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Regional endemism and cryptic species revealed by molecular and morphological analysis of a widespread species of Neotropical catfish

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The lower Central American landscape was fully emergent approximately three million years ago, an event which marked the beginning of the Great American biotic interchange. Freshwater fishes participated in the biotic interchange. Because primary freshwater fishes are restricted to freshwater, they provide an excellent system for investigating the interplay of historical and recent processes on the assembly, structure and diversity of the regions' aquatic ecosystems. We focused on examining the history of diversification for a species of catfish (*Pimelodella chagresi*) whose distribution spans multiple, isolated drainage basins across the Isthmian landscape and into north-western South America. Analysis of mitochondrial DNA haplotypes and morphological traits indicated that *P. chagresi*, as currently recognized, comprises a species complex. In addition, along the Pacific slope of Panama, repeated dispersion, diversification, extinction and possibly hybridization are thought to underlie a complex distribution of haplotypes. Overall, the results underscore the tremendous importance of historical processes on regional biodiversity.

Keywords: Neotropical; catfish; phylogenetics; biodiversity

1. INTRODUCTION

Lower Central America (LCA), including Panama and Costa Rica, presents an ideal landscape for investigating the interaction of dispersal, diversification and extinction on regional biodiversity. The region is recent, limiting the complexity of biogeographical patterns caused by the superimposition of ancient and recent divergence and dispersal characteristic of other continental systems (e.g. Joseph *et al.* 1995; Taberlet *et al.* 1998). LCA is relatively large and topographically complex. Consequently, local catastrophic events are unlikely to play a large role in shaping regional diversification as is often the case with well-studied island systems (e.g. the Caribbean) (Spiller *et al.* 1998). For freshwater fishes, additional advantages exist. It is likely that the birth of the Isthmian landscape witnessed a rapid and extensive spread of freshwater fishes such that the entire region was colonized more or less simultaneously (Bermingham & Martin 1998). In addition, most if not all primary freshwater fishes invaded Central America from a common source region in north-western South America (Miller 1966; Myers 1966; Bussing 1985). The rapid spread of multiple lineages and a common source region constrain the possible historical effects on the species diversity of LCA. Finally, the landscape is arranged as two linear transects of adjacent drainages from South America into Costa Rica which flow on either side of a central cordillera. This latter feature closely approximates a stepping-stone organization of populations and, therefore, provides an excellent means for gauging the frequency and magnitude of alongshore and cross-cordillera dispersal.

Many species of freshwater fishes in LCA have distributions which span multiple, isolated drainages (Loftin 1966; NEODAT 1998). Given that the dispersal of freshwater fishes is limited by direct connections between drainages (Darwin 1859), the observation of wide-ranging species suggests several alternative hypotheses. The region may have been recently colonized and isolated populations have not had sufficient time to manifest unique traits which would warrant recognition as distinct species. Depending on the nature of selection, population size and the levels of genetic variation, divergence of form may take a long time. Alternatively, sufficient gene flow may exist to maintain morphological and genetic cohesion across the wide and dissected distribution. Finally, significant genetic divergence between currently isolated populations may exist, which is not evident from analysis of phenotypes (see, for example, Schneider & Moritz 1999). Lack of phenotypic divergence may indicate strong stabilizing selection or some sort of canalization of development underlying the expression of morphological traits or simply reflect that the distinction between populations is subtle and requires careful scrutiny in order to discern significant differences.

If widespread species of freshwater fishes do consist of multiple, incipient species, this suggests that isolated drainage basins may harbour endemic assemblages of species comprising aquatic communities. On the other hand, invoking episodic, chance dispersal events creates opportunities of mixing together divergent forms, which may have varied consequences depending on reproductive compatibility. In some cases divergent forms may coexist with minimal ecological overlap, while in other cases divergent forms may enter the same gene pool, resulting in increased genetic and phenotypic variance. These alternative scenarios have dramatically different ecological and evolutionary implications. In addition, resolution of the alternative hypotheses has implications for deciphering the relative importance of the historical and

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Figure 1. Map showing most of the distribution of *P. chagresi*. The number and letter codes identify particular rivers and correspond to the bold characters of the taxon names in figure 2. The dots represent collections of *P. chagresi*. Light grey lines represent the continental divide (the cordillera) and dark grey lines indicate the river systems.

contemporary processes on the assembly, structure and diversity of ecological communities (Ricklefs & Schluter 1993).

