiation among populations, amphidromy may impede speciation in these fishes, possibly explaining why the Hawaiian freshwater fish fauna is depauperate compared to other species-rich Hawaiian faunas. It may also provide them with a kind of evolutionary flexibility atypical of other, more isolated island faunas and allow natural restocking to occur in streams that have been restored to suitable conditions. Comparisons of restriction site and sequence data suggested similar population genetic conclusions for all species except *S. stimpsoni,* for which the restriction site data is questioned.

The extent of geographic differentiation within animal species varies (Avise et al. 1987) and appears to be negatively correlated with mobility and dispersal ability (Ball et al. 1988; Ward et al. 1992). This variety is evident in fishes, which exhibit a wide array of life-history strategies, many affecting dispersal potential (Gyllensten 1985; Shulman and Bermingham 1995; Vrijenhoek et al. 1985; Waples 1987). Several authors have shown that freshwater species with a catadromous life stage and marine species with a pelagic larval stage exhibit higher dispersal and lower levels of interpopulation differentiation than strictly freshwater populations or marine species lacking a pelagic larval stage (Allibone and Wallis 1993; Doherty et al. 1995; reviewed in Avise 1994). However, the population structure of strictly freshwater species that have a brief marine stage, not for the purpose of breeding, is uncertain. Such amphidromous species migrate from fresh water to sea (and vice versa) during a defined stage in the life cycle, but not to breed, as in catadromous and anadromous species (Myers 1949; McDowall 1988, 1992). Documenting the geography of genetic variation of amphidromous species would strengthen our understanding of the interplay between life-history strategy, gene flow, population structure, and speciation potential.

The native Hawaiian freshwater fish fauna offers opportunities to explore the relationship between amphidromy and genetic population structure. The fauna consists of only five indigenous species [three endemic gobies (*Stenogobius hawaiiensis, Lentipes concolor, Sicyopterus stimpsoni*), one nonendemic goby (*Awaous guamensis*), and one endemic eleotrid (*Eleotris sandwicensis*)], all of which are amphidromous (Ego 1956). Each of the five species occurs on all five main high islands—Kauai, Maui, Molokai, Oahu, and Hawaii (Devick et al. 1992; Higashi and Yamamoto 1993). The species are likely not each other's closest relatives because each belongs to a different genus, with congeners found in the Pacific and Indian Oceans (McDowall 1988); therefore each represents an independent test of amphidromy's effects on genetic population structure. Taken together, these tests facilitate a more general examination of amphidromy's potential influence on speciation rates. In particular, our studies may shed light on why the Hawaiian freshwater fish fauna is depauperate. Because the species

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are found on islands of known age, many of the confounding effects of continental systems are minimized (Grant 1986), and correlations between genetic patterning, island distance, and island age may be revealed (Zink et al. 1996).

Knowledge of how genetic variation is partitioned geographically also helps determine whether populations should be managed as separate evolutionary units or as single metapopulations (Allendorf and Leary 1988; Moritz 1994; Ryder 1986; Vrijenhoek 1989). To elucidate these patterns of genetic variation within and among island populations of the Hawaiian gobies we sequenced both coding and noncoding regions of mitochondrial DNA (mtDNA): cytochrome b, NADH dehydrogenase subunit 6, and the noncoding control region (henceforth cyt b, ND6, and CR). MtDNA has several properties advantageous for such analyses (Avise 1986; Meyer 1993; Wilson et al. 1985). Because noncoding regions generally evolve faster than coding regions, we compared genetic variation in cyt b and ND6 with that of the control region. We used phylogenetic methods to analyze mtDNA sequences (coding and noncoding combined) and the resultant phylogeny was superimposed over islands to reveal spatial patterns of differentiation—"phylogeography" (Avise 1994; Avise et al. 1987). We compared our results to those of Zink et al. (1996) who used mtDNA restriction fragment length polymorphisms (RFLPs) to study all five species. Because RFLP analysis is often considered a coarse-grained method (Wilson et al. 1989), our sequencing results offer finer phylogeographic resolution and a comparison of direct sequencing and RFLP analysis.

Ecology and Behavior of the Hawaiian Freshwater Gobies

All four of the goby species occur most commonly on the windward (northeastern) sides where perennial streams are maintained by yearly rainfall ranging from nearly 100 to over 300 in. (Devick et al. 1992). Only *A. guamensis* is nonendemic, occurring throughout the western Pacific. All four gobies possess pelvic fins that are fused into a sucker disk that allows them to grasp the substrate firmly and thus resist the movements of strong freshwater currents. Consequently they can climb upstream upon returning from the sea as larvae; *L. concolor* has been found at elevations of nearly 1 km, above a waterfall with a drop of 130 m, and above a series of falls with a combined drop of nearly 600 m (Fitzsimons and Nishimoto 1990). *S. hawaiiensis,* on the other hand, is a coastal species occurring in brackish water, freshwater ponds, and the lower reaches of streams below the first waterfall. *A. guamensis* and *S. stimpsoni* typically occur in the middle reaches of streams up to 350 m elevation.

The marine larval stage ranges from several weeks to a few months, although exact data are not available (Radtke et al. 1988). Fitzsimons and Nishimoto (1990, 1991) and Nishimoto and Fitzsimons (1986) found no distinct differences in reproductive behavior within each species between populations from Hawaii and Kauai. Thus there seem to be no premating isolating mechanisms to prevent breeding between fish from the extreme ends of their range (Devick et al. 1992; Fitzsimons et al. 1993).

