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Colonization, population expansion, and lineage turnover: phylogeography of Mesoamerican characiform fish

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We present a phylogeographical analysis of four genera of Mesoamerican primary freshwater fish (*Brycon*, *Bryconamericus*, *Eretmobrycon*, and *Cyphocharax*). Three hundred and thirty-nine individuals were genotyped into one of 31 operational taxonomic units (OTUs) based on the nucleotide sequence of their mitochondrial *ATPase 6* & *8* genes (842–839 bp). Contrary to inference based on the species-level taxonomy of these genera, molecular data identified only a single case of sympatry between closely related OTUs, despite extensive parapatry. Polytomies dominate the mtDNA-based phylogenies and demonstrate multiple, noncontemporaneous waves of rapid expansion across Mesoamerica from South American sources. Analyses based on genetic distances observed among congeneric species of Mesoamerican primary freshwater fishes in comparison to divergence between transisthmian marine fishes permit the strong inference that the Pliocene rise of the Panama land bridge provided the first opportunity for the colonization of Mesoamerica by Characiform fishes. We develop a priority-effect model, based on the assumption that genetically closely related OTUs share similar ecological niches, to reconcile the general lack of contemporary sympatry between closely related OTUs with the substantial historical connectivity among Mesoamerican drainages demonstrated by the rapid expansion of *Brycon*, *Bryconamericus*, and *Cyphocharax*. Finally, in most cases, we infer that the westerly limits of freshwater fish distributions in Mesoamerica are more consistent with being defined by ecological factors rather than by dispersal limitation. *Biological Journal of the Linnean Society*, 2006, **88**, 235–255.

ADDITIONAL KEYWORDS: *ATPase* – mitochondrial DNA – niche-overlap – phylogeography – polytomies – primary freshwater fish – priority effect – synonymous substitution.

INTRODUCTION

During 200 years of scientific investigation into the determinants of spatial and temporal distributions of plants and animals, focus has centred on deep history and across broad geographical expanses (Buffon, 1761; Candolle, 1820; Darwin, 1859; Wallace, 1894; Darlington, 1957). This focus has resulted from the spatial and temporal perspective provided by fossils and from the use of highly inclusive taxonomic categories (often subfamily or above), which permit biogeographical comparison across a diverse array of organisms. The best-recognized determinant of organismal distribution operating at broad spatial scales is continental drift (Wegener, 1966), but less celebrated models have also contributed importantly to our understanding of factors affecting the distributions of animals and plants.

A relevant case in point is provided by the classic study of freshwater fish distributional patterns by Myers (1938). On the basis of fossil data and the contemporary distribution of taxa, Myers separated freshwater fish families into different divisions,

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reflecting inferred modes or routes of dispersal. For example, primary division fish families were designated on the basis of their purely continental distributions (i.e. absent from islands never connected to continents by Tertiary land bridges), putatively reflecting dispersal limitation due to physiological intolerance to salt water.

Here, we apply an approach similar to that of Myers, but at a very much more restricted spatial and temporal scale, to identify biotic and abiotic factors that influence the formation and persistence of species' ranges. Unsurprisingly, Myers' macrozoogeographical paradigm, which relates the dispersal of primary division fish to direct terrestrial connections linking drainage basins, is also supported by the data presented here at the geographical scale of Mesoamerica. However, the more recent evolutionary dimension of our study, accessed through mitochondrial DNA phylogeography in place of fossil age and distribution, permits identification of additional biological and physical factors that have played a clear role in defining the distribution patterns of freshwater fish, but that could not be discerned at the taxonomic and geographical scale utilized by Myers.

The analysis presented here is based on phylogenetic appraisals of geographical populations representing four genera of primary division Mesoamerican fishes: *Brycon*, *Bryconamericus*, and *Eretmobrycon* in the family Characidae, and *Cyphocharax* in the family Curimatidae. These four genera represent five distinct evolutionary lineages in Mesoamerica, each of which is recently and independently derived from South America (see below). Comparative phylogeographical analysis of the five colonizing lineages was based on the contemporary geographical distribution of mtDNA haplotypes (Bermingham & Avise, 1986; Avise *et al*., 1987), resulting from each lineage's expansion and subsequent diversification across the Mesoamerican landscape. By this approach, we were able to identify physical features, such as volcanoes and river anastomosis at low sea level stands, that have clearly influenced the regional distribution of primary freshwater fishes. Because the dispersion events under scrutiny are recent, there is no long history of extinction and re-colonization to obscure the physical features and biological processes responsible for establishing and maintaining the distributional breakpoints that separate the geographical lineages resulting from their expansion across the Mesoamerican landscape. As a result, we were able to infer ecological and demographic factors that have probably played a predominant role in establishing the distributional reach and diversification of Mesoamerican freshwater fishes. We have developed a simple model that sets a higher probability for the founding of immigrant populations at the leading-edge of colonizing waves compared to the turnover of resident lineages by immigrants, explaining the paradoxical lack of sympatry among closely related evolutionary lineages, in spite of the relative ease of dispersal among Mesoamerican drainages demonstrated by the rapid expansion of primary division fish colonists. Our model provides an explanation for both the mode of expansion and subsequent patterns of diversification described here for Mesoamerican freshwater fishes by distinguishing different probabilities of immigration associated with expanding lineages that encounter residents with similar fundamental niches, vs. those that do not (see also Bermingham & Martin, 1998; Perdices *et al*., 2002). Thus, we propose that niche-overlap between resident and immigrant populations may be an important (MacArthur & Levins, 1967; Brown, 1995) but generally overlooked factor in the establishment and maintenance of phylogeographical breaks in a wide variety of organisms.

Systematic background

Of the 17 valid Mesoamerican species included in the present study, only *Eretmobrycon bayano* and *Cyphocharax magdalenae* are easily identified using morphological criteria. By contrast, identification of *Brycon* and *Bryconamericus* species is not trivial and relies principally on meristics, which have overlapping counts among closely related species. Furthermore, the geographical distribution of individual Mesoamerican *Brycon* and *Bryconamericus* species has not been carefully documented. Although relating genetically defined groupings with the geographical distributions of described species is not an aim of the present study, there are striking correspondences (Fig. 1). Consequently, we describe the geographical distribution of each named species using the extensive regional collection of Mesoamerican fishes maintained at the Smithsonian Tropical Research Institute (STRI; Bermingham *et al.*, 1997a; see Supplementary Material). The geographical descriptions presented here, and informed by the molecular data, demonstrate considerably less sympatry within species groups, and regions of endemism that are less restricted in comparison to previous descriptions (Meek & Hildebrand, 1916; Hildebrand, 1928; Loftin, 1965). The STRI collection maintains catalogued DNA voucher specimens and associated lots representing the specimens reported in the present study. Locations of named geographical areas are shown in Figure 1A.