We have previously inferred the area relationships of LCA based on a combined analysis of gene trees for three widespread species of primary freshwater fishes, *Brachyhypopomus*, *Roeboides* and *Pimelodella* (Bermingham & Martin 1998). In this paper we focus our analysis on one of these three species, the common and widespread species *Pimelodella chagresi*. Our previous study indicated that there have been multiple episodes of invasion of *Pimelodella* into LCA from South America and some evidence for the dispersal and extinction of lineages across the LCA landscape (Bermingham & Martin 1998). In this paper we have concentrated on inferring the evolutionary dynamics of divergence and dispersal across the whole range of *P. chagresi*. In particular, we are interested in quantifying the extent to which isolated drainages harbour unique lineages. This is accomplished using a phylogenetic analysis of mitochondrial sequences, geographical analysis of mitochondrial (mtDNA) restriction fragment length polymorphism (RFLP) haplotype frequencies and an assessment of morphological divergence for several unique mitochondrial haplotypes. The sympatry of two divergent haplotypes in many of the Pacific slope rivers provides an opportunity of assessing the degree of morphological distinction in areas where there is mixing of lineages. These data should provide a metric for establishing the significance of observed mtDNA diversity.

2. MATERIAL AND METHODS

We sampled individuals from across the entire range of *P. chagresi* (figure 1), including the Pacific slope of eastern Costa Rica, both slopes of Panama, north-western Colombia (the Choco region), the Magdalena valley of Colombia and north-western Venezuela (the Maracaibo basin). In addition, we also sampled species of *Pimelodella* from Trinidad and the Amazon and Orinoco basins (in Peru and Guyana, respectively). Species were identified using Eigenmann (1922), except for specimens collected from Venezuela and Peru which were identified by L. Page (Illinois Natural History Survey) and H. Ortega (Museo de Historia Natural, Universidad Nacional Mayor de San Marcos), respectively. Fishes were collected using a Smith–Root electrofisher, cast-nets or seines. Gill arches and filaments and small pieces of muscle were preserved at ambient temperature in a saturated salt solution (NaCl) of dimethyl sulphoxide (DMSO) and disodium ethylenediamine tetra-acetate (EDTA) (Seutin *et al.* 1991). The whole fish was labelled with a number corresponding to the tissue sample and preserved in buffered formalin and later transferred to 70% ethanol. The samples were collected, exported and imported under appropriate permits. Venezuelan fishes were provided as tissue loans by the