Table 1. Haplotype distribution by island based on combined dataset of all sequenced gene regions

S. hawaiiensis		*L. concolor*		*S. stimpsoni*		*A. guamensis*	
Hapl ID #	Location	Hapl ID #	Location	Hapl ID #	Location	Hapl ID #	Location
1	Kauai	1	Kauai	1	Kauai	1	Kauai
2	Kauai	2	Kauai		Oahu	2	Kauai
3	Kauai	3	Kauai		Maui	3	Oahu
	Molokai	4	Oahu		Hawaii (2)	4	Oahu
4	Oahu	5	Oahu	2	Kauai (2)	5	Molokai
5	Oahu	6	Oahu		Oahu	6	Molokai
6	Oahu	7	Molokai		Maui	7	Maui
7	Molokai	8	Molokai		Molokai (3)	8	Maui
8	Molokai	9	Molokai		Hawaii	9	Maui
9	Maui	10	Maui			10	Hawaii
10	Maui	11	Maui			11	Hawaii
11	Maui	12	Maui				
12	Hawaii	13	Hawaii				
13	Hawaii (2)	14	Hawaii				
		15	Hawaii				

Numbers in parentheses indicate when more than one individual from a single island possessed the same haplotype.

Materials and Methods

Specimens were collected on each of the five high islands. All samples (Table 1) were collected from streams on the northeast side of each island by divers using nets, spears, or hook and line. After removal of tissues, voucher specimens were fixed in 10% formalin, placed in 70% EtOH and cataloged into the collection of fishes at the Louisiana State University Museum of Natural Science, Baton Rouge, Louisiana.

Closed-circular mtDNA was purified from heart, liver, and body muscle tissue using CsCl-ethidium bromide density gradient centrifugation (Dowling et al. 1990; Lansman et al. 1981). MtDNA samples were the same as those used by Zink et al. (1996). Because we used "purified" mtDNA, we suggest that there was relatively low probability that we accidentally amplified nuclear copies of mtDNA regions (Zhang and Hewitt 1996). Using the polymerase chain reaction, mtDNA was symmetrically amplified in 50 μl reactions containing two 1000 ng template DNA, 5 mM Tris-HCL (pH 8.3), 25 mM KCl, 0.2 mM dNTPs, 1.75–2 mM $MgCl_2$, 0.6 μM of each primer, and either 1.25 units *Thermus aquaticus* polymerase (Boehringer Mannheim) or 0.7 units of Tfl DNA polymerase (Epicentre Technologies). Gene regions were amplified for 30 cycles using the following temperature regime: 94°C for 40 s, 50°C for 1 min, and 72°C for 1 min. A final extension at 72°C was carried out for 10 min. Electrophoresis of 6 μl of amplification mixture in a 1% agarose gel (Seakem LE, FMC) verified the presence of each desired amplified product.

For amplification and sequencing of cyt b in all four species, primers L14841 (Kocher et al. 1989) and H15299 (Hackett 1992) were used (Figure 1 and Table 2). Primers C-Leu3 and C-Glu (Park et al. 1993) were used to amplify ND5 and ND6 in *S. hawaiiensis* and *L. concolor.* Primer C-Glu was then used to sequence 220 bp of the ND6 heavy strand in these two species (Figure 1 and Table 2). When it was apparent the ND6 sequences exhibited no higher variation than cyt b, we did not sequence it in the remaining two species.

For amplification of the control region in *S. hawaiiensis,* primers L16007 in the tRNA proline (Kocher at al. 1989, as modified by Shedlock et al. 1992) and H1248 in the tRNA phenylalanine (Tarr 1995) were used to amplify an approximately 950 bp section (Figure 1, Tables 2 and 3). After sequencing approximately 500 bp in the center of this segment, two new primers—L0500 and H0600—were designed inside

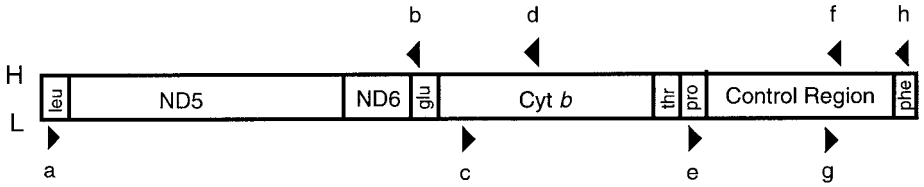

Figure 1. Primer locations for PCR and sequencing: a = C-Leu; b = C-Glu; c = L14841; d = H15299; e = L16007; f = H0600; g = L0500; h = H1248. See Table 2 for references.

the control region approximately 300 bp upstream of the tRNA phenylalanine for internal sequencing of *S. hawaiiensis* (see Table 2 for primer sequences). In *L. concolor,* however, amplification of the entire control region was unsuccessful, so primers L0500 and H1248 were used to amplify and sequence the 331 bp section directly upstream and running into the tRNA phenylalanine (Table 3). For *A. guamensis* and *S. stimpsoni,* primers L16007 and H1248 were used to amplify the control region, and primer L0500 was then used to sequence internally the 300 bp immediately upstream of tRNA phenylalanine (Table 3).

