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Phylogeography of the pipefish, *Urocampus carinirostris*, suggests secondary intergradation of ancient lineages

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Abstract We assayed the pattern of mitochondrial DNA evolution in the live bearing, seagrass specialist pipefish, *Urocampus carinirostris*, in eastern Australia. These life history attributes were predicted to result in strong phylogeographic structure in *U. carinirostris*. Phylogenetic analysis of cytochrome *b* sequences detected two monophyletic mtDNA clades that differed by 8.69% sequence divergence – a large level of intraspecific divergence for a marine fish. The geographical distribution of clades was non-random and resembled clinal secondary intergradation over a 130-km stretch of coastline. Contrary to phylogeographic predictions, this large phylogeographic break does not occur across a traditionally recognised biogeographic boundary. Analyses of historical demography suggested that individuals belonging to the most widespread clade underwent a population expansion from a small refuge population during the Pleistocene.

Introduction

Molecular systematics has facilitated several extensions of our understanding of the interplay between the genetic differentiation of populations and geographic speciation. Particularly noteworthy is the undeniable influence of historical environmental changes on patterns of gene flow among conspecific populations (Avise 1994). Extrinsic forces such as glaciation and sea level changes appear to have affected the pattern of neutral molecular evolution in many codistributed species in similar ways regardless of subtle differences in intrinsic life history characteristics (e.g. Australia's wet tropics: Schneider and Moritz 1999; and coastal marine taxa in the southeastern United States: Avise 1994). However, there are exceptions where life history attributes appear to be the predominant factor leading to genetic differences among populations. For example, very strong genetic differences are maintained among populations of the coral reef fish, *Acanthachromis polyacanthus,* which is exceptional among coral reef fishes due to its lack of a pelagic larval phase. Compared with other presently codistributed coral reef fishes that exhibit pelagic larval dispersal, *A. polyacanthus* exhibits significantly stronger genetic differentiation among Great Barrier Reef populations (Doherty et al.1995). Because these codistributed species are likely to have experienced similar historical biogeographic forces, these patterns suggest that the unique life history of *A. polyacanthus* is a major determinant of population genetic patterns.

It has been suggested that patterns of genetic differentiation among populations of marine fishes may be associated with degree of habitat specialisation (Smith and Fujio 1982), and life history dispersal characteristics (Waples 1987). Marine fishes display phylogeographic structures that span a continuum, from the apparent panmixis displayed by Atlantic eels (Avise et al. 1986) to the secondary intergradation of divergent lineages in the menhaden, *Brevoortia tyrannus* (Bowen and Avise 1990). However, more often than not, marine fishes display weak phylogeographic structuring, and intraspecific mitochondrial DNA clades are shallow compared with freshwater fishes (Shulman and Bermingham 1995; Avise et al. 1998).

Hairy pipefish (*Urocampus carinirostris* Castelnau) are small, cryptic fish from the family Syngnathidae,

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which also includes seahorses and seadragons. *U. carinirostris* is the most common syngnathid along much of the eastern Australian coast (Howard and Koehn 1985; Gray et al. 1996). It shares a specialised reproductive strategy with other syngnathids, involving the female passing eggs to the underside of the male, in the case of *U. carinirostris* into a brood pouch under the abdomen (Dawson 1985). Young juveniles hatch from the pouch fully formed. *U. carinirostris* is typically found in shallow meadows of the seagrass, *Zostera capricorni*, in estuaries and sheltered embayments. The lack of a dispersive egg and larval phase and the weak swimming ability of adults suggest that movement among estuaries is limited and that closure of the life cycle commonly occurs over a very localised geographical scale.

We have assayed mitochondrial DNA variation in eastern Australian populations of *U. carinirostris,* a marine species representing an extreme in habitat specialisation and limited dispersal potential, to determine if such an extreme suite of intrinsic biological attributes has resulted in strong phylogeographic patterns. The absence of such a pattern would further emphasise the potency of extrinsic environmental forces in determining the genetic structure of marine populations.