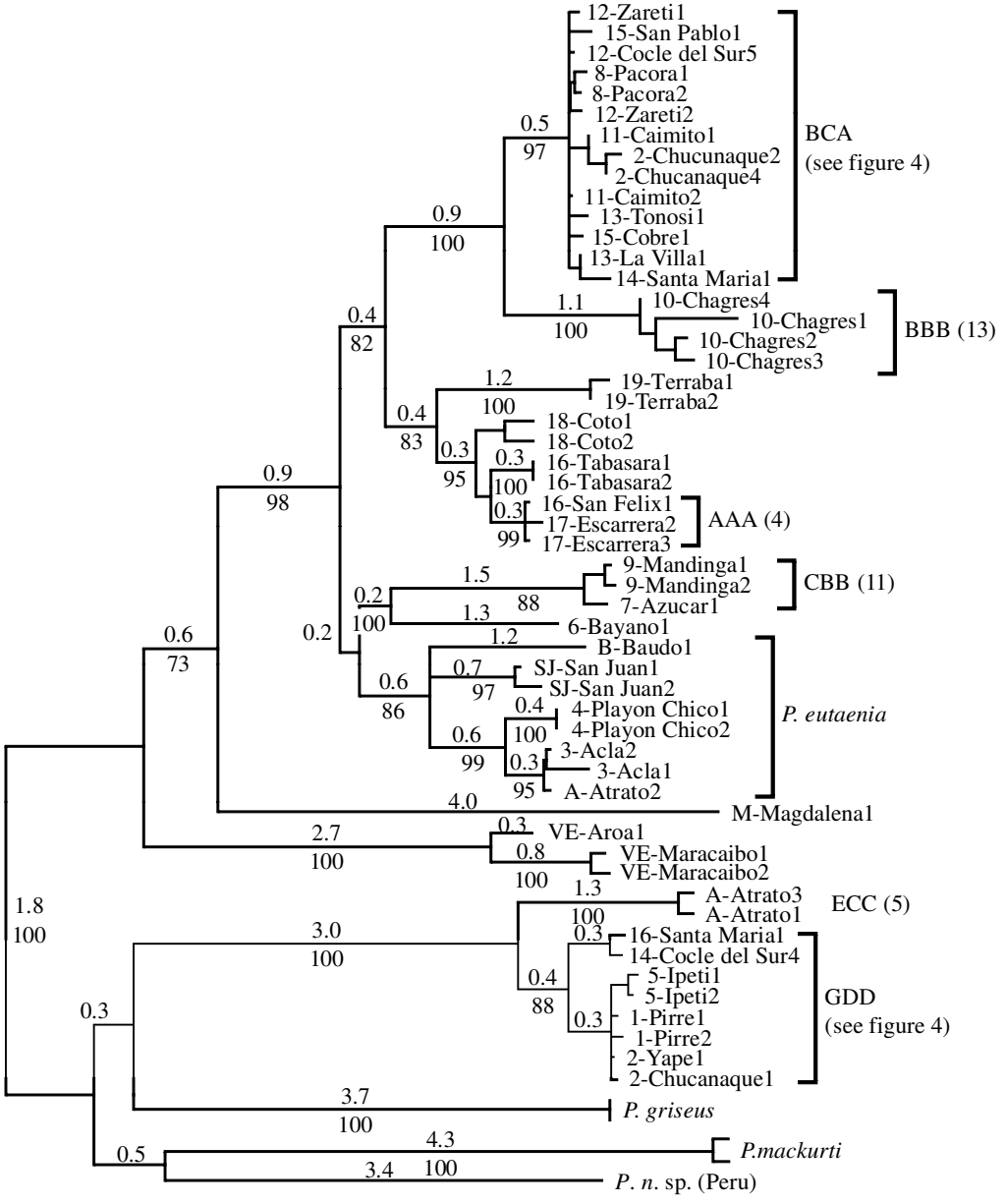

Figure 2. Phylogram of a bootstrap tree based on the neighbour-joining analysis of the Kimura two-parameter-corrected genetic distances. The numbers above branches are the estimated numbers of substitutions per 100 sites. The numbers below branches are bootstrap values. The three-letter codes are composite haplotypes based on three endonucleases (*Hae*III, *Hin*fI and *Msp*I). The numbers in parentheses indicate the number of individuals surveyed for the RFLP haplotype. The taxon labels included a number corresponding to collection sites in figure 1, the name of the river and the individual sequence number.

Illinois Natural History Survey. All DNA sequences reported herein have a numbered voucher specimen (see electronic Appendix A available on The Royal Society Web site).

We chose a limited number of individuals for direct sequence analysis of the ATPase 6/8 gene using the geographical distributions of the specimens collected, qualitative morphological assessment of the voucher specimens and the prevailing *Pimelodella* taxonomy. In all cases we sequenced at least two individuals per drainage, a replication strategy which served to establish confidence in the mtDNA sequences used in our phylogenetic analyses. Additional individuals were sequenced for drainages with multiple haplotypes. We extended our survey to include more individuals by amplifying the ATPase gene and cutting the amplified product with three endonucleases (*Hae*III, *Hin*fI and *Msp*I) which were diagnostic for haplotypes defined by sequence analysis. Unique haplotypes defined by the RFLP analysis were sequenced so that they could be included in the construction of gene trees. In total we determined complete ATPase 6/8 sequences for 58 individuals and surveyed the RFLP patterns for 72 additional individuals.

