Mitochondrial Phylogeography of the Intertidal Isopod *Excirolana braziliensis* on the Two Sides of the Isthmus of Panama

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**ABSTRACT.** The intertidal isopod *Excirolana braziliensis* Richardson possesses limited means of dispersal; there is no larval stage, and adults remain sedentary under the sand. It is represented on the two coasts of Panama by three morphs, two in the Pacific (P and C') and one in the Atlantic (C). Previous work has quantified morphometric differences between the morphs, found that there are multiple allozyme differences between them, and produced indirect evidence that they are reproductively isolated from each other. Here we report comparisons of 345 bp of 12S and 678 bp of cytochrome oxidase I (COI) mitochondrial DNA (mtDNA) from three populations of each morph. The mtDNA sequences from the three morphs are reciprocally monophyletic, strengthening the case for recognizing them as separate species. As in morphology and isozymes, the C morph and the C' morph are sister clades, and the P morph is an outgroup. In contrast to what was previously supposed, the C and C' morphs neither are the result of a recent introduction from one ocean to the other, nor were separated at the final stages of the completion of the Isthmus of Panama three million years ago, but rather are anciently separated sister clades that now exist on separate shores. Patterns of mitochondrial gene flow between populations of the same morph vary. The C and C' morphs show large genetic differences between local populations, as would be expected from an organism with such limited vagility. In the P morph, on the other hand, populations from localities 5 km apart are identical in mitochondrial DNA, even though they differ in one allozyme locus, suggesting the possibility of sex-biased migration.

**INTRODUCTION**

Many marine organisms are capable of dispersing over large distances at some point of their life cycle. The population genetics of such organisms usually reveal a genetic neighborhood size in the order of thousands or tens of thousands of kilometers. Some marine species, however, provide a contrast to this picture of wide dispersal in that they lack any means of transferring their genes by either vagile adults or free-swimming larvae, yet have wide geographic ranges. How much gene flow may occur between noncontiguous populations of such species and whether species cohesion is maintained in the face of limited vagility is of special interest to population genetics. The tropical isopod *Excirolana braziliensis* is an example of a species apparently spread over the tropical seas of Americas, even though it lacks the means of maintaining genetic contact between distant populations.
Excirolana braziliensis Richardson is a common isopod of intertidal beaches on both sides of the Americas, from the Pacific coast of Mexico (30°N) to S. Chile (40°S) and from the northern Caribbean (31°N) to Uruguay (25°S) (Cardoso and Defeo, 2003). It is a small (approximately 3–4 mm in length), dioecious species, which reaches its highest abundance just above the high-tide mark, where it lives buried in the sand during low tide and rises to the water column at high tide to feed on live and dead fishes and invertebrates (Brusca and Iverson, 1985). E. braziliensis has very limited means of dispersal. The female carries broods of 4 to 17 offspring per reproductive event. Young are released directly into the adult habitat (Klapow, 1970). The frequency of reproduction is such that a population may turn over every four months (Brusca and Iverson, 1985). In Panama, recruitment occurs throughout the year (Dexter, 1977). Dispersal in E. braziliensis may occur as a result of feeding events, during which individuals of this genus have been observed attached to fish or other prey items for several minutes (Brusca, 1980). This behavior may represent the only means of transport of this organism between beaches because free-swimming individuals are likely to be eaten by fish.

Weinberg and Starczak (1988, 1989) reported the existence of three morphological variants of E. braziliensis from Panama. Two similar and presumably closely related types, termed C and C’, are found on the Caribbean and Pacific coasts, respectively. The third type (P) is morphologically distinct from C and C’; its distribution overlaps with that of C’ throughout most of its range (Weinberg and Starczak, 1989). In general, Pacific beaches contain only one of the two morphotypes. Nevertheless, 2 of 43 beaches sampled by Weinberg and Starczak (1989) were found to contain C’ and P morphs in approximately equal numbers. The geographic patterns of morphotype distribution and genetic composition remain stable over time, but there are occasional complete replacements of entire beaches by a different morph, presumably as the result of extirpation and subsequent recolonization (Lessios et al., 1994). Morphological and genetic divergence (based on allozyme data) between morphs are highly correlated and large enough to suggest that the P morph constitutes a distinct species (Lessios and Weinberg, 1994). The allozyme data are also consistent with the hypothesis that the C and C’ morphotypes are geminate species that resulted from the rise of the Panamanian Isthmus three million years ago (Lessios and Weinberg, 1994).