Prior to sequencing, PCR products were treated with exonuclease I and shrimp alkaline phosphatase (United States Biochemical) to remove excess dNTPs and primers. They were then sequenced using the Sequenase PCR Product Sequencing Kit (United States Biochemical). Sequenced products were electrophoresed through 0.4 mm, 6% polyacrylimide gels with a top buffer of 0.8× TTE and a bottom buffer of 1× TTE. Both heavy and light strands of cyt b were sequenced for most fish. When only heavy strands were sequenced, several were sequenced twice to ensure results. Both heavy and light strands were sequenced in the control region. Because the ND6 region was contained in such a long fragment (>2 kb), only its heavy strand was sequenced; several samples were sequenced twice to check results. Sequences were visualized by autoradiography (^{35}S).

Sequences of all four goby species were read manually and aligned using the sequence of loach (*Crossostoma lacustre*) (Tzeng et al. 1992) and carp (*Cyprinus carpio)* (Chang et al. 1994) for cyt b and ND6. The control region proved too variable to align with other published sequences, so it was aligned within the four species of gobies themselves. Each distinct sequence (all genes combined) was designated a separate haplotype; only one representative of each haplotype was used for phylogenetic analysis. Complete sequences are available in Genbank (AFO32749-32861).

Descriptive statistics were generated using the computer program MEGA (Kumar et al. 1993). Haplotype diversity (h) for each species was calculated using

$$h = 1 - \Sigma f_i^2$$

where f_i is the frequency of the ith haplotype (Avise 1994, p. 21). This value measures the extent of haplotypic diversity among all individuals, not the magnitude of sequence divergence between haplotypes. In rapidly evolving genomes such as animal mtDNA, haplotypic diversities can approach 1.0 (Avise et al. 1989). The percentage of variable nucleotide sites between individuals and haplotypes was calculated by dividing the number of variable nucleotide sites by the total number of nucleotides and multiplying this value by 100. Average percent nucleotide divergence within each species was then computed by taking the mean of the pairwise comparisons.

To assess the presence of phylogenetic signal, we computed the g1 statistic of 1000 random trees generated by PAUP in which significant left skewness to the distribution signifies informative structure (Hillis 1991). We recognize that this approach is controversial (Kallersjo et al. 1992). Haplotype trees were computed using maximum parsimony (branch & bound algorithm in PAUP; Swofford 1990), overall distance (neighbor-joining in MEGA), and maximum likelihood (PHYLIP; Felsenstein 1993) approaches; variable nucleotide positions were analyzed as unordered in the parsimony analysis. Parsimony and distance trees were rooted arbitrarily at a random haplotype from Kauai because no species we studied, including the other Hawaiian gobies themselves, were genetically close enough to act as suitable outgroups (Smith 1994). A Kauai haplotype was chosen because Kauai is the oldest extant high island at an estimated 5 million years (McDougall and Tarling 1963), and thus would likely contain the most basal populations if populations were geographically structured. Equally parsimonious trees were summarized using a "50% majority-rule" consensus tree.

To test alternative phylogenetic trees, we used Kishino and Hasegawa's (1989) method for comparing the log likelihood of two tree topologies assuming all nucleotide sites are independent and equivalent. This test is implemented in PHYLIP and calculates the mean and variance of log-likelihood pairwise differences between trees, taken across sites (Felsenstein 1993). If the mean is more than 1.96 standard deviations different, then the trees are said to be significantly different. For each species we compared likelihoods of a minimum-length parsimony tree, the maximum likelihood tree found by PHYLIP, and a tree with artificially imposed geographic structure (Figure 2). Although we recognize that other area cladogram topologies signify geographic structure, we tested our data against the progressive clades pattern (Figure 2) because it is

Table 2. Primer sources for PCR and sequencing of ND6, cytochrome *b,* and the control region

Gene	Primer	Reference
ND5/ND6	C-Leu 3	Park et al. 1993
	C-Glu	Park et al. 1993
Cyt *b*	L14841	Kocher et al. 1989
	H15299	Hackett 1992
Control region	L16007	Kocher et al. 1989, as modified by Shedlock et al. 1992
	H0600	CTGTTAACCTTAGCGCTG
	L0500	CAGCGCTAAGGTTAACAG
	H1248	Tarr 1995

Sequences of goby-specific primers are printed 5′ to 3′.

Table 3. Primer combinations used for PCR and sequencing of the control region

Species	PCR	Sequencing
S. hawaiiensis	L16007	L0500
	H1248	H0600
L. concolor	L0500	L0500
	H1248	H1248
S. stimpsoni	L16007	
	H1248	H1248
A. guamensis	L16007	
	H1248	H1248