Characidae, *Bryconinae*, *Bryconini, Brycon* Müller & Troschel 1844

The taxonomy of the genus *Brycon* was described as a 'pretty mess' by Géry (1977) and stands as an

accurate assessment of Mesoamerican *Brycon* species. Two lower Mesoamerican *Brycon* species groups, '*argenteus*' and '*striatulus*', are generally recognized. *Brycon guatemalensis* Regan 1908 is either included in the *striatulus* group or left to stand on its own; the former convention is adopted here. The *argenteus* and *striatulus* species groups were found to correspond, respectively, to the *Brycon* eastern (BRE) and western (BRW) mtDNA major-lineages described in the Results section.

All three species in the *argenteus* group have distributions that include both Pacific and Atlantic slope populations. *Brycon argenteus* Meek & Hildebrand 1913 is the easternmost species in the group and is found in the Tuira and Bayano drainages on the Pacific slope and along the Atlantic San Blas coast west to the Rio Cascajal (slightly east of the Panama canal; Fig. 1A). It is replaced immediately to the west by *Brycon petrosus* Meek & Hildebrand 1913 in the Chagres system on the Atlantic slope and in the Rio Pacora and adjacent rivers on the Pacific slope. *Brycon obscurus* Hildebrand 1938 is the westernmost species in this group and is found in the rivers of central Panama: Rio Indio and Rio Cocle del Norte on the Atlantic slope and Rio Cocle del Sur on the Pacific slope. The species in the *argenteus* group are considered to be closely related to the *trans*-Andean *Brycon oligolepis* Regan 1913 of Colombia and Ecuador.

The *striatulus* group includes five species in Mesoamerica. *Brycon striatulus* (Kner 1863) is restricted to Pacific drainages of Colombia to central Panama. To the west, along the Pacific slope, there is a gap in the distribution of the *striatulus* group until *Brycon behreae* Hildebrand 1938 is encountered in the rivers west of the Panama's Azuero Peninsula and continuing north until Costa Rica's Gulf of Nicoya. On the Atlantic slope of Panama, *Brycon chagrensis* Kner & Steindachner 1863 is found in the Rio Chagres and adjacent drainages and *Brycon* sp. nov. (Howes, 1982) is found in the rivers of Bocas del Toro (far western Panama). *Brycon guatemalensis* is distributed from Costa Rica to Mexico, which led Bussing (Bussing, 1976; Bussing, 1985) to include the *striatulus* group in his 'Old-Southern' element (see Discussion). The *striatulus* species group demonstrates a considerably more extensive, but also more discontinuous, geographical distribution than the *argenteus* group. Members of the *striatulus* group exhibit close morphological relationships to *Brycon meeki* Eigenmann & Hildebrand 1918 and to *Brycon dentex* Gunther 1860, both distributed along the Pacific slopes of Colombia and Ecuador. The cis-Andean *Brycon stolzmanni* Steindachner 1879 from Peru is included in the present study as a geographical and taxonomic outgroup.

Characidae, Tetragonopterinae, Tetragonopterini, Bryconamericus Eigenmann 1907

From the molecular systematic perspective provided below, an orderly association of Mesoamerican *Bryconamericus* species with geography is not possible. This is not surprising given the described geographical distributions of some species in the genus. For example, the described distribution of *Bryconamericus scleroparius* Regan 1908 includes the Atlantic slopes of Nicaragua, Costa Rica, and western Panama, a single Pacific slope population in Costa Rica, and the Amazon and Pacific slopes of Ecuador (Barriga, 1991), but is absent from Colombia and most of Panama. *Bryconamericus scleroparius* is considered to be closely related to *Bryconamericus terrabensis* Meek 1914, endemic to the Terraba drainage on Costa Rica's Pacific slope, and *Bryconamericus ricae* Eigenmann 1908, sympatrically distributed with the former along the Atlantic slope of Costa Rica (Rio Reventazon) to western Panama (Bocas del Toro). Excluding the undetermined status of *Br. scleroparius* populations from Ecuador, these species are all considered members of the '*scleroparius*' species group (extending from Costa Rica to western Panama), which corresponds to the ARW mtDNA major-lineage (see Results). The westerly distribution of this species group led Bussing (Bussing, 1976; Bussing, 1985) to include it within his 'Old-Southern' element (see Discussion).

The remaining *Bryconamericus* species in Mesoamerica are placed in the '*emperador*' species group (ARE mtDNA major-lineage). This group includes several endemic species with narrow distributions, and the widespread *Bryconamericus emperador* Eigenmann & Ogle 1907 found in the Tuira and Bayano drainages of eastern Pacific Panama, and along the Atlantic slope from the central San Blas region of eastern Panama, west to the Rio Calovebera at the eastern limit of Panama's westernmost Bocas del Toro province. *Bryconamericus cascajalensis* Meek & Hildebrand 1916 is considered endemic to the Rio Cascajal, located midway along the Atlantic slope distribution of *Br. emperador*. *Bryconamericus zeteki* Hildebrand 1938 is considered endemic to the Pacific versant of the El Valle volcano in central Panama and represents the westernmost Pacific slope population of *Bryconamericus* in Panama. To the east along Panama's Pacific slope, there are no other *Bryconamericus* populations until *Br. emperador* is encountered in the Cabra drainage. The *emperador* group is closely related to two species from the Colombian Choco: *Bryconamericus ortholepis* Eigenmann 1913 found in the Atrato and San Juan rivers draining the Atlantic and Pacific slopes of the Choco, respectively; and