Allozyme analyses indicate that the three morphotypes of E. braziliensis are probably reproductively isolated, because they form few hybrids even when they co-occur at the same beach. Even within morphotypes, gene flow between populations from different beaches is low, as deduced from the predominance of distinct alleles in one or more loci, even among beaches situated less than 5 km apart. However, dispersal (as measured by individuals homozygous for alleles that otherwise occur on a different beach) is rather high, suggesting that some form of reproductive isolation prevents them from mating with individuals from the local population (Lessios and Weinberg, 1993).

The purpose of the present study is to investigate the phylogenetic and phylogeographic relationships within and between the three morphotypes of Excirolana braziliensis using sequences of mitochondrial DNA (mtDNA). Specifically, we are addressing the following questions: (1) Does mtDNA show patterns of genetic divergence, phylogeny, and geographic distribution congruent with those suggested by isozymes and morphology? (2) When did the three lineages diverge? (3) What are the patterns of population genetic structure? Do mtDNA data show similar levels of gene flow as isozymes within and between morphotypes? (4) What processes can explain mtDNA discrepancies between patterns from mtDNA and allozyme markers?

MATERIALS AND METHODS

SAMPLE COLLECTION

Excirolana braziliensis were obtained from nine locations along the Pacific and Caribbean coasts of Panama (Figure 1). Each of the three morphotypes was represented in our collections by three populations. Isopods were collected on beaches during low tide. The top 10 cm of sand at haphazard locations above the high-tide mark were sifted through a 500 µm sieve, and isopods were placed in plastic bags with wet sand. The collected isopods were brought alive to the laboratory and frozen at −80°C. The majority of samples used in this study were from collections made in 1988, the same collections used to assay isozymes (Lessios and Weinberg, 1993, 1994). Additional individuals were collected in 1998 from Isla Culebra. Specimens of Excirolana mayana were also collected at Isla Culebra to be used as outgroups.

DNA EXTRACTION, POLYMERASE CHAIN REACTION, AND mtDNA SEQUENCING

Genomic DNA was extracted using a standard phenol/chloroform protocol (Sambrook et al., 1989) with ethanol precipitation. For amplification and sequencing
of 345 base pairs (bp) of the 12S mtDNA gene, we used the universal primers 12Sa and 12Sb (Simon et al., 1994). A 678 bp fragment of cytochrome oxidase I (COI) was amplified and sequenced with combinations of the forward primers BWBK (5' /H11032 -GAG CTC CAG ATA TAG CAT TCC-3' /H11032 ) and ISO-F1 (5' /H11032 -CYC TTT TAT TAG GRA GGG GG-3' /H11032 ), and the reverse primers BWBJ (5' /H11032 -CAA TAC CTG TGA GTC CTC CTA-3' /H11032 ) and ISO-R2 (5' /H11032 -ACR GCA ATA ATT ATG GTA GC-3' /H11032 ). The following conditions were used for polymerase chain reaction (PCR): initial denaturation for 2 min at 94°C, then 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 50°– 53°C, extension for 1 min at 72°C, and final extension for 10 min at 72°C. PCR products were cleaned for sequencing using silica gel purification columns. Cycle sequencing was carried out in both directions, with the ABI PRISM d-Rhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystematics). Sequences were obtained on an ABI 377 automated sequencer and verified and aligned by eye in the program Sequencher (Gene Codes Corporation). 12S was sequenced in 104 individuals; a subset of 22 individuals was also successfully sampled for COI, whereas the rest failed to amplify for this locus.