Materials and methods

Collections

We surveyed previously mapped and identified seagrass meadows for *U. carinirostris* using seine nets. The sampling covered the east coast distribution from Lake Macquarie in the south to the known northern extent of the range (Fig. 1) Samples were analysed from nine different seagrass beds spanning a total of 1,200 km of coastline (Fig. 1).

Molecular methods

Our molecular analysis involved two phases. First, to identify major lineages in the intraspecifc mtDNA phylogeny of *U. carinirostris,* for each of ten individuals per population, we sequenced a portion of the mitochondrial cytochrome *b* gene. Mitochondrial DNA was isolated using the alkaline lysis technique outlined by Tamura and Aotsuka (1988). A fragment of the mitochondrial cytochrome *b* gene was amplified via the polymerase chain reaction using primers GLU-L and CB2H (Palumbi et al. 1991) which correspond to positions 14725 and 15175 in the human mtDNA genome. The thermal profile for PCR amplifications involved 40 cycles of 30 s at 940 C, 30 s at 550 C and 1 min at 720 C. Sequencing of both heavy and light strands was carried out on an Applied Biosystems 377 automated sequencing machine using the Big Dye® Terminator chemistry. For each individual, a minimum of 300 bp of high-quality sequence data were obtained from the approximately 450 bp amplified fragment. This sequence data provided numbers sufficient for estimating nucleotide diversity and allowed statistical tests of historical demography to be performed.

Second, to better characterise the geographical distribution of two major mtDNA lineages that were detected, a diagnostic restriction enzyme assay was developed and performed on an additional 135 individuals (15 per site) (sensu Slade et al. 1993). The restriction enzyme *Rsa1* cleaved the analysed fragment at position 292 in all clade A individuals and did not cleave any positions in clade B individuals. The enzyme *Alu1* cleaved position 139 in all clade B individuals and did not cleave any positions in clade A individuals. Two restriction digests were carried out on all individuals (one per enzyme) using 10µl of PCR product following the manufacturer's directions. Digests were later run on agarose gels to determine the presence or absence of a diagnostic restriction site. To allow unbiased comparisons among sites, during both phases of the analysis, an equal number of individuals were analysed from each site.

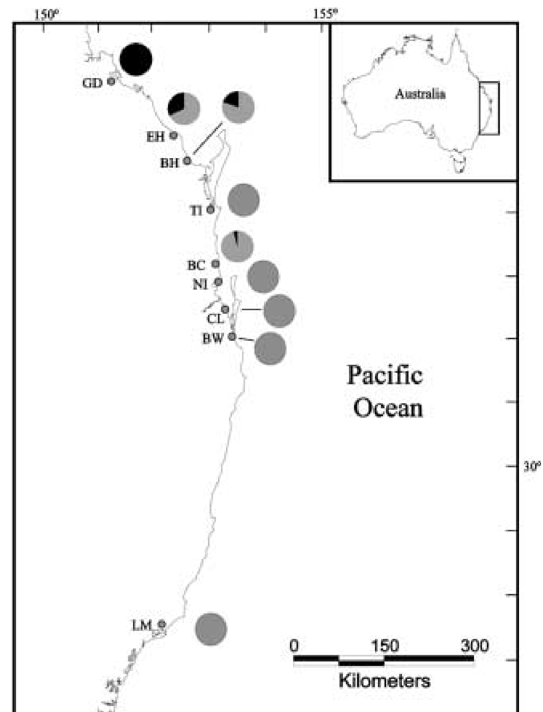

Fig. 1. Map of seagrass beds sampled for *Urocampus carinirostris*. Pie diagrams are frequency distributions of two major mtDNA clades *A* (grey) and *B* (black) based on samples of 25 individuals per site assayed by direct sequencing of the cytochrome *b* gene and subsequent diagnostic PCR- RFLP assay. Site codes are *GD* Gladstone Harbour; *EH* Elliott Heads; *BH* Burrum Heads; *TI* Tin Can Bay; *BC* Bells Creek; *NI* Ningi; *CL* Cleveland; *BW* Broadwater; *LM* Lake Macquarie

Statistical methods

Sequences were aligned by eye. Identical sequences were assigned the same haplotype identity. Phylogenetic analyses were performed using distance-based, (neighbor-joining; Saitou and Nei 1987) maximum parsimony, and maximum likelihood (quartet puzzling; Strimmer and von Haeseler 1996) methods. The mtDNA diversity distributed within and among populations was calculated using the package REAP (McElroy et al. 1992). A Mantel's test was used to test for a relationship between geographic distance and nucleotide divergence among sites (Mantel 1967).