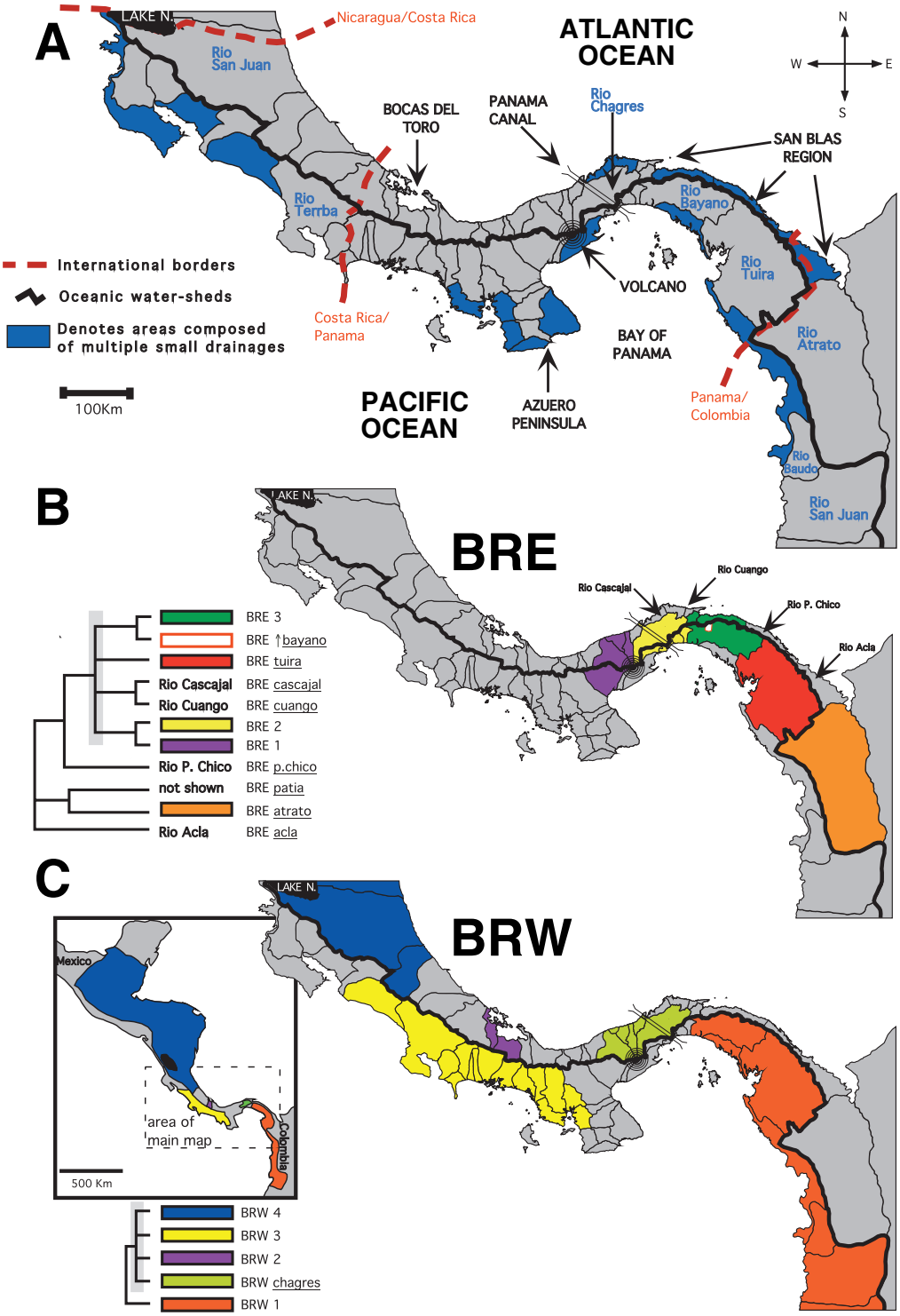

Figure 1. A, physical and geographical features of Lower Mesoamerica, enclosed areas in grey denote single river drainages, or areas in which a single river drainage dominates (except where stated otherwise). The bold line marks the separation between the Pacific and the Atlantic drainages. Names of larger drainages are shown, as are the locations of the El Valle volcano, the Panama Canal, regional names, and international borders. The southernmost tip of Lake Nicaragua is labelled 'Lake N.'. Maps (B) to (F) represent in colour the geographical ranges of the constituent operational taxonomic units of major-lineages. Cartoons of phylogenetic area relationships based on the trees shown in Figure 2 are inset in each map and drawn to an approximately similar scale.