Phylogenetic Analysis

For phylogenetic analysis, identical haplotypes from multiple individuals were collapsed. We applied the program Modeltest 3.0 (Posada and Crandall, 1998) to calculate the goodness of fit of various models of DNA evolution. The selected model for the 12S data was that of Tamura and Nei (1993), with equal base frequencies, a gamma distribution with a shape parameter of 0.443, and the following substitution rates: [A—C] = 1.00; [A—G] = 6.00; [A—T] = 1.00; [C—G] = 1.00; [C—T] = 11.71; and [G—T] = 1.00. The selected model for the COI sequence was a transversional model (TVM + I; Posada and Crandall, 2001), with a proportion of 0.69 of invariant sites and the following substitution rates: [A—C] = 8908.76; [A—G] = 258211.81; [A—T] = 26092.37; [C—G] = 0.0001; [C—T] = 258211.82, and [G—T] = 1.00. A partition homogeneity test, executed in version 4.0b10 of PAUP* (Swofford, 2000), indicated that phylogenetic signals in the COI and 12S data were not significantly different (P = 0.256). The best fitting model for the combined 12S and COI data was HKY (Hasegawa et al., 1985) with a transition/transversion ratio of 11.46 and a gamma distribution shape parameter of 0.782. Employing these parameters, we ran phylogenetic analyses for 12S and COI separately and with the two DNA regions concatenated. We used the BioNJ algorithm (Gascuel, 1997) and heuristic searches in maximum parsimony and maximum likelihood with PAUP* (Swofford, 2000). Bootstrap confidence values for distance and likelihood trees were calculated in 5,000 and 500 iterations, respectively. Bayesian phylogeny inference was carried out in the program MrBayes v.3.04b (Huelsenbeck and Ronquist, 2001). Bayesian analyses on the COI and the combined data sets were run for 800,000 generations, of which the first 20,000 (2,000 trees) were discarded. For 12S, 2,760,000 generations were run, and 67,500 (6,750 trees) were discarded. Convergence of chains was determined by average standard deviations of split frequencies less than 0.01 and by potential scale reduction factors approximately equal to 1.0. The trees were rooted on sequences of Excirculana mayana. Clock-like evolution of sequences was tested with likelihood ratio tests. The tests were carried out in PAUP* 4.0b10 by calculating the difference in log-likelihood of the neighbor-joining trees (see above) with and without the enforcement of a molecular clock and comparing the likelihood ratios to the χ² distribution.

Geographic Distribution of Genetic Variation within Morphs

Genealogical relationships of haplotypes within species may be better represented by networks than trees, as
ancestral haplotypes may still be present in the population (Crandall and Templeton, 1993; Posada and Crandall, 2001). We calculated unrooted parsimony haplotype networks based on 12S for each of the three morphotypes separately, using the computer program TCS (Clement et al., 2000). In this method the parsimony limit (the maximum number of differences among haplotypes as a result of single substitutions) is calculated with 95% statistical confidence, and haplotypes are connected in order of increasing number of substitutions. To investigate the population genetic structure within each morphotype, we applied analysis of molecular variance (AMOVA; Excoffier et al., 1992) to the 12S data. Genetic variation for this analysis was assessed based on the Kimura (1980) two-parameter distance between haplotypes. The significance of fixation indices was tested by 10,000 rearrangements of haplotypes between populations. Calculations were carried out in version 2000 of the computer program ARLEQUIN (Schneider et al., 2000).