The historical demography of each major mtDNA clade was investigated using mismatch distributions, which are the distribution of pairwise genetic differences among individuals in a population (Rogers and Harpending 1992). We used simulations to test the significance of the raggedness statistic *r*, for each mismatch distribution (program provided courtesy of H. Harpending). Populations that have undergone a large expansion are expected to exhibit smooth, unimodal mismatch distributions and low raggedness values. More ragged mismatch distributions tend to result from large stable populations (Harpending 1994).

Results

mtDNA diversity within and among populations of U.carinirostris

Sequencing of 300 bp of the cytochrome *b* gene for 90 individual *U. carinirostris* yielded 49 distinct haplotypes defined by 54 variable nucleotide sites. Sequences have been submitted to GenBank under accession numbers (AF155426–AF155474). Although most nucleotide substitutions were silent changes, owing to the high level of sequence divergence among haplotypes (maximum of 12.14%), there was also a number of amino acid substitutions.

Nucleotide diversity was lowest in the Gladstone Harbour (GD) sample which was at the end of the sampled range and is also close to the northernmost record of *U. carinirostris* in eastern Australia (Dawson 1985). Diversity rose at least 13-fold in the two next northernmost populations, Elliott Heads (EH) and Burrum Heads (BH), only 130 km from GD. Most other sites had moderate values of diversity and any extreme values were primarily due to either presence or absence of both highly divergent but shallow mtDNA lineages (Table 1).

Divergence among populations was also similarly influenced by the geographical distribution of divergent haplotypes. Comparisons of GD with other sites produced divergence values ranging between 6% and 10%. Other comparisons were zero and were primarily driven by the sharing of identical mtDNA haplotypes or ones that differed by only a single nucleotide substitution. Apart from the large divergence values involving the GD sample, nucleotide divergence was not significantly associated with geographical distance among sites (Mantel's test $P = 0.185$).

Phylogeographic patterns

All phylogenetic analyses resolved two major mtDNA clades (Fig. 2). Correcting for within clade genetic diversity suggests that these clades differ by 8.69% in their cytochrome *b* sequences, an extremely large value for a marine fish. Typing of a further 135 individuals using diagnostic PCR-RFLP analysis to boost our sample sizes confirmed that GD contained only clade B individuals. Clade B was detected in lower frequencies in the next two northernmost populations EH and BH. The southernmost detection of an individual bearing a clade B mtDNA haplotype was at Bells Creek (BC) where a single individual was detected (Fig. 1). The geographical distribution of clades was more structured than expected by chance alone ($P < 0.001$; randomised chi-square, Roff and Bentzen 1989) and suggests clinal interchange of highly divergent mtDNA clades within the sampled range.

Assuming that in fishes, cytochrome *b* lineages diverge at between 1% and 2.5% per million years (Irwin et al. 1991; Martin et al. 1992), individuals of each clade last shared a common ancestor between 3.5 and 8.6 million years ago. This places the timing of population separation sometime during the early Pliocene or late Miocene. All individuals assayed at Lake Macquarie (LM), the southernmost site, appear to form a small shallow clade within clade A.