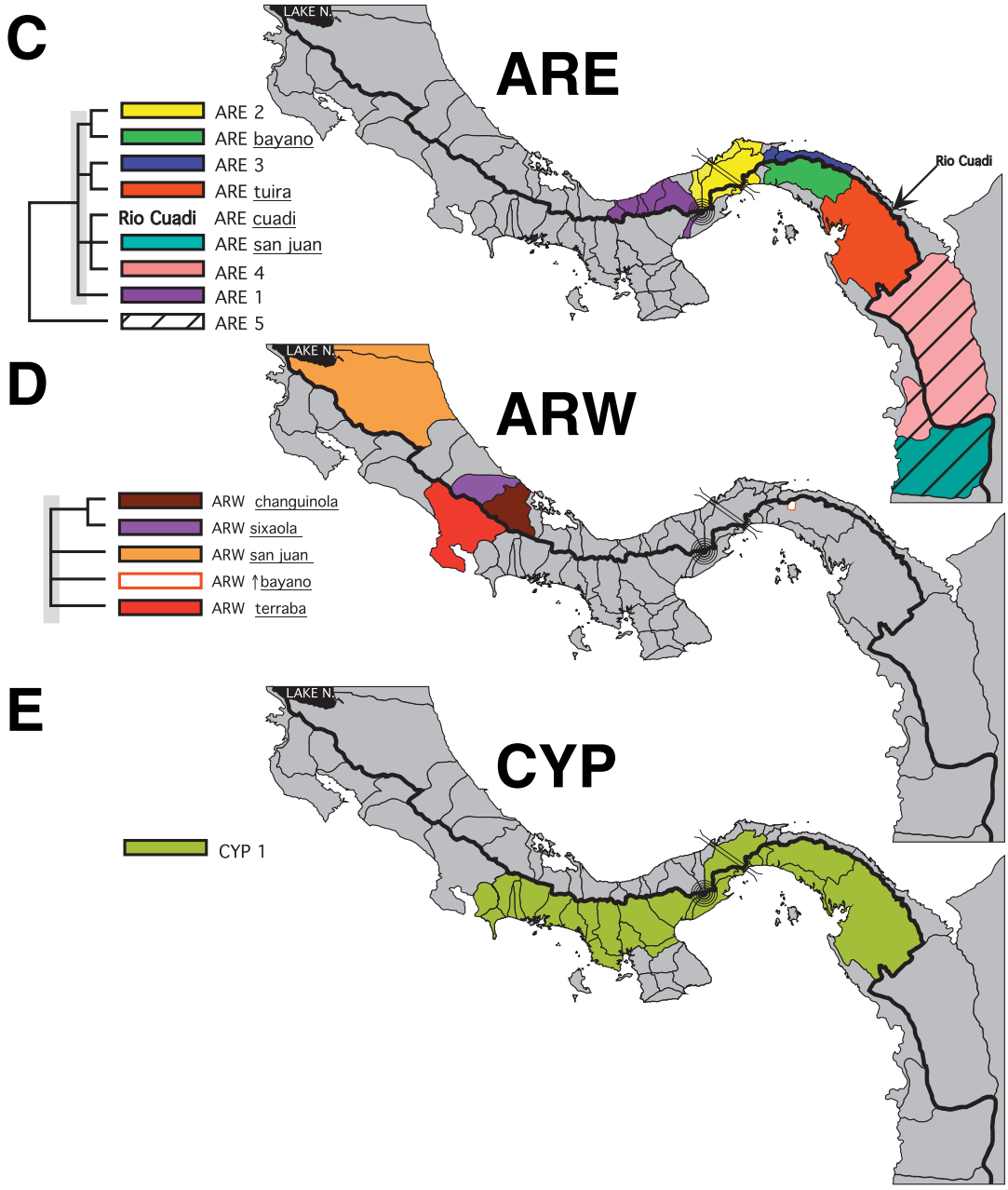

Figure 1. *Continued*

Bryconamericus baudoensis Fowler 1944 endemic to the Rio Baudo of the Pacific slope. The *emperador* group is also related to the more widespread *Bryconamericus scopiferus* Eigenmann 1913 along the Atlantic and Pacific slopes of Colombia. *Bryconamericus galvisi* (Román-Valencia, 2000) and *Bryconamericus caucanus* Eigenmann 1913 from the Amazon and Magdalena basins, respectively, were used as outgroups.

Characidae, Tetragonopterinae, Tetragonopterini, Eretmobrycon Fink 1976

Eretmobrycon bayano Fink 1976, the sole representative of this monotypic genus, is endemic to the Rio Bayano on the Pacific slope in eastern Panama. Although morphologically distinctive, based on the molecular analysis presented here, *E. bayano* represents a geographically isolated member of the *Brycon-*

americus '*scleroparius*' species group (ARW). Although we will retain the name *E. bayano*, it is included with other *Bryconamericus* species and populations for analytical purposes.

Curimatidae, Curimatinae, Cyphocharax Fowler 1906

Cyphocharax magdalenae (Steindachner 1879) is distributed from the Rio Magdalena and Rio Atrato of the Atlantic versant of north-western Colombia and along the Pacific slope of Panama and Costa Rica. The Rio Chagres record of *C. magdalenae* reported here represents the first reported observation of this species from the Atlantic versant of Mesoamerica. It is the only member of its family in Mesoamerica and is closely related to *Cyphocharax aspilos* Vari 1992 of the Lago Maracaibo basin of Venezuela.

MATERIAL AND METHODS

Sample collection

Fish were collected using cast nets, seines and an electro-fishing unit. Sampling effort varied among drainages, and only occasionally was it possible to sample along an altitudinal gradient. However, for most of the large Panama river systems (e.g. Tuira, Bayano, Chagres), several widespread locations were sampled. Collection latitude and longitude and drainage locations are listed in the Supplementary Material. In the field, gill arches with filaments, or in some cases small pieces of muscle, were preserved at ambient temperature in a saturated salt solution (NaCl) of 20% dimethyl sulphoxide and 0.5 M disodium ethylenediamine tetraacetate (EDTA) (Seutin, White & Boag, 1991). Uniquely tagged morphological voucher specimens were retained for all individuals sampled for DNA analysis. Voucher samples were fixed in 10% Formalin (V/V) and later transferred to 70% ethanol (V/V).

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from 0.1–0.5 g of tissue using a standard phenol/chloroform extraction procedure (Sambrook, Fritsch & Maniatis, 1990). The DNA was collected by ethanol precipitation, washed in 70% ethanol (V/V), resuspended in a 50–150 μL volume of 1/10 TE (1 mM Tris, 0.1 mM EDTA), and stored at –80 °C.

The entire *ATPase 6* & *8* genes for *Bryconamericus* (842 bp), *Cyphocharax (*842 bp), and *Brycon* (839 bp) were sequenced. The polymerase chain reaction (PCR) reactions were performed using 2.5 U Amplitaq polymerase (Perkin-Elmer) or 2.5 U Tfl polymerase (Epicentre Technologies), 2.0 mM $MgCl_2$, 200 μM of each dNTP, and 0.5 μM of each primer. Reactions were performed in 25–50 μL volumes and were initiated with 1 μL of template. PCR was performed at 93 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min for 35 cycles. The primers (designed by Shawn McCafferty) used for amplification and sequencing were 8.2 (5′-AAAGCR TYRGCCTTTTAAGC-3′) and 3.2 (5′-GTTAGTGGTCA KGGGCTTGGRTC-3′), which have 3′ base positions of L 8842 and H 9751, respectively, relative to the complete carp mtDNA genome (*Cyprinus carpio;* GenBank: X61010; Chang, Huang & Lo, 1994). The entire PCR product was electrophoresed in a low melting TAE gel and manually excised from 1% gels. The DNA contained in the resulting gel slice was purified using either GeneClean II (Bio101) or 1 μL Gelase digestion (Epicentre). GeneClean products were dried down and resuspended in 25 μL of water for sequencing; gelase products were sequenced directly. A 1–7-μL aliquot of cleaned DNA was cycle-sequenced using either the PRISM Reaction Ready Mix (ABI), dRhodamine Reaction Ready Mix (ABI), or kTaquenase (Rematech) following the respective manufacturers' recommendations. In addition to the 8.2 and 3.2 PCR primers described above, we also used an internal sequencing primer 8.3, 5′-AAYCCTGARACTGACCATG-3′, which has a 3′ base position of L 9035 relative to carp (GenBank: X61010). Cycle-sequencing products were purified using CentriCep G-50 columns (Princeton Separations) and then dried to completion. The resulting product was resuspended in formamide/EDTA and run on either an ABI 373A or 377 automated sequencer following the manufacturer's recommendations. The resulting chromatograms were checked and aligned by eye using SeqEdit (ABI) or Sequencher 3.1 (Gene Codes Corp.) utilizing IUB ambiguity codes where necessary. Due to the out of phase overlap (10 bp) between the *ATPase 6* & *8* reading frames, a extra nucleotide position with the ambiguity code 'N' was inserted immediately upstream of the first bases of the ATPase 6 start codon to maintain the same reading frame for both genes in all analysed files.