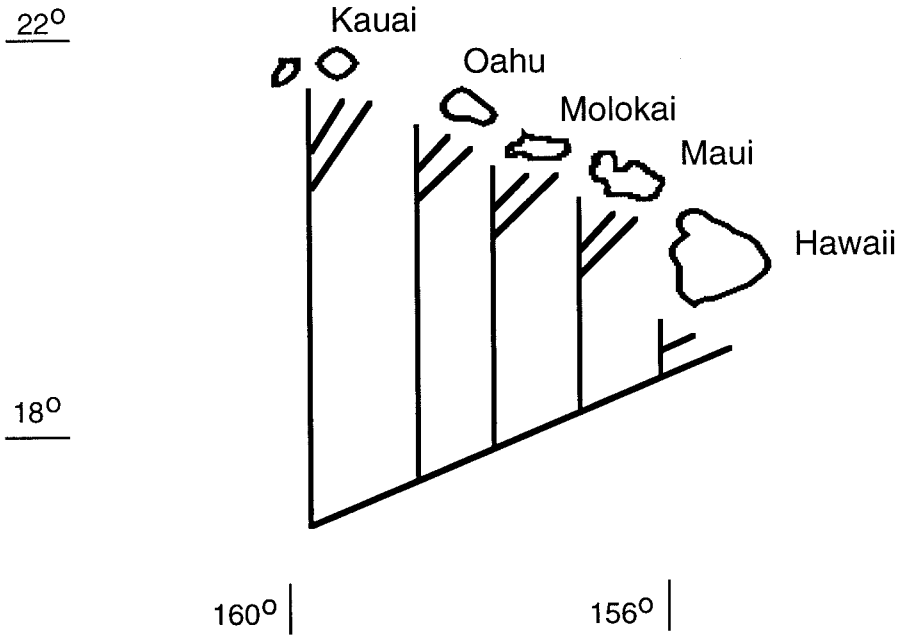

Figure 2. Hypothetical phylogenetic tree showing phylogeographic pattern of mtDNA haplotypes. All haplotypes from one island are more closely related to each other than any is to one from another island population. The tree is rooted at the oldest extant high island, Kauai, which assumes that its populations are most basal, whereas those of Hawaii—the youngest high island—are most derived. If a given species did not have three haplotypes from an island, the observed number was used.

found in many other Hawaiian lineages (Funk and Wagner 1995).

Results

Stenogobius hawaiiensis

With the combined sequence from four gene regions (1391 base positions), 13 haplotypes were observed among 15 individuals. Haplotype 3 was found on two islands, whereas all other haplotypes were restricted to single islands (see Table 1). Two individuals from Hawaii shared the same haplotype (haplotype 13). Average nucleotide sequence divergence among haplotypes and among individuals was 0.4% in both cases (haplotype range 0.1–1.1%), and the haplotype diversity (h) was 0.91. The three gene regions surveyed (ND6, cyt b, and CR) showed slight differences in their levels of variation among individuals (Table 4), with ND6 showing the most divergence among haplotypes (1.9%), and the control region showing the least (0.4%). Overall, 21 sites were variable, showing a transition:transversion ratio of 3:1 in ND6, a 2:1 ratio in cyt b, and a 4.5:1 ratio in CR. No amino acid substitutions occurred in coding regions. Of the variable sites, 13 were potentially phylogenetically informative.

The g1 statistic (−2.615) indicated significant skewness and therefore the possible presence of phylogenetic signal. Maximum parsimony analysis produced eight equally parsimonious trees of 25 steps (CI = 0.880, RI = 0.857). The consensus tree showed structure, but the structure was not geographically partitioned. Haplotypes from Maui, Oahu, Hawaii, and Molokai clustered together in a bifurcating clade, and most other haplotype positions were unresolved (Figure 3).

No geographic structure was revealed by the neighbor-joining tree (not shown); however, the maximum likelihood tree (not shown) clustered all three haplotypes found on Molokai (3, 7, and 8) in a clade. Haplotype 3, however, was also found on Kauai. The log likelihood differences between a most parsimonious tree (log L = −2095.947), the maximum likelihood tree (log L = −2101.969), and an artificially forced geographically structured tree (log L = −2197.132) revealed that the parsimony tree was the most likely of the three trees and was significantly better than the artificially structured tree, confirming lack of strong phylogeographic structure.

Lentipes concolor

From 890 bp total, each of the 15 individuals sequenced was found to be a separate haplotype (see Table 1). Average nucleotide divergence between haplotypes was 0.6% (range 0.1–1.1%). Haplotype diversity (h) was 0.98. ND6, cyt b, and CR showed slight differences in their levels of genetic variation, with ND6 showing the least average divergence among haplotypes (0.7%) and cyt b showing the most (0.9%) (Table 4). In all there were 22 variable sites with only one transversion, which was found in the control region. No amino acid substitutions were found in either coding region. Of the variable sites, 10 were potentially phylogenetically informative.

The significant g1 statistic, −0.621, revealed a skewed random tree distribution, indicating possible phylogenetic signal. Maximum parsimony analysis produced

Table 4. MtDNA sequence information for each species and gene region, including number of bases sequenced, absolute number of transitions/transversions (s/v), number of haplotypes, average percent sequence divergence among haplotypes, and sample sizes

	ND6	Cyt *b*	CR	$tRNA^{phe}$	Combined
S. hawaiiensis					
# bases	210	312	833	36	1391
s/v	3/1	4/2	9/2	0/0	16/5
# haplotyp.	2	6	10	10	13
% diverg.	1.9	0.7	0.4	0	0.4
Sample size	15	16	15	15	15
L. concolor					
# bases	222	337	295	36	925
s/v	3/0	13/0	5/1	0/0	21/1
# haplotyp.	4	14	7	7	15
% diverg.	0.8	0.9	0.7	0.0	0.6
Sample size	15	16	15	15	15
S. stimpsoni					
# bases	—	359	308	—	667
s/v	—	1/1	4/3	—	5/4
# haplotyp.	—	2	2	—	2
% diverg.	—	0.6	2.3	—	1.3
Sample size	—	14	14	—	13
A. guamensis					
# bases	—	340	298	—	638
s/v	—	2/2	10/0	—	12/2
# haplotype.	—	4	10	—	11
% diverg.	—	0.7	1.0	—	0.7
Sample size	—	13	13	—	11