Historical demography of mtDNA clades

Following the line of reasoning that the two mtDNA clades A and B have independent geographical origins, we tested the mtDNA sequences in each clade for signal of past demographic processes. Clade A has a unimodal mismatch distribution (Fig. 3). Using the raggedness test, the hypothesis of no population growth was rejected ($P < 0.01$) but the hypotheses of 100-fold and 1,000-fold growth could not be rejected ($P > 0.05$). The estimate of the time since expansion in mutational units, τ, was 3.45. $\tau = 2ut$ where u refers to the aggregate mutation rate over the DNA region studied (Rogers and Harpending 1992). For DNA sequence data $u = M_T \mu$ (Rogers and Harpending 1992), where M_T is the number of nucleotides assayed per individual and μ is the mutation rate. Assuming that cytochrome *b* gene sequences diverge between 1% and 2.5% per million years, values of μ range between 0.5×10^{-8} and 1.25×10^{-8}. Scaling for

Table 1. Percent nucleotide diversity, π (bold along diagonal), the average number of nucleotide substitutions within pairs of populations, d_{xy} (above the diagonal) and net nucleotide divergence between populations corrected for within population diversity, d_a (below the diagonal) for nine populations of *Urocampus carinirostris* (Nei 1987) Sample sizes for DNA sequence data appear below site codes, see Fig. 1 for details of site codes

	GD (10)	EH (10)	BH (10)	TC (10)	BC (10)	NI (10)	CL (10)	BW (10)	LM (10)
GD	**0.32**	8.56	8.66	11.04	9.88	10.62	10.85	10.79	10.38
EH	6.18	**4.43**	4.17	3.09	3.63	3.06	3.06	3.11	3.26
BH	6.18	–0.36	**4.64**	3.07	3.70	3.13	3.10	3.09	3.32
TC	10.43	0.42	0.30	**0.90**	2.06	1.16	0.93	0.95	1.34
BC	8.12	–0.19	–0.21	0.01	**3.19**	2.20	2.09	2.13	2.42
NI	9.78	0.17	0.13	0.02	–0.08	**1.37**	1.20	1.21	1.58
CL	10.17	0.33	0.26	–0.03	–0.02	0.01	**1.03**	0.97	1.43
BW	10.11	0.38	0.26	–0.01	0.02	0.01	–0.06	**1.02**	1.44
LM	9.91	0.74	0.69	0.58	0.51	0.60	0.61	0.62	**0.61**