The sequence data were aligned by eye and subjected to tree-building algorithms under different assumptions and models of evolution using PAUP* 4.0 (Swofford 1998). Genetic distances were corrected for multiple hits using a Kimura two-parameter model of sequence evolution and the relationships between haplotypes estimated by a 50% majority bootstrap consensus tree (based on 300 replications) generated by the neighbour-joining clustering algorithm. The tree was rooted using confamilial *Rhamdia* species. Nearly identical results were obtained when parsimony was adopted as the objective criteria, with the differences occurring in the basal portions of the tree without relatively high bootstrap support. Because our interest was in the

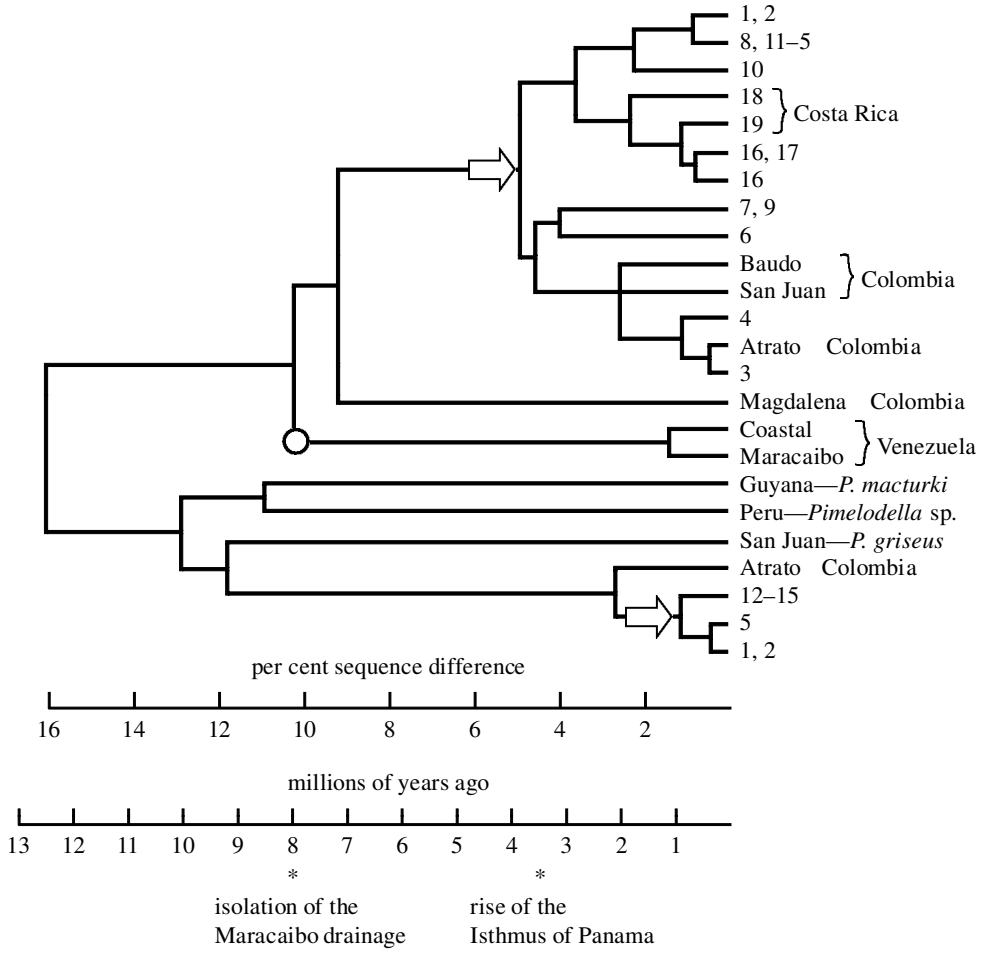

Figure 3. Area cladogram based on the ATPase 6/8 gene tree. The numbers correspond to the rivers shown in figure 1. The tree is drawn with branch lengths proportional to time based on fitting the sequence data to the bootstrap tree using an HKY + G maximum-likelihood model with a molecular clock constraint. The numbers refer to localities from figure 1. Open arrows indicate inferred colonization of fishes from South America into LCA. The time-scale was established by assuming that the isolation of the Maracaibo fishes occurred 8 Myr ago and is marked by an open circle. Based on the temporal scale of diversification, the basal divergence of Panamanian populations occurred at the time of the rise of the Isthmus in the Late Pliocene (*ca.* 3.5 Myr ago). The sequence divergence observed at this level of diversification is similar to the genetic distances for the same gene estimated for a number of species of transgeminate marine fishes (see Bermingham *et al.* 1997).

history of diversification of *P. chagresi* within LCA and not on the relationships between distinct species of *Pimelodella*, we adopted the neighbour-joining tree as our working hypothesis. Qualitatively identical and quantitatively indistinguishable results would have been obtained if we had adopted the parsimony tree as our working hypothesis of evolutionary history.

The tree was pruned so that only distinct haplotypes representing geographical areas were included. The temporal scale of diversification was estimated by fitting a HKY + G likelihood model of sequence evolution with a molecular clock constraint to the data using the neighbour-joining tree (Hasegawa *et al.* 1985). Parameters for a maximum-likelihood model of sequence evolution were estimated from the data (assuming the neighbour-joining tree) and the molecular clock tested using a log-likelihood ratio test (Huelsenbeck & Rannala 1997). An absolute time-scale was determined by assuming that the Maracaibo fishes were isolated eight million years (Myr) ago (Hoorn *et al.* 1995; Lovejoy *et al.* 1998).