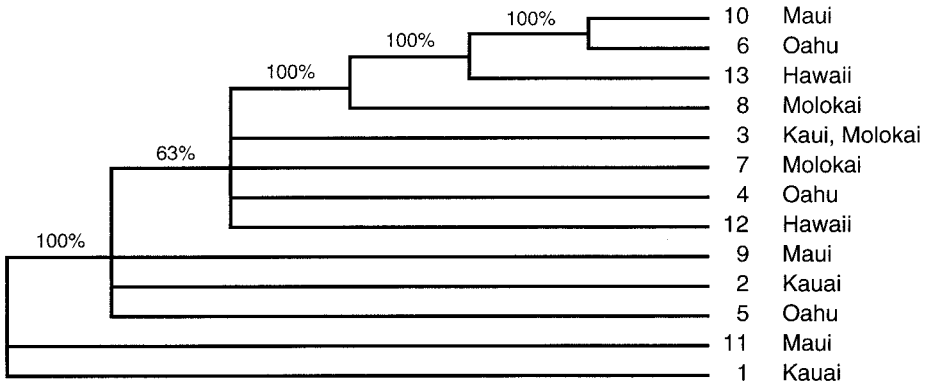

Figure 3. Majority-rule consensus of eight equally most parsimonious trees for *S. hawaiiensis* based on 1391 bp from cyt b, ND6, and CR. Haplotype identification numbers are also shown.

218 equally parsimonious trees of 33 steps (CI = 0.697, RI = 0.545). The consensus tree lacked geographic structure (Figure 4). Neither the neighbor-joining tree (not shown) nor the maximum likelihood tree (not shown) revealed geographic structure, and the log-likelihood comparison between a parsimony tree (log L = −1447.684), a maximum likelihood tree (log L = −1449.864), and an artificially structured tree (log L = −1501.844) showed that the parsimony tree was the most likely and was significantly more likely than the forced geographically structured tree.

Sicyopterus stimpsoni

Two haplotypes were observed among 13 individuals in 667 bp of sequence (cyt b and CR) . Haplotype diversity (*h*) was 0.47. Five fish had haplotype 1, and eight fish had haplotype 2 (see Table 1). The distribution of the haplotypes among islands was unstructured except that all three fish from Molokai had haplotype 2. The nucleotide divergence between the two haplotypes was 1.3%. Cyt b showed the least genetic divergence among haplotypes (0.6%) and CR showed the most (2.3%) (Table 4). Overall there were nine variable sites, showing a transition:transversion ratio of 1:1 in cyt b (only two variable sites) and a 4:3 ratio in CR. There were no amino acid substitutions in cyt b. All nine variable positions were potentially phylogenetically informative. Because our data resolved only two haplotypes, the possibility exists that we sampled nuclear copies of mitochondrial genes (Zhang and Hewitt 1996). However, our sequence gels lacked shadow bands and difficult-to-read areas that often occur with mixed nuclear and mtDNA copies. It seems unlikely that an undetected set of haplotypes revealing strong structure was unsampled both within and among species by our sampling regime.

Because there were only two haplotypes, phylogenetic analysis using the sequence data was impossible. However, RFLP data from Zink et al. (1996) were used to generate a maximum likelihood tree (log L = −329.702) that was then compared to Zink et al.'s parsimony tree (log L = −326.767) and the forced artificially structured tree (log L = −351.825) using the log likelihood ratio test. As in the other goby species, the unstructured parsimony tree was the most likely of the three and was significantly better than the geographically structured tree.

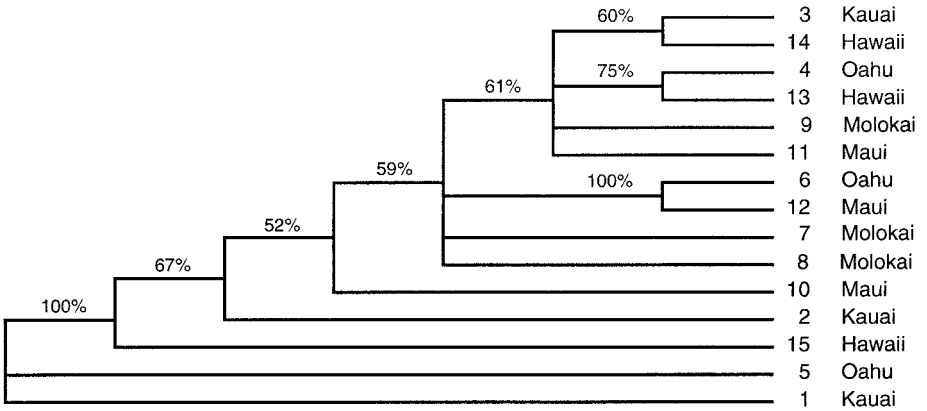

Figure 4. Majority-rule consensus of 218 equally most parsimonious trees for *L. concolor* based on 925 bp from cyt b, ND6, and CR. Haplotype identification numbers are also shown.