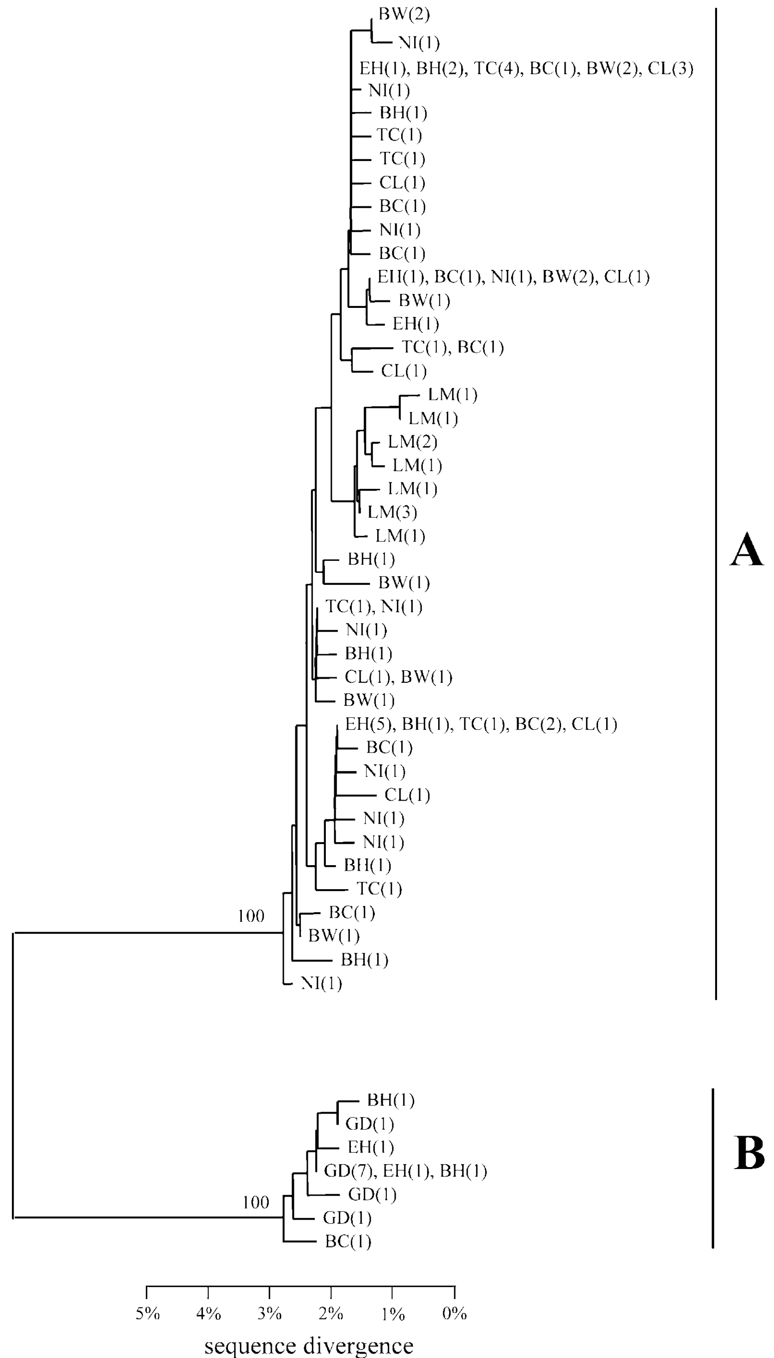

Fig. 2. Neighbor-joining tree with results of 500 bootstrap replicates indicating two major mtDNA lineages, *A* and *B*, in *U. carinirostris*. Distances among haplotypes were calculated assuming a two-parameter model of nucleotide substitution (Kimura 1980). The two clades were also detected in other phylogenetic analyses – maximum likelihood: quartet puzzling (Strimmer and von Haeseler 1996) assuming a Tamura and Nei (1993) model of nucleotide substitution and parsimony analysis: 50 replications of an heuristic search with random sequence addition. *Numbers in brackets* represent the number of individuals detected bearing a particular haplotype

300 nucleotides, μ ranges from 1.5×10^{-6} to 3.75×10^{-6}. Thus the estimated τ value of 3.45 implies that the expansion occurred during the Pleistocene between 1,150,000 and 490,000 years ago (assuming a generation time of 1 year).

Clade B does not exhibit a strong a pattern of population expansion (Fig. 3). Although the hypotheses of 100-fold and 1,000-fold growth could not be rejected with the raggedness test, the hypothesis of no population growth could not be rejected either. However, considering that the power of the raggedness test is very sensitive to sample size (H. Harpending, personal communication), and that only 15 clade B sequences were detected compared with 75 for clade A, we cannot rule out limited statistical power to detect a past population expansion in this clade.

Discussion

In *U. carinirostris* we have detected two mtDNA lineages that have independent evolutionary histories and are distributed in a non-random pattern across the sampled range. For a marine teleost, *U. carinirostris*