PCR-restriction fragment length polymorphism (RFLP) genotyping

ATPase 6 & *8* sequences representing *Brycon*, *Eretmobrycon*, and *Bryconamericus* of the entire *ATPase 6* & *8* genes were surveyed for potential restriction site polymorphism using MacVector 4.5 (Kodak SIS). A variety of restriction enzymes were employed to generate operational taxonomic unit (OTU)-specific digestion profiles. In cases where we could not distinguish between closely related OTUs using restriction enzymes, we genotyped all individuals using the full *ATPase 6* & *8* sequence. Full details of predicted OTU restriction sites are given in the Supplementary Mate-

rial. Five μLs of the PCR-amplified *ATPase* gene regions were digested using the conditions recommended by the manufacturer for each restriction enzyme employed. Typically, we used 1 U of enzyme to digest the DNA for ≥ 3 h. The resulting DNA digestion products (PCR-RFLPs) were visualized on 1.5–3% agarose TBE mini-gels. Individuals presenting novel PCR-RFLP profiles were sequenced.

Genetic distance metrics and tree construction

Genetic distance estimates were calculated using Sequencer 6.1 (shareware written by B. Kessing and available at: http://nmg.si.edu/sequencer/). To exclude the impact of any heterogeneity in the rate of nonsynonymous substitution between lineages, only estimates of synonymous substitutions per synonymous site were utilized for phylogenetic reconstruction. The widely applied method of Li, Wu & Luo (1985) was modified to ensure that the transition-transversion ratio (*W*) used to estimate the number of synonymous and nonsynonymous sites at two-fold degenerate positions is specified for each tree building matrix, rather than being effectively set at 0.5 as in the original method. Instantaneous maximum-likelihood estimates of *W* were generated from complete mtDNA *ATPase* data sets for each major-lineage (including available outgroups) with PAUP4B4 (Swofford, 1998) based on a neighbour-joining tree constructed using HKY85 distances (Hasegawa, Kishino & Yano, 1985) calculated from substitutions at third codon positions. This modified genetic distance is referred to here as K_s. Unlike the related method described by Pamilo & Bianchi (1993), which estimates *W* for each separate pairwise comparison, the described modification is less prone to anomalous estimates between very closely related taxa (due to small numbers of substitution types having occurred) or highly divergent taxa (where *W* is generally underestimated due to saturation). We used a value of $W = 11$ for comparisons between major-lineages and within CYP (where due to the low level of divergence it was not possible to meaningfully estimate *W*). This value represents the average transition-transversion ratio within BRE, BRW, ARE, and ARW which had values of 6, 16, 11, and 11, respectively.

Nexus format K_s matrices were exported from Sequencer 6.1 directly into PAUP4B4. The heuristic option in PAUP4B4 with minimum-evolution as the objective criterion was used to search for minimum-length trees. For two of the five major-lineages (BRW and BRE) multiple shortest trees were found, but differed only in the relationships of extremely closely related individuals (one or two bases different). Consequently, we selected one tree at random. Trees were midpoint rooted unless stated otherwise. To provide a crude estimate of the duration of time represented by polytomies and subsequent divergence, ultrametric phylograms were constructed from K_s matrices using the method of Fitch & Margoliash (1967) method implemented in PHYLIP 3.572c (Felsenstein, 1989). Data matrices can be downloaded from TREEBASE.

Diagnosis and naming of OTUs

Phylogenetic analyses (see below) identified five evolutionarily divergent clades (ARW, ARE, BRW, BRE, and CYP), which we term mtDNA major-lineages (Table 1). Major-lineages could be further subdivided using phylogenetic criteria into OTUs, which are defined as the most inclusive monophyletic grouping of individuals for which K_s estimates were ≤ 2.0%. The value of 2% was selected on the largely arbitrary basis that it appeared to identify meaningful phylogeographical groupings at a suitably refined scale. Individuals exhibiting genetic divergence > 2.0% from all other specimens were also designated OTUs. The OTU criterion was consistently applied across the entire taxonomic range of the study with two minor exceptions in the main data set (see Results) and two in the re-analysed data set of Bermingham & Martin (1998). OTUs sampled from a single drainage were given the name of that drainage (underlined), prefixed by the abbreviation of the major-lineage to which they belong

Table 1. Genetic distances within and between Mesoamerican primary freshwater fish mtDNA major-lineages

	OTUs (*N*)	BRW	BRE	ARW	ARE	CYP
BRW	5	0.07	0.55	0.51	0.64	0.69
BRE	11	*0.25*	0.26	0.71	0.72	0.56
ARW	5	*0.31*	*0.34*	0.11	0.23	0.46
ARE	9	*0.34*	*0.35*	*0.09*	0.17	0.43
CYP	1	*0.36*	*0.34*	*0.25*	*0.24*	0.01

Average estimated pairwise distances between major-lineages, utilizing exemplar individuals (identified in Fig. 2 with asterisks) from all operational taxonomic units (OTUs), are shown above and below diagonal for K_s and HKY85 (in italics), respectively. Values on the diagonal represent the maximum divergences observed within the respective major-lineages for K_s. It is suggested that, on the basis of the failure of distance estimates to reflect the fact that CYP is in a different family to other major-lineages, distance between major-lineages (with the exception of ARE/ARW) should be regarded with caution. A value of $W = 11$ was used to calculate K_s between major-lineages. BRW, *Brycon* western; BRE, *Brycon* eastern; ARW, *Bryconamericus* western; ARE, *Bryconamericus* eastern; CYP, *Cyphocharax*.

(e.g. BRE tuira; from the Rio Tuira drainage). OTUs representing multiple drainages were arbitrarily numbered, again prefixed by their major-lineage membership (e.g. BRE 1).

Geographic distribution maps

The geographical distributions of the five mtDNA major-lineages and their constituent OTUs are presented on maps of Mesoamerica (Fig. 1B–F). Our sampling design generally permitted us to identify the distributional endpoints for each OTU, but left some geographically intermediate drainages unsampled. In these cases, the geographical ranges of OTUs were interpolated from the sample drainages. Most geographical divisions on the drainage maps represent single drainages or regions dominated by a single large drainage. However, a number of areas (identified in Fig. 1A) composed of multiple small adjacent rivers could not easily be represented on our maps and are shown as single geographical units.