Awaous guamensis

For 638 bp (cyt b and CR), 11 haplotypes were observed among 11 individuals (see Table 1). Average nucleotide divergence among individuals and among haplotypes was 0.7% (range 0.2–1.4%), with the cyt b region showing the least variation among haplotypes (0.7%) and the control region showing the most (1.0%) (Table 4). Haplotypic diversity (*h*) was 0.91. In all there were 14 variable sites showing a transition:transversion ratio of 1:1 in cyt b and a 10:0 ratio in CR. One amino acid substitution was found in cyt b; six individuals possessed a codon for arganine, and at the same position seven had a codon for alanine, indicating first and second base position changes. Eight of the variable sites were potentially phylogenetically informative.

Parsimony analysis yielded 26 equally parsimonious trees with 19 steps (CI = 0.737, RI = 0.722). The majority-rule consensus tree showed no geographic structure (Figure 5). One node with 77% support linked two haplotypes from Maui (8 and 9), two from Hawaii (10 and 11), and one from Molokai (5). Another consensus node with 100% support linked a haplotype from Maui (7) with one from Oahu (4). All other haplotype positions were unresolved. The neighbor-joining tree and the maximum likelihood tree lacked geographic structure as well. When the maximum likelihood tree (log L = −1010.399) and parsimony tree were compared to an artificially structured tree in the log likelihood test, the parsimony tree (log L = −1006.860) proved to be the most likely of the three and was significantly better than the geographically structured tree (log L = −1061.143).

Restriction Site Analysis Versus Direct Sequencing

Analysis of restriction sites revealed comparable values of mtDNA variation for *L. concolor* and *S. hawaiiensis* (Table 5). Comparison of the two datasets revealed a major discrepancy between RFLP and sequence analysis for *S. stimpsoni*; only two relatively divergent haplotypes were found in the sequence analysis, whereas restriction sites defined nine haplotypes differing by 0.70% from each other. Although both RFLP and sequence data re-

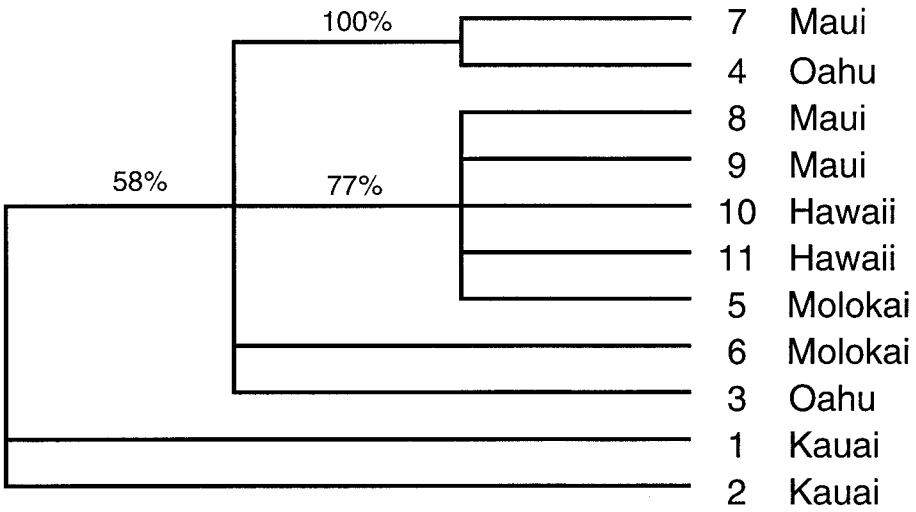

Figure 5. Majority-rule consensus of 26 equally most parsimonious trees for *A. guamensis* based on 638 bp in cyt b and CR. Haplotype identification numbers are also shown.

vealed high haplotype diversity for *A. guamensis,* diversity among haplotypes estimated from restriction sites was 50% of that estimated from sequence analysis.

Discussion

Genetic Variation Within and Among Island Populations

No strong phylogeographic structure was found in the four species of freshwater Hawaiian gobies. Using restriction sites, Zink et al. (1996) also found no phylogeographic structure for the gobies and the other indigenous species unstudied here, *E. sandwicensis.* Although our samples of each species were relatively small, it is unlikely that we failed to detect phylogeographic structure. For all but *S. stimpsoni,* we detected over 11 haplotypes, and resultant phylogenetic trees were unstructured geographically. It seems unlikely that an undetected set of haplotypes revealing strong structure was unsampled both within and among species by our sampling regime. Such trees within each of the five species of Hawaiian freshwater fishes are strong evidence of a type I phylogeographic pattern (Avise et al. 1987), consistent with a lack of isolating barriers at least in the recent past (Bermingham and Avise 1986). Given estimates of effective population size at 100,000, and assuming a generation time of 2 years for each species (Fitzsimons JM and Nishimoto RT, unpublished data), monophyly of haplotypes by island should occur in less than 1 million years of isolation [i.e., within 4Ne (effective population size for females) generations; Avise 1994]. Because all but one island is at least 1 million years old (Hawaii is dated at approximately 500,000 years), sufficient time has probably elapsed for island haplotypes to have formed distinct clades. Thus we believe that our data are most consistent with an absence of phylogeographic pattern.