RESULTS

DESCRIPTIVE STATISTICS AND PHYLOGENETIC ANALYSIS

Although we sampled many more individuals of *Excirolana braziliensis* for 12S than for COI, trees based on the former DNA region (Figure 2) displayed less resolution than the combined analysis of both genes together. Despite this, all analyses of the 12S segment alone resulted in three distinct lineages, which correspond to the previously described C, C’, and P morphs. The 12S sequences of each morph were monophyletic in all analyses. The node joining C and C’ was well supported by maximum-parsimony analysis but fairly weakly supported by neighbor-joining, maximum-likelihood, and Bayesian analysis. The three main lineages were present in more than one beach, but each beach contained representatives of only one lineage. Although Santelmo had previously been found to contain a mixture of C’ and P morphotypes and the allozymes corresponding to these morphs (Weinberg and Starczak, 1989; Lessios and Weinberg, 1994), all nineteen 12S sequences from Santelmo, differing from each other by a maximum of three substitutions, belonged to the C’ morph. The tree based on fewer sequences of COI (not shown) and the tree based on the combined data (Figure 3) were well resolved and gave strong support to the expected grouping of the C and C’ lineages as sister groups, irrespective of the type of phylogenetic algorithm used.

The 12S Tamura and Nei average distances ranged between 11% and 18% between morphs (Table 1). Within morphs, distances varied between 0% and 2.3%. For COI, average distances (TMV) among lineages were 17.4%–26.1% and within lineages 0%–1.5%. There were five amino acid changes in the COI fragment, of which four were substitutions of nonpolar for nonpolar residues (Met/Ileu; Val/Ileu) and one was a nonpolar for a polar residue (Ala/Thr). Three of the changes differentiate the C/C’ and P lineage; one groups C and P, versus C’, and one is shared between C’ and P, compared to C. Likelihood ratio tests failed to reject the hypothesis of clock-like evolution of either the 12S or the COI sequences (P > 0.05).

GENETIC VARIATION WITHIN MORMPHS

Parsimony haplotype networks showed that populations of the C and C’ morphs, but not the P morph, were genetically structured (Figure 4). The most common and (presumably) ancestral haplotype of the P morph was shared by all three populations. Two derived haplotypes were also shared, one between all populations and the other between two populations. In the C’ morph two haplotypes, including the ancestral one, were shared between Santelmo and Isla Culebra. Although Isla Adentro contained three haplotypes not found in any other population, the majority of specimens from this island were of a single haplotype, leading to a low haplotype diversity compared to other populations (H = 0.14). The population at Bocas del Toro (C morph), was characterized by high nucleotide diversity compared to other populations (π = 0.0077 ± 0.0045). Haplotypes from Bocas del Toro were differentiated from Maria Chiquita and Shimmy Beach by one to eight substitutions whereas the latter two populations shared the ancestral haplotype.

In the C morph, AMOVA (Table 2) found that 67.44% of genetic variance was partitioned among populations; population pairwise FST comparisons (Table 3) showed that all populations of this morph were significantly differentiated from each other. In the C’ morph, 35.62% of the variance was the result of differences between populations. The population at Adentro had significantly high FST values when compared to both Santelmo and Culebra, whereas the latter two were not significantly different from each other (Table 3). In the P morph, all the genetic variance was contained within populations, a result in stark contrast with high levels of population subdivision seen in the other two morphs.
FIGURE 2. 12S mitochondrial DNA (mtDNA) maximum-likelihood bootstrapped consensus tree relating three morphotypes (C, C', and P) of *Excirolana braziliensis*. Numbers above branches indicate maximum-likelihood bootstrap confidence values; numbers below branches refer to posterior probabilities (Bayesian analysis), neighbor-joining bootstrap support, and maximum-parsimony bootstrap support, respectively, from top to bottom. Support values <50% are not shown. Locality codes of specimens: prco = Perico; csw = Causeway; lb = Lab; bca = Bocas del Toro; mcq = Maria Chiquita; shmy = Shimmy Beach; ade = Isla Adentro; bs = Isla Culebra; selm = Santelmo; xbs = Isla Culebra (xbs specimens were collected in 1998; all other samples were collected in 1988). See Figure 1 for the position of each locality.
DISCUSSION