Histograms of pairwise genetic distance estimates

The two most divergent individuals representing each OTU were used to construct the histograms of K_s distances. In the sole instance (ARE san juan) where a single individual represented an OTU, we duplicated the individual's sequence to maintain balance in the construction of the histograms. Although histograms utilizing patristic distances (distances reconstructed from the phylogenetic trees) produced more homogeneous estimates across nodes, we used observed genetic distances to provide a more accurate reflection of the variance of divergence estimates from sequence data. We compared the freshwater fish genetic distances to *ATPase 6* & *8* sequence divergence calculated for seven of eight geminate marine species pairs that Bermingham, McCafferty & Martin (1997b) posited were split by the final closure of the Isthmus of Panama approximately 3.1 Mya.

RESULTS

We genotyped the mtDNA of 339 individuals representing *Brycon*, *Bryconamericus*, *Eretmobrycon*, and *Cyphocharax*. Fish were assigned to one of 31 OTUs based on their complete *ATPase 6* & *8* sequences ($n = 167$), or PCR-RFLP assay of diagnostic *ATPase* polymorphisms that distinguish between the OTUs ($n = 172$). Collection localities and data type summaries for all individuals are presented in the Supplementary Material. The *ATPase* sequences have GenBank Accession Numbers: *Brycon* (AF412628–AF412727); *Bryconamericus* & *Eretmobrycon* (AF412573–AF412627); and *Cyphocharax* (AF412930–AF412941). The mtDNA *ATPase 6* & *8* results provided below are fully corroborated by the analysis of the complete mitochondrial cytochrome *b* gene (1140 bp) and partial cytochrome oxidase subunit *I* gene (630 bp) performed for a phylogenetically informative subset of the individuals included here (data not shown).

General features of mtDNA major-lineages

On the basis of the phylogenetic results presented below, the mtDNA major-lineages sampled in each genus were descriptively named to simplify the presentation of results and the ensuing discussion. For Mesoamerican *Brycon*, two major-lineages were identified: ***Br****ycon* **e**astern (BRE), and ***Br****ycon* **w**estern (BRW). *Bryconamericus* also comprised two major-lineages: *Brycon****a****m****e****r****i****cus* **e**astern (ARE) and *Brycon****a****m****e****r****i****cus* **w**estern (ARW). The genus ***Cyp****hocharax* comprised a single mtDNA major-lineage: CYP. Table 1 clearly illustrates the magnitude of mtDNA divergence between the defined major-lineages and Figure 1 presents their geographical distribution.

Molecular characteristics of *ATPase 6* & *8* sequences

Brycon utilizes 'TTG','CTG', or 'GTG' as initiation codons for *ATPase* 6, in addition to the more typical 'ATG' start codon observed in *Bryconamericus, Eretmobrycon*, and *Cyphocharax*. Furthermore, all *Brycon* have an amino acid deletion in *ATPase 8* in comparison to the other three genera considered in the present study or the *ATPase 8* gene from the complete mitochondrial *Cyprinus carpio* genome reported in GenBank: X61010. The position of the deleted AA residue relative to other *ATPase 8* sequences is unclear due to the very low levels of amino acid sequence similarity across taxa, excepting the short conserved regions at the C and N termini.

Biogeographical characteristics of mtDNA major-lineages

We present five results common to the biogeographical analysis within each of the five named mtDNA major-lineages, followed by the results specific to each major-lineage. First, only a single example of sympatry was observed west of the Panama/Colombia border (Fig. 1A) between OTUs representing the same mtDNA major-lineage. Second, all OTUs were geographically contiguous and distributions were never interrupted by a second OTU. Third, phylogenetically defined sister OTUs were always geographically adja-

cent (or potentially so, allowing for unsampled areas). Fourth, when basal lineages of mtDNA major-lineages could be phylogenetically identified (e.g. ARE, BRE, and BRW), the geographical distribution of the basal OTU(s) always included South American locations. Fifth, the Mesoamerican OTUs form polytomies within each mtDNA major-lineage, suggesting the roughly contemporaneous divergence of geographical populations across the Mesoamerican range inhabited by each major-lineage.

Brycon eastern mtDNA major-lineage (BRE)

The complete *ATPase 6* & *8* genes were sequenced for 54 individuals from the *B. argenteus* group collected from 17 river drainages extending west from the Colombian/Ecuador border to central Panama (Fig. 1B) and phylogenetic analysis identified 11 OTUs (Fig. 2). The basal BRE lineages are represented by OTUs with Colombian (BRE atrato and BRE patia) or eastern Panama (BRE acla) distributions. BRE p.chico, representing the region immediately to the west of BRE acla, is sister to a group of seven BRE OTUs uniting in a polytomy (shaded grey in Fig. 2). This group of seven OTUs is contiguously distributed to the west of the basal BRE lineages (Fig. 1B). It is noteworthy that a shared *ATPase 6* 'CTG' initiation codon and two sequential *ATPase 8* 'TAA' termination codons unite BRE p.chico and the group of seven OTUs, while the basal BRE acla & BRE atrato utilize a 'TTG' initiation codon and a single 'TAA' termination codon. The sampled South American *Brycon* outgroup utilize the more usual 'ATG' initiation codon and a single 'TAA' termination codon. The graphical representation of all pairwise K_s estimates between OTUs shown in Figure 3 clearly illustrates the genetic separation between basal and derived Mesoamerican BRE OTUs and also shows the similarity of genetic distances across the seven OTUs included in the derived polytomy.

Notwithstanding the generally unresolved relationships among the seven Panamanian BRE OTUs, our analysis supported a number of unambiguous sister OTUs representing geographically adjacent regions. It is also noteworthy that the westernmost BRE OTUs have distributions spanning Panama's Central Cordillera; BRE1 spans the divide in the region of the El Valle volcano and BRE 2 crosses the lowest point of the continental divide in the Panama canal region (locations of geographical features are given in Fig. 1A). BRE 3 and BRE ↑bayano, separated by 3.3% K_s genetic distance, represent the single Mesoamerican example of OTU sympatry within a mtDNA major-lineage.