Although species exhibited fairly high haplotype diversity, the genetic variation between haplotypes was low. Average nucleotide divergence between haplotypes ranged from 0.4 to 1.3%. *S. stimpsoni,* compared to the other three species, had a lower *h* value of 0.47 (two haplotypes were found across the five main islands). However, because each haplotype was relatively frequent, *S. stimpsoni*'s haplotype diversity exceeds zero. Somewhat unexpectedly, the coding regions and the control region did not differ noticeably in their overall levels of nucleotide divergence within each species, except within *S. stimpsoni* where control region divergence between haplotypes was comparatively high (2.3%). Thus the control region segments that we sequenced in these fishes do not appear to evolve at a significantly higher rate than the coding regions we studied. A similar result was reported by Shedlock et al. (1992), who concluded that most of the control region of salmonids may evolve at a rate similar to coding regions in the mitochondrial genome.

Low intraspecific mtDNA sequence variation within species is not uncommon among fishes (Beckenbach et al. 1990; Johansen et al. 1990; Shedlock et al. 1992; Thomas et al. 1986; Wilson et al. 1985) and stands in contrast to data on mammal populations (Avise and Lansman 1983; Lansman et al. 1981). Fishes might exhibit lower mtDNA variation compared to other taxa due to functional constraints imposed by ectothermy (Rand 1994; Thomas and Beckenbach 1989); however, relatively low rates of variation also have been observed in avian mtDNA control regions (Zink and Blackwell, submitted). Either slow rates of mtDNA evolution and/or recent bottlenecks may explain the low levels of intraspecific variation that have been observed in Atlantic cod (Johansen et al. 1990) and several salmonids (Beckenbach et al. 1990; Shedlock et al. 1992; Thomas et al. 1986; Wilson et al. 1985). Both low levels of mtDNA variability among haplotypes and lack of geographic differentiation have been revealed in North American eels (Avise et al. 1986), chum salmon (Park et al. 1993), and the Hawaiian freshwater gobies. These two outcomes together could be caused by several phenomena, singly or in combination: (1) bottlenecks following colonization events and their attending genetic drift, (2) a "selective sweep" that maintains a well-adapted gene complex across all locations (Rand et al. 1994), (3) insufficient time since isolation of island populations to show differentiation, and/or (4) high levels of gene flow.

Bottlenecks and selective sweeps could lead to similar phylogeographic patterns, in that both would "restart" molecular divergence. In all five species, maximum haplotype divergence was less than 1.5%.

Table 5. Comparison of mtDNA RFLP from Zink et al. (1996) and sequence data for Hawaiian freshwater fishes

	RFLP data					Sequence data				
Species	*N*	No. restriction sites	No. haplotypes	h	n	*N*	bp	No. haplotypes	h	n
S. hawaiiensis	29	98	14	0.80	0.35	15	1391	13	0.91	0.40
L. concolor	26	104	18	0.89	0.58	15	890	15	0.98	0.60
S. stimpsoni	23	92	9	0.70	0.40	13	667	2	0.47	1.3
A. guamensis	21	102	17	0.90	0.34	11	638	11	0.98	0.70

"h" refers to haplotype diversity and "n" refers to average diversity (percent sequence divergence) among haplotypes.

It seems unlikely that selective sweeps would have occurred nearly simultaneously in all five species. Thus a bottleneck affecting all species seems more plausible, given that the species occur in similar habitats. Shulman and Bermingham (1995) developed a similar argument regarding the likelihood of bottlenecks in several species of Caribbean fishes.

Haplotype trees exhibited geographically diffuse topologies or "stochastic patterns" (Funk and Wagner 1995), with most-similar haplotypes often occurring on different islands. According to Slatkin and Maddison (1989), a stochastic topology indicates that, as in the previous RFLP (Zink et al. 1996) and allozyme studies (Fitzsimons et al. 1990), the most likely hypothesis for the lack of geographic structuring in the Hawaiian gobies' mtDNA is gene flow between populations. In this case, gene flow is mediated by the gobies' larval life stage—amphidromy. In particular, either annually or within a period of decades, larvae from one island are recruited as breeders to other islands.

Life History Implications for Levels of Genetic Differentiation and Rates of Speciation

Several authors have suggested that diadromous life history influences levels of intraspecific variability and geographic differentiation. According to Thomas et al. (1986), the anadromy of pacific salmon and their ability to migrate and colonize new areas might constrain intraspecific divergence of mtDNA. Likewise, Shedlock et al. (1992) articulated that the linkage between genetic divergence and geographic distance in anadromous salmonids may not be as tight as would be expected in strictly freshwater fishes. However, geographic differentiation has been revealed in several anadromous salmonids that exhibit natal homing, a behavior that reduces gene flow between populations (Bermingham et al. 1991; Ryman and Utter 1987).

Gyllensten (1985) attributed differences in geographic differentiation between freshwater, anadromous, and marine species to differences in the occurrence of geographic barriers between populations; strictly freshwater species experience the most barriers, marine species the least, and anadromous species an intermediate amount. Consequently freshwater populations showed the most genetic divergence between populations and marine the least, whereas anadromous species, according to Gyllensten, showed a distribution of variability similar to that of marine species. Among some marine congeners, species with extended larval stages can exhibit little differentiation between populations, whereas the sister species with brief or absent pelagic larval stages can be highly structured genetically (Ehrlich 1975; McMillan et al. 1992; Waples 1987). It appears that amphidromy in the freshwater Hawaiian gobies—manifested as a highly dispersive marine larval stage—causes their population structure to resemble that exhibited by other diadromous freshwater species and marine species with extended larval stages.