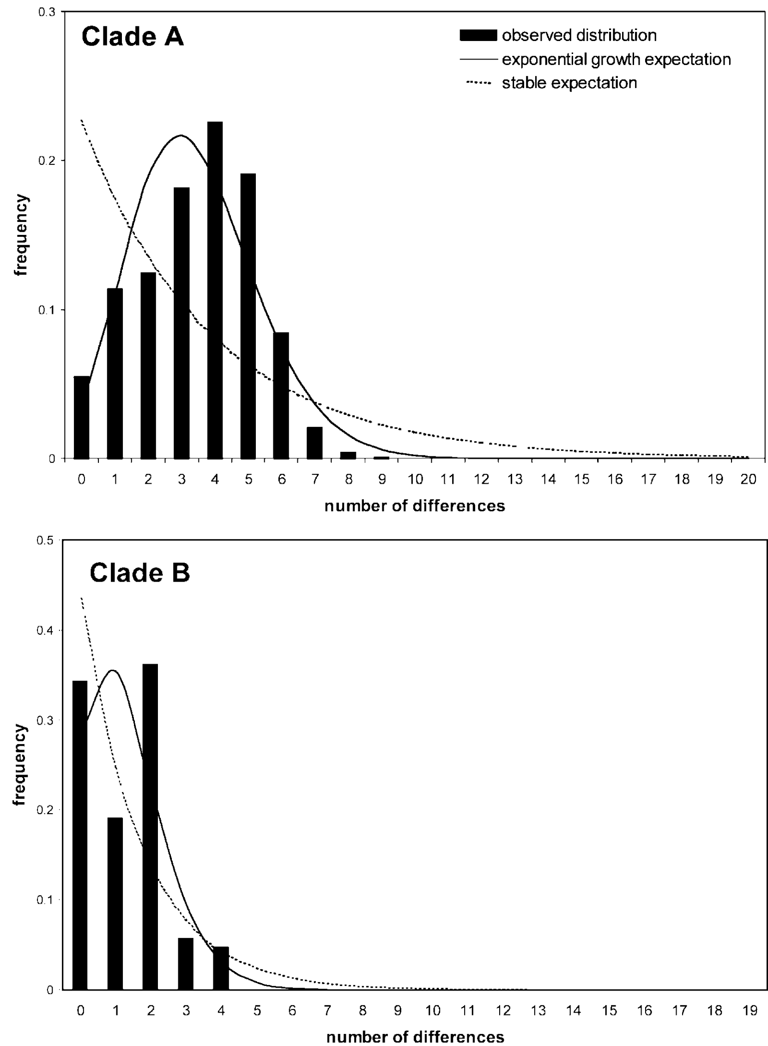

Fig. 3. Mismatch distributions for mtDNA clades *A* and *B*. *Vertical stacks* are the observed mismatch distributions, the *short dashed lines* are the geometric expectation of a stationary population, while *long dashed lines* are Poisson expectations for an expanded population

exhibits an extremely large level of mitochondrial DNA sequence divergence between the two monophyletic clades A and B of 8.69%. In a review of phylogeographic patterns in the vertebrates, Avise et al. (1998) found that for fishes, the largest level of mtDNA sequence divergence among intraspecifc clades was 8.4% in the bluegill sunfish, *Leopomis macrochirus*, a freshwater species. In fact, the largest eight levels of sequence divergence in the 24 fishes surveyed were freshwater species. This is not surprising given the well-documented phenomenon of higher levels of allozyme variation among populations of freshwater fishes than among populations of marine fishes (see Gyllensten 1985 and Ward et al. 1994 for reviews).

The deepest level of intraspecific divergence among marine fishes reviewed by Avise et al. (1998) was in the killfish, *Fundulus heteroclitus*. *F. heteroclitus* exhibits 3.4% mtDNA RFLP differences among intraspecific clades which intergrade between northern and southern populations along the North American Atlantic coast (Gonzalez-Vilansenor and Powers 1990). However, although it tolerates full salinity, *F. heteroclitus* is primarily a freshwater or brackish water species. Among truly marine species the levels are usually much lower (less than 1%) (Graves 1998). An exception has been reported for the deep-sea species *Cyclothone alba* (Miya and Nishida 1997), where intraspecific divergence was 11.1%. However, these differences occurred between populations from different oceans, not from the same sites, as we report here. The level of divergence in *U. carinirostris* suggests that populations were isolated for an extremely long time, between 3.5 and 8.6 million years assuming the range of cytochrome *b* divergence rates reported for fishes (Irwin et al. 1991; Martin et al. 1992). Thus a likely historical scenario for the sampled populations of *U. carinirostris* is one of a vacariant event that lead to a cessation of gene flow between northern and southern populations sometime during the late Miocene or early Pliocene followed by exponential population growth in the south during the late Pleistocene (as suggested by mismatch analysis).

Phylogeographic theory predicts that genetic breaks within species are most likely to occur across traditionally recognised biogeographic boundaries (Avise 1994). The phylogeographic patterns in *U. carinirostris* are contradictory to this prediction. There is no well-recognised

biogeographical boundary spanning the zone of interchange between GD and BC sites. The only marine biogeographic boundary within the sampled range is between the Solanderian and Permian provinces which occurs between the two southernmost sites Broadwater (BW) and LM (Poore 1994). Although there is some evidence of genetic structuring between these two sites, it is nowhere near the scale of the major break between clades A and B. It is noteworthy that the northernmost population GD lies at the same latitude as the beginning of the Great Barrier Reef. North of this point along the coastline, the continental slope becomes very shallow and extends eastwards for up to 100 km. South of GD, the continental shelf is much closer to the mainland and often lies only tens of kilometres off the coastline.