In addition to the 54 BRE individuals sequenced directly, the *ATPase* genotypes for an additional 84 fish were determined using restriction endonuclease analysis of PCR amplified *ATPase 6* & *8* genes (PCR-RFLP). In total, the *ATPase* genotypes and OTUs for 138 BRE individuals were determined (see Supplementary Material). Owing to the moderately large number of individuals sequenced, the *Brycon* BRE major-lineage best illustrates the general pattern of low levels of intra-OTU *ATPase* polymorphism. Furthermore, larger sample sizes provide increased support for the general absence of sympatry among BRE OTUs, and a more precise determination of the geographical relationship between the sympatric Rio Bayano BRE OTUs, ↑bayano and BRE3. BRE ↑bayano ($n = 38$) was only observed in two adjacent high elevation tributaries, whereas BRE 3 ($n = 33$) was found in seven widely separated tributaries of the Bayano, and included six individuals syntopically collected with BRE ↑bayano.

Brycon western mtDNA major-lineage (BRW)

The complete *ATPase 6* & *8* genes were sequenced for 46 individuals from the *B. striatulus* group collected from 27 drainages extending from the Colombia/Ecuador border north to Mexico (a distance of approximately 2500 km; Fig. 1C), and phylogenetic analysis identified five Brycon BRW OTUs (Fig. 2). All methods of analysis, including parsimony and maximum likelihood, indicated that the branch separating BRW 1 from the remaining four OTUs contained approximately twice the number of substitutions compared to the other branches (Figs 2, 3). The lack of closely related outgroup taxa prevented confident, phylogenetically based determination of whether or not BRW 1 was basal with respect to the remaining *Brycon* BRW OTUs. However, on the basis of its geographical distribution, the putative systematic relationships to *Brycon* species on the Pacific slope of Ecuador and Colombia (see Systematic Background), and the geological history of the area, we posit a basal position for BRW 1. The inclusion of the individual STRI-7584 within BRW 1 represents the first of the two exceptions to our OTU criterion (see Materials and Methods) because it diverged by 2.3–2.7% K_s. It is included in BRW 1 rather than as the sole representative of a distinct OTU to highlight the phylogeographical similarity of this sample, from the Colombia/Ecuador border, with Mesoamerican taxa (Fig. 1C). The estimated pairwise sequence divergence between all BRW OTUs was extremely small (Fig. 2), as was intra-OTU nucleotide diversity, excepting BRW 1. For example, no more than three substitutions were observed (1.3% K_s) between the ten BRW 3 individuals collected from eight drainages over a geographical area that encompasses an additional ten or more significantly sized river systems. The results

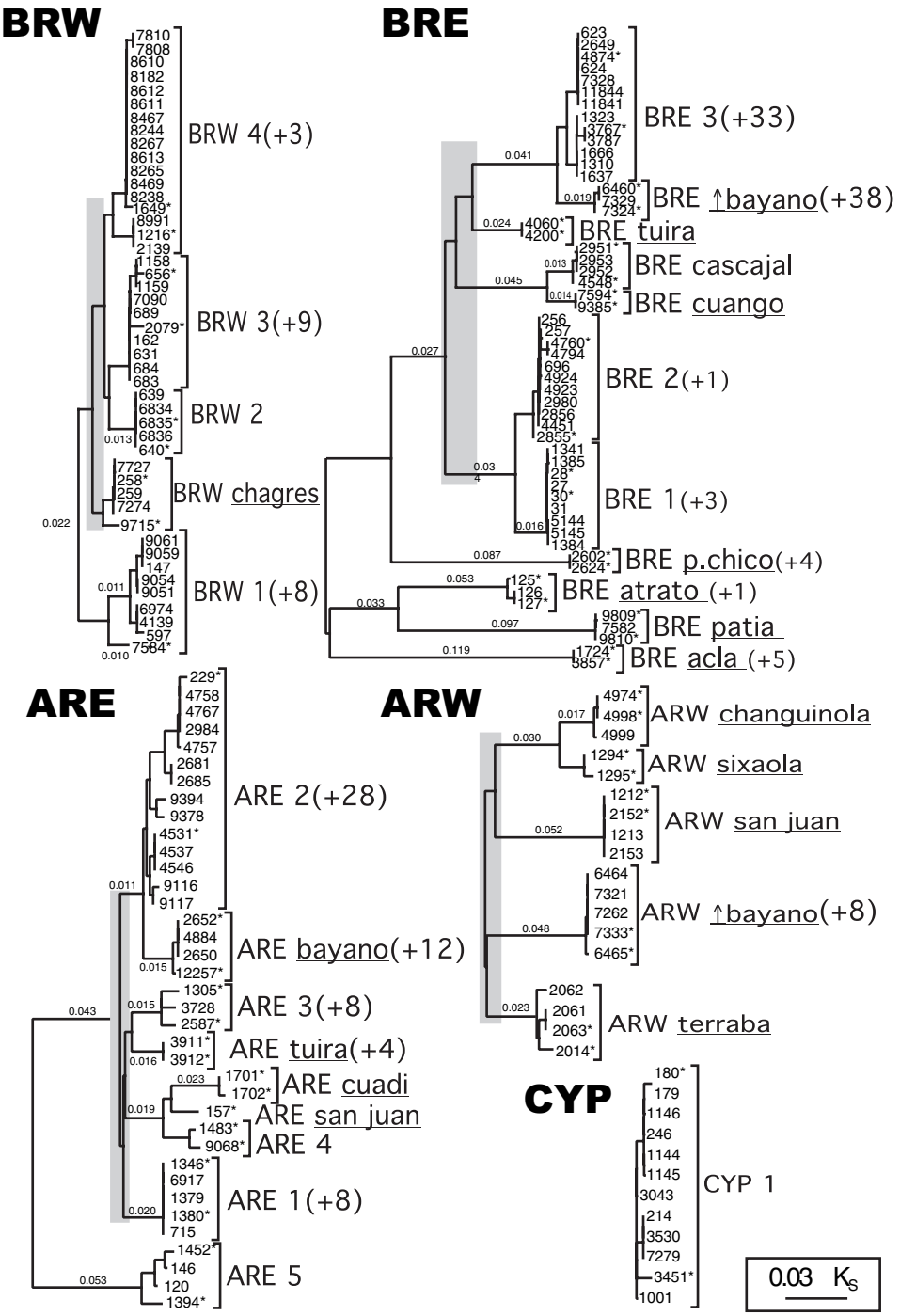

Figure 2. Minimum-evolution K_s phylograms based on sequences of the mitochondrial *ATPase 6* & *8* genes (839–842 bp) for five evolutionary lineages of Mesoamerican primary freshwater fishes: BRE, BRW, ARE, ARW, and CYP mtDNA major-lineages. The five phylograms are drawn to a common genetic distance scale. Sequenced individuals are represented by the numerical identification codes listed in the Supplementary Material. Operational taxonomic unit (OTU) definition and labels are described in the Materials and methods. Individuals (two per OTU) marked by asterisks indicate OTU exemplars utilized in the analysis represented in Figure 3. The numbers of additional individuals assigned to OTUs on the basis of polymerase chain reaction-restriction fragment length polymorphism are shown in parenthesis beside OTU labels. Grey shaded areas represent nodes of the trees between which branch lengths are uniformly short and which we refer to as hard polytomies (see Results). ARE and ARW phylograms are rooted using outgroups that are not shown; the remaining major-lineages are mid-point rooted owing to the absence of sampled outgroups < 0.40 K_s.