If a marine life stage induces gene flow among island populations, speciation rates should be affected. In marine invertebrates, the fossil record indicates that species with high-dispersal pelagic larvae have broader geographic ranges, greater species durations, and slower rates of speciation than similar species with low dispersal (Jablonski 1986). In diadromous vertebrates like salmonids, geographic isolation in the form of landlocking is often required for speciation to occur (McDowall 1988). Whereas several nonamphidromous freshwater complexes have exhibited rapid divergence into closely related "species flocks" (Echelle and Echelle 1984; Echelle and Kornfield 1984), the native Hawaiian freshwater fauna includes just five species. It seems these fishes illustrate, in five independent "replications," a link between amphidromous larvae, high levels of gene flow, low genetic differentiation, and their existence in a species-poor fauna. Genetic mixing of the goby populations has potentially prevented "rapid" speciation often exhibited in island environments; other Hawaiian fauna, for example, have shown tremendous adaptive radiations (Carson and Kaneshiro 1976; Freed et al. 1987; Tarr and Fleischer 1993; Wagner and Funk 1995).

By mixing island populations and thus maintaining a larger gene pool than would exist if each island population was landlocked, amphidromy may provide a kind of genetic "insurance" against extinction that is not present in smaller, isolated populations more prone to drift and demographic stochasticity. In desert topminnows, for instance, small remnant populations in Arizona showed less genetic variation than the more widespread populations of Mexico (Vrijenhoek et al. 1985). Because amphidromy may maintain the gobies' status as ecological "generalists," it might decrease their vulnerability to demographic stochasticity and thus increase their chances of survival over time. Other diadromous species have been shown to be more generalized in both morphology and behavior/habitat than nondiadromous species (McDowall 1988). The fact that many island freshwater fishes have a marine stage suggests that loss of it increases the likelihood of extinction.

Conservation Implications

Although *L. concolor* may warrant threatened or endangered status (Sierra Club Legal Defense Fund, Inc., and U.S. Fish and Wildlife Service, unpublished, 1990), previous results (Fitzsimons et al. 1990; Zink et al. 1996) combined with the present study indicate that reasonable levels of genetic variation exist in *L. concolor* and in the other three species of gobies, and that there is a diversity of haplotypes within extant populations. Although caution should be used in employing these data for management purposes because they are based on only a small part of the genome and on small samples, the replication of results across studies and across species with similar life histories lends confidence to the findings.

These genetic data, in conjunction with behavioral and morphological findings (Fitzsimons and Nishimoto 1990, 1991; Nishimoto and Fitzsimons 1986), suggest that recruitment to all islands is from a common offshore pool of plankton, and thus each species might be regarded as a single, genetically similar stock. For management purposes this means that amphidromy may allow natural restocking to take place into streams that have been restored to suitable habitat conditions, as has been shown in some diadromous species in New Zealand (McDowall 1996). Because *L. concolor* was recently rediscovered on Oahu, natural restocking may already be occurring (Higashi and Yamamoto 1993). It is important to note, however, that certain streams and perhaps certain islands might contribute more than others to the common pool of larvae (Devick et al. 1992). Thus understanding more about the patterns of recruitment and the potential existence of favored source streams will be an ongoing challenge in the management of the Hawaiian freshwater fishes, because local demographic fluctuation is as critical a concern to conservation as genetic composition (Gilpin and Soule 1986; Lande 1988). In the Hawaiian freshwater fauna especially, it has been suggested that the amount of dispersal that is sufficient to maintain genetic similarity among populations may

not be sufficient to overcome local demographic processes (Hodges 1992). More research, which would complement the mtDNA sequence and RFLP data, is needed to explore these possibilities. Despite this cautionary note, we believe that amphidromy in these fishes provides reason for optimism in their preservation as long as stream conservation and restoration of degraded streams continues to be a high priority.

Resolving Power of Different Datasets

As researchers switch from restriction sites to direct sequencing, comparisons of population genetic inferences derived from each dataset are of interest (Table 5). In two of our study species, *L. concolor* and *S. hawaiiensis,* one would reach the same population genetic conclusions. For *A. guamensis,* a shallower haplotype tree is implied by restriction sites, but both datasets indicate high haplotype diversity. For *S. stimpsoni,* the two datasets suggest different levels of haplotype and nucleotide diversity. Zink et al. (1996) noted problems in resolving restriction site patterns for goby mtDNA; gel banding patterns were often faint and some extrapolation was reported. Therefore we are inclined to suggest that the sequence data represent a better picture of mtDNA variation. This leaves unexplained the existence of only two relatively old haplotype lineages in *S. stimpsoni*; the possibility of nuclear gene contamination in this species exists (Zhang and Hewitt 1996). In the other three species, however, both restriction site and sequence data suggested similar phylogeographic patterns (lack of structure). Thus although there are reasons to prefer sequence data, it does not mean that inferences from RFLP studies are necessarily biased.

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