A subset of individuals was surveyed for morphological variation by measuring four characters which can discriminate between different populations of *P. chagresi*: (i) the proportion of the pectoral spine with posterior projecting teeth, (ii) the length of the pectoral spine (i.e. the length from the base of the pectoral fin to the tip), (iii) the caudal peduncle length (i.e. the length from the posterior insertion of the adipose fin to the beginning of the dorsal fin ray of the caudal fin), and (iv) the caudal peduncle depth (i.e. the length from the posterior insertion of the adipose fin). In addition, the standard length from the tip of the snout to the centre of the base of the caudal fin (the end of the peduncle) was measured for each individual. An individual lacking knowledge of haplotypes made the measurements. Proportional values were subjected to arcsine square-root transformation. All other measurements were log transformed. Kolmogorov–Smirnov tests for a normal distribution were insignificant for all log-transformed data. The log-transformed variables (with the exception of the proportion of the pectoral spine with teeth) were regressed against the log of the standard length and the residuals used for testing whether different haplotypes exhibit different morphologies. Significant differences between haplotypes were estimated using an *a posteriori* test for planned comparisons (the least-squares difference (LSD) test) (Sokal & Rohlf 1981, pp. 243–244). Distinct groups defined by the LSD test were assigned a letter code. In addition, the residuals from the regression of the three log-transformed variables against the

log-transformed standard lengths and the transformed proportion data were subjected to factor analysis. Factor scores for the first principle component were tested for a difference in morphology using ANOVA. Tests of differentiation were performed between the two widespread haplotypes and between rivers within haplotypes.

3. RESULTS

We determined complete ATPase 6/8 sequences for 58 individuals and surveyed the RFLP patterns for 72 additional individuals. Figure 2 depicts the phylogenetic relationships between the 41 distinct haplotypes. A remarkable level of mtDNA diversity is buried within the taxon *P. chagresi*. The maximum corrected sequence divergence within *P. chagresi* approaches 16%. In addition, *P. chagresi* is polyphyletic. Individuals identified as *Pimelodella eutaenia* comprise a derived clade within one of the major clades of individuals identified as *P. chagresi*, whereas other samples of *P. chagresi* group together with *Pimelodella griseus* and *Pimelodella mackurti*.