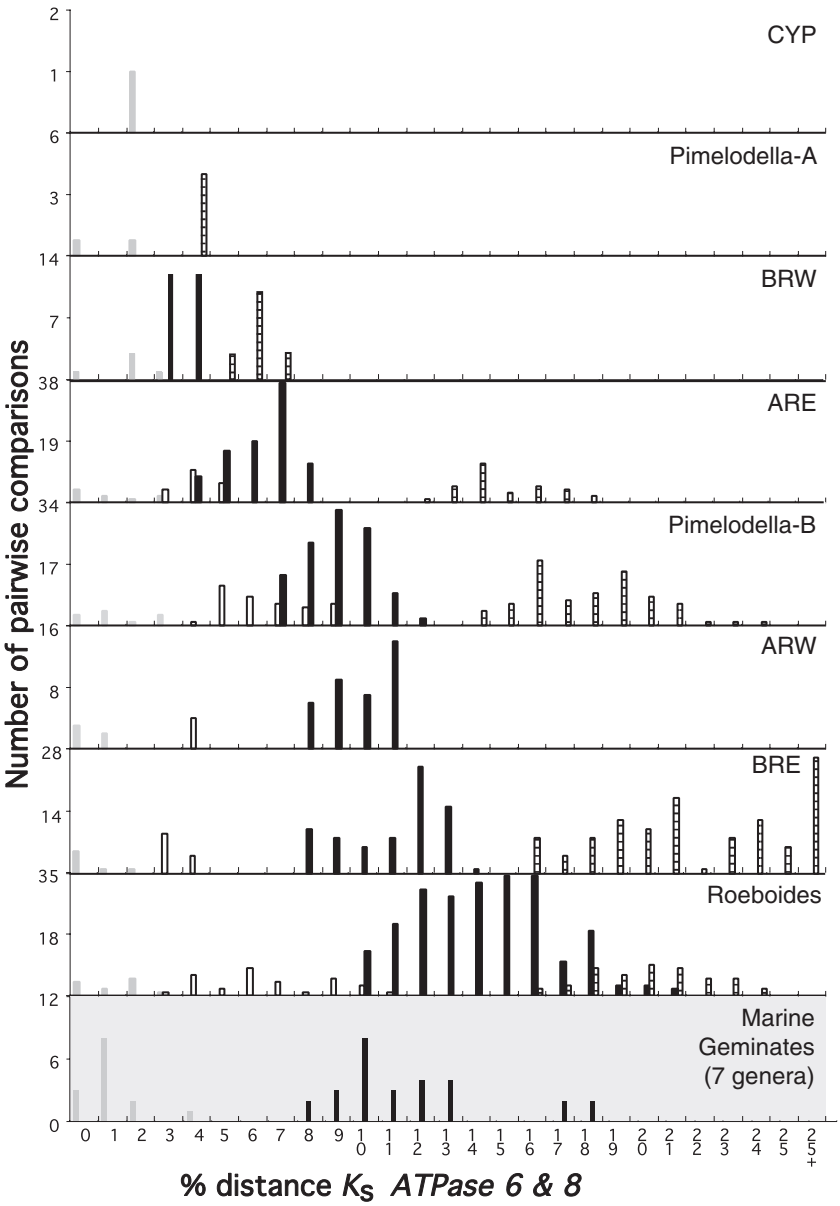

Figure 3. Histograms of pairwise K_s distances among sampled operational taxonomic units (OTUs) representing Mesoamerican primary freshwater fishes (see Materials and methods for full details). Intra-OTU comparisons are shown in grey, inter-OTU comparisons not across tree nodes considered to be part of a polytomy are in white. Inter-OTU comparisons across polytomy nodes are in black. Comparisons of Mesoamerican OTUs (or South American OTUs clearly derived from the latter) with basal South American or Eastern San Blas OTUs are hatched. Major-lineages abbreviations are detailed as in the text. Pairwise K_s distances estimates between seven pairs of marine geminate fish species hypothesized to have been separated by the final closure of the isthmus of Panama by Bermingham *et al*. (1997b) are also shown. Intra-oceanic comparisons are shown in grey, with interoceanic comparisons between geminates shown in black. Note that the *y*-axis frequency scale varies between histograms and that all axes are shown from 0.

presented above were supported by 20 additional fish genotyped using PCR-RFLP analysis of the *ATPase 6* & *8* genes.

BRW individuals utilize a 'GTG' *ATPase* 6 initiation codon, excepting two individuals (STRI-9051 and STRI-9054) from the Rio Jurado of Pacific Colombia, which use 'ATG'. Interestingly these two individuals differ by only a single additional synonymous substitution from BRW 1 individuals from the Rio San Juan that utilize the 'GTG' initiation codon.

BRYCONAMERICUS EASTERN MAJOR-LINEAGE (ARE)

Thirty-seven individuals representing the *Bryconamericus* '*emperador*' species group were sequenced from 19 drainages distributed from the Colombian Choco to Lake Nicaragua, and nine OTUs were diagnosed on the basis of the complete *ATPase 6* & *8* genes. The most basal *Bryconamericus* in this major-lineage, ARE 5, has an entirely Colombian distribution. The remaining eight OTUs define a polytomy (Fig. 2) distributed contiguously from Colombia to Central Panama (Fig. 1D). *Bryconamericus* ARE 5 was found sympatrically with ARE 4 (Rio Atrato and Rio Baudo) and ARE san juan (Rio San Juan) in Colombia. The six OTUs in the ARE mtDNA major-lineage with Panamanian distributions were not observed sympatrically, and sister OTUs are