The mtDNA data presented here confirm the results from analysis of both morphology (Weinberg and Starczak, 1988, 1989; Lessios and Weinberg, 1994) and allozymes (Lessios and Weinberg, 1994) that *Excirolana braziliensis* populations from the Pacific and Caribbean coasts of Panama consist of three distinct lineages. Allozymes suggest that these lineages are reproductively isolated (Lessios and Weinberg, 1993) and should, therefore, be considered separate species. Although mtDNA data agree with morphological and allozyme data on the grouping of the C and C’ lineages as sister groups with respect to P, the relative magnitude of the measures of differentiation in the three sets of characters is different. Mahalanobis generalized distance from morphometric characters and Nei’s D from allozymes indicate that the P morphotype is three times more distant from C and C’ than the latter are from each other (Lessios and Weinberg, 1994). Mitochondrial DNA, on the other hand, gives a P/(C, C’) distance that is only 1.2 to 1.3 times higher than that between C/C’.

A review of molecular divergence across the Isthmus of Panama in 34 lineages likely to have been separated by the final closure of the Isthmus of Panama (Lessios, 2008) has shown that during 3 million years of independent evolution (Coates and Obando, 1996; Coates et al., 2005), crustacean COI has accumulated genetic distances ranging from 4.1% to 8.7% (Knowlton and Weigt, 1998; Schubart et al., 1998; Williams et al., 2001; Morrison et al., 2004) and 12S ranging from 2% to 3% (Robles et al., 2007). Based on these calibrations, and given the differences we determined in COI and 12S, the divergence of the P morph from the two C morphs occurred between 9 and 25 million years ago and that of C from the C’ morph between 6 and 17 million years ago. Thus, in contrast to what was surmised by Lessios and Weinberg (1994) on the basis of isozymes, mtDNA data do not support the idea that the C and C’ morphotypes were isolated at the final stages of the closure of the Panamanian Isthmus, 3 million years ago, but rather that their populations were separated well before the final closure. On the basis of molecular divergence, this appears to be also the case in 73 other amphio-isthmic sister lineages of crustaceans, sea urchins, fishes, and mollusks (Lessios, 2008).

The combination of large mitochondrial differences and evidence for reproductive isolation from allozyme data (Lessios and Weinberg, 1993, 1994; Lessios, 1998) rules out the hypothesis that C’ merely represents a recently
FIGURE 4. Parsimony network of 12S mtDNA haplotypes of the three morphs (clades) of *Excirolana braziliensis*. Each large oval represents a unique haplotype, boxes represent ancestral haplotypes, and small ovals indicate hypothetical, intermediate haplotypes not observed in the populations. The size of each shape represents the frequency of each haplotype. Numbers within each symbol indicate the number of individuals bearing each haplotype. Localities: Prco = Perico; Lab = Lab; CSW = Causeway; ADE = Isla Adentro; BS = Isla Culebra; SELM = Santelmo; MCQ = Maria Chiquita; BCT = Bocas del Toro; SHMY = Shimmy Beach.
introduced population of C from the Caribbean into the Pacific, as had been suggested by Weinberg and Starczak (1988, 1989) and strengthens the case that each of the three lineages represents a distinct species.

**Population Structure and Dispersal**

Populations of the C and C’ morphs were characterized by population subdivision, as illustrated by high $F_{ST}$ estimates (overall values of 0.67 and 0.36, respectively), whereas samples from different localities of the P morph can be considered as belonging to the same genetic population ($F_{ST} = -0.02$). Populations from Isla Adentro (C’ morph) and from Bocas del Toro (C morph) stand out for their lack of alleles shared with individuals from other localities. Maria Chiquita and Shimmy Beach (C morph) also have significantly different allele frequencies, whereas the populations at Santelmo and Isla Culebra, as well as at Perico, Lab and Causeway, are not significantly differentiated. The two populations most divergent from others in the same morph, Adentro and Bocas del Toro, are also the most geographically distant from other localities containing individuals of their respective morphs, raising the possibility that dispersal to and from these localities is restricted as a result of physical distance. With only three populations per morph, statistical verification of a correlation between geographic and genetic distances is not meaningful.