The evolutionary history of *Pimelodella* was estimated using a reduced gene tree in which the branch lengths were scaled assuming a molecular clock (figure 3). The molecular clock assumption was justified by a log-likelihood ratio test ($-2\log\Lambda = 12.43$, $p > 0.9$ and d.f. = 22) (see Huelsenbeck & Rannala 1997). An absolute (in millions of years) time-scale of diversification was established by assuming that the isolation of the Maracaibo fishes occurred 8 Myr ago (Lovejoy *et al.* 1998). Two results are particularly noteworthy. First, LCA appears to have been colonized from South America by two widely divergent lineages at two different times (see also Bermingham & Martin 1998). The hypothetical ancestor of most LCA lineages existed 3–4 Myr ago, suggesting that the colonization and diversification of ancestral *P. chagresi* in LCA was coincidental with the rise of the Isthmus of Panama. The second putative invasion of a completely different lineage occurred sometime *ca.* 1 Myr ago. Second, different drainages harbour multiple mitochondrial lineages which vary in their degree of genetic differentiation. Some drainages have relatively closely related lineages (e.g. Costa Rica, the Western Pacific slope of Panama and most Atlantic drainages), whereas others harbour divergent mtDNA lineages (rivers of the Central and Eastern Pacific slope). The former case presumably reflects recent divergence within drainages (or recent genetic exchange between neighbouring rivers) while the latter case reflects the colonization and dispersal of a unique lineage which diverged from other LCA *P. chagresi* prior to the origin of the Isthmian landscape. In the putative source region (i.e. the Atrato) multiple *Pimelodella* species exist, even under the prevailing taxonomy.

The developing picture of the biogeographical history of LCA shows a strong signal of diversification driven by vicariant events coupled with episodic dispersal and local extinctions. This dynamic process appears to be operating across smaller geographical scales as well. This is particularly evident for the Pacific slope rivers which drain into the Bay of Panama. There is clear evidence of recent dispersal for the BCA and GDD haplotypes (figure 4). Nevertheless, the two haplotypes are not homogeneously distributed. Remarkably, two adjacent drainage basins

Table 1. *Summary of the differences in the morphological traits for different mitochondrial haplotypes*

(Haplotypes with the same letter code are not significantly different as determined by an *a posteriori* test for planned comparisons. The significance values were adjusted for multiple comparisons. *n*, number of individuals included in the analysis; pele, caudal peduncle length; CPD, caudal peduncle depth; sple, pectoral spine length; tele/sple, proportion of the pectoral spine with teeth. The composite score provides a metric for establishing morphological distinction across all measurements.)

haplotype	*n*	pele	CPD	sple	tele/sple	composite
AAA/CR	9	b	c	ab	a	bc(ab)a
BCA	45	a	a	ab	a	aa(ab)a
CBB	13	c	b	b	b	cbbb
GDD	44	a	d	bc	b	ad(bc)b
ECC	5	a	e	d	b	aedb
P. eutaenia	15	a	b	ab	b	ab(ab)b

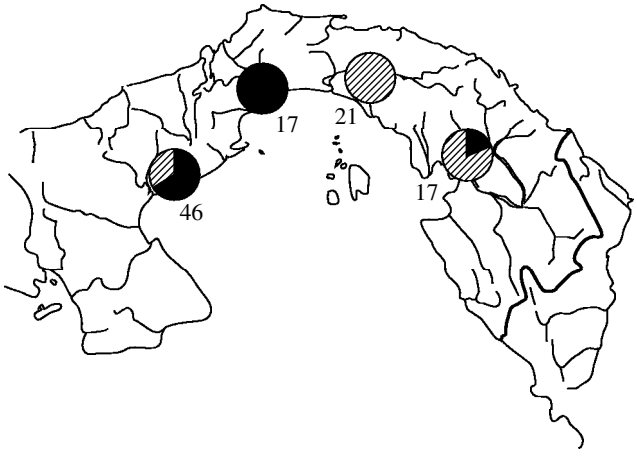

Figure 4. Map of central and western Panama showing the proportion of GDD (diagonal stripes) and BCA (black) haplotypes. The numbers are the sample sizes.

appear to harbour one or the other but not both haplotypes. All of the fishes sampled from the Bayano have the GDD haplotype, whereas the fishes inhabiting the adjacent drainage to the east have the BCA haplotype. This pattern may reflect biased (non-random) sampling, habitat selection or differential extinction of haplotypes. It is unlikely that the pattern reflects biased sampling because we used identical sampling methods across localities and typically sampled multiple streams within drainages. We also attempted to sample all habitats at any given collection locality. Thus, we favour the hypothesis that the observed pattern reflects differential extinction.