That the southern population has undergone an expansion since populations were sequestered could mean that the geographical location of the barrier is now blurred. Strictly, the raggedness test only considers population growth per se and does not necessarily suggest a range expansion. However, given the typically small amount of seagrass meadows available within a catchment, we believe that the genetic signature of population expansion most likely coincided with a range expansion in *U. carinirostris*.

Is it possible that the barrier did indeed stem from the biogeographical boundary between LM and BW sites? It is difficult to predict the location of the original ancestral population but it would be expected to contain the highest diversity and number of clade A haplotypes. The sample from Ningi (NI) has the highest nucleotide diversity, and the largest number of clade A haplotypes. If NI is indeed near to the location of the founding population, the result still implies that the historical barrier was not at the site of the biogeographical boundary between LM and BW sites. Such lack of concordance between intraspecific phylogeographic breaks and biogeographical province boundaries has been reported in a number of marine species. It appears that the concordance between intraspecific and interspecific breaks may only occur where the interspecific breaks are between closely related taxa (Burton 1998).

The level of cytochrome *b* divergence between *U. carinirostris* clades is of similar order that separates congeners in fishes (Johns and Avise 1998). In fact only 21% of comparisons among sister species of fishes exhibit cytochrome *b* sequence divergence larger than the divergence among intraspecific clades in *U. carinirostris* (Johns and Avise 1996). Allozyme data suggest interbreeding among clade types where the two clades are sympatric. Allozyme variation at six loci in *U. carinirostris* exhibits no major deviations from Hardy-Weinberg equilibrium expectations at BC and EH sites where the two clades are sympatric (J.M.H. and R.C.C., manuscript in preparation). Deficiencies of heterozygotes at these sites would suggest that individuals from each clade are reproductively isolated. It is possible that our test lacked the power to identify reproductive isolation, which may occur if allele frequencies at the loci assayed are similar in the two groups. For example, two sympatric species of marine snail *Nucella emarginata* and *N. ostrina* exhibit only one fixed difference at nine polymorphic loci (Marko 1998); yet breeding studies indicate they are reproductively isolated (Palmer et al. 1990). Nevertheless, the divergence between the two pipefish clades is very high for populations that are morphologically so similar that traditional taxonomy places them in the same species.

Secondary contact of non-reproductively isolated lineages is not uncommon in marine fishes. The marine teleost, *Lates calcarifer*, does not exhibit any evidence of reproductive isolation despite a period of allopatry separating Pacific from Indian Ocean populations (Chenoweth et al. 1998), although in that case the levels of divergence were much lower (0.47% at mtDNA protein coding genes). Similarly, Magoulas et al. (1996) reported no evidence for reproductive isolation following secondary contact among Aegean and Mediterranean populations of the anchovy, *Engraulis encrasicolus*, which are separated by 3.75% sequence divergence (estimated from RFLP data). Gonzalez-Vilansenor and Powers (1990) concluded that secondary contact was the best explanation for the phylogeographic pattern exhibited by the killifish, *F. heteroclitus*. Although subtle differences in egg size and body form were noted between the historically differentiated populations, there was no evidence for the evolution of reproductive isolation between groups.

Our goal was to assess the degree of phylogeographic structure displayed in *U. carinirostris,* a fish that has seemingly limited potential gene flow and represents an extreme in the presence of specialisation. We detected evidence not only for strong phylogeographic structure but also for gene flow as many haplotypes within clades are shared across distant locales (Fig. 2). The specialised life history of *U. carinirostris* appears to have played a part in shaping its phylogeographic patterns in that the species is susceptible to environmental perturbations that may reduce gene flow via habitat loss. However, it is also evident that these features were not sufficient to prevent a post-vicariance range expansion during the Pleistocene.

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