We observed several differences in the degree of population subdivision when comparing mtDNA and allozyme markers (Lessios and Weinberg, 1994). Based upon mtDNA sequence, the populations at Bocas del Toro and Shimmy Beach were the most different of all ($F_{ST} = 0.71$), whereas their allozyme allele frequencies were rather similar ($F_{ST} = 0.097$, as calculated from data in Lessios and Weinberg, 1993). On the whole, mtDNA data suggest a higher divergence between the morphs, but a lesser degree of subdivision between populations of the same morph, compared to data on allozymes. These results support Lessios and Weinberg’s (1993) findings that dispersal among populations is much higher compared to gene flow, because even individuals of the same morph show some sort of reproductive isolation. According to their estimates, up to 2.5% of individuals in a locality consist of new immigrants that do not inject their genes into the host population, indicating that some form of reproductive isolation exists between populations of the same morph, even at the scale of a few kilometers. The data from Santelmo are interesting in this connection: This is the only locality in which two morphs, P and C’, coexist (Lessios and Weinberg, 1993, 1994). The number of hybrids between them, as judged by allozymes, is lower than would be expected from random mating (Lessios and Weinberg, 1993), but hybrids do exist. However, all 19 mitochondrial haplotypes from this locality belong to the mtDNA clade that corresponds to the C’ morph, despite having been sampled from the same collections as the allozymes. Barring the unlikely possibility of a sampling accident, this finding indicates that some individuals with a P nuclear genotype, as manifested in morphology and isozymes, actually carry

**Table 2.** Analysis of molecular variance (AMOVA) of Panamanian populations of *Excirolana braziliensis* based on 12S mtDNA sequences. Partitioning of genetic variance within and between populations (beaches) was estimated for each morph (clade) separately. The significance of fixation indices was tested by 10,000 permutations.

<table>
<thead>
<tr>
<th>Morph</th>
<th>Between populations</th>
<th>Within populations</th>
<th>$\Phi_{CT}$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>67.44</td>
<td>32.56</td>
<td>0.67</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C’</td>
<td>35.62</td>
<td>64.38</td>
<td>0.36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P</td>
<td>-2.39</td>
<td>102.39</td>
<td>-0.02</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

**Table 3.** *Excirolana braziliensis* population pairwise $F_{ST}$ values from 12S mtDNA sequences. Bold values are significant at the $P < 0.01$ level.

<table>
<thead>
<tr>
<th>P Morph</th>
<th>Lab</th>
<th>Perico</th>
</tr>
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<tbody>
<tr>
<td>Perico</td>
<td>-0.02</td>
<td>-0.02</td>
</tr>
<tr>
<td>Causeway</td>
<td>-0.02</td>
<td>-0.04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C Morph</th>
<th>Maria Chiquita</th>
<th>Bocas del Toro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bocas del Toro</td>
<td>0.69</td>
<td>-</td>
</tr>
<tr>
<td>Shimmy Beach</td>
<td>0.27</td>
<td>0.71</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>C’ Morph</th>
<th>Isla Adentro</th>
<th>Isla Culebra</th>
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</thead>
<tbody>
<tr>
<td>Isla Culebra</td>
<td>0.58</td>
<td>-</td>
</tr>
<tr>
<td>San Telmo</td>
<td>0.50</td>
<td>-0.02</td>
</tr>
</tbody>
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a C’ mitochondrial DNA. This, in turn, suggests that hybridization between the morphs, when it occurs, is successful in only in one direction, that is, only if the mother belongs to the C’ clade.

In conclusion, *E. braziliensis* in Panama consists of at least three lineages (C, C’, and P), which diverged well before the final closure of the Isthmus and warrant separate species status. Populations that are more than 30 km distant from each other (C, C’) are genetically divergent, whereas those at less than 5 km (P) are panmictic in mtDNA, even though they are different in at least one allozyme locus (Lessios and Weinberg, 1994). It remains to be seen whether population structure is a result of isolation by physical factors or whether the three species have inherently different dispersal potential, and whether the higher degree of gene flow in mtDNA relative to isozymes is the result of sex-biased migration.

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**LITERATURE CITED**