Our morphological measurements were meant to serve as a marker for phenotype in the same way as mtDNA haplotypes are markers for genotype. All distinct haplotypes for which we were able to measure a number of individuals were morphologically distinct (table 1). Importantly, the morphology of the two sympatric haplotypes (designated BCA and GDD) is significantly

different across Panama ($F = 158.1$ and $p < 0.0001$) and within the same river (the Cocle del Sur) ($F = 12.9$ and $p = 0.003$). The one exception to this general pattern is the Rio Pacora (locality 8 in figure 1). Although all of the fishes sampled from Pacora had the BCA haplotype, they were morphologically more similar to fishes with the divergent GDD haplotype ($F = 0.13$ and $p = 0.722$) than fishes with the same haplotype ($F = 63.2$ and $p < 0.0001$). Fishes with identical GDD haplotypes in different rivers did not exhibit significant morphological differentiation for the characters examined (for GDD haplotypes $F = 1.448532$ and $p = 0.23$). In contrast, the morphological comparisons between two populations with the BCA haplotypes (Rio Caimito and Cocle del Sur) suggested that morphological differentiation has taken place ($F = 11.4$ and $p = 0.002$), despite a lack of mtDNA differentiation.

4. DISCUSSION

Representatives of the taxon *P. chagresi* have been collected from numerous localities representing most of the species' range, which extends from northern Ecuador to Costa Rica on the Pacific slope and from north-western Venezuela to central Panama along the Caribbean versant (NEODAT 1998). Perusal of the mitochondrial gene tree indicated that the species as currently recognized harbours an immense amount of genetic variation. The genetic differences between haplotypes coupled with evidence for morphological differentiation suggest that *P. chagresi* is a species complex. Species descriptions of the genetic and morphologically distinct taxa identified in this study are forthcoming. Nevertheless, an important message from this study is that the current estimates of species diversity for Neotropical fishes may be severely underestimated.

Beyond descriptions of biodiversity, *P. chagresi* provides an instructive example of the complex history which underlies local species diversity. The strong geographical structure of distinct haplotypes argues that dispersal is rare and, in many cases, drainages are completely isolated and appear to have been for millions of years. Similar results are evident for other species (Bermingham & Martin 1998), implying that many drainage basins harbour endemic taxa.

The mosaic distribution of two divergent lineages along the Pacific slope is particularly interesting. We hypothesize that the two haplotypes (BCA and GDD) are descendants of two temporally displaced biotic invasions of fishes into Panama from putative source regions in north-western South America. The BCA lineages are more ancient members of the LCA landscape, arriving *ca.* 3–4 Myr ago. The GDD lineages are newcomers, arriving in the last million years or so and they appear to be spreading from east to west along the Pacific slope. In some places, the newest invader appears to have completely replaced the 'older' BCA lineage. The peculiar mosaic pattern may reflect differential lineage extinction due to the stochastic processes or contingency associated with the dispersal and establishment of populations. Alternatively, the pattern may reflect some deterministic process, such as competitive exclusion or introgressive hybridization, implying that the two species interact in streams and rivers in which they are sympatric. It is very likely that the species do interact because they appear to be ecological equivalents. The observation of fishes which are morphologically similar to the newest invader but possess the older BCA haplotype in the Rio Pacora suggests that introgressive hybridization may be occurring. Additional studies are necessary in order to test these alternative hypotheses. Whatever the explanation for the distribution of the mitochondrial and morphological variation in *P. chagresi*, our study clearly demonstrates a significant historical component underlying the local species diversity, even in relatively new and simple systems such as the Isthmian landscape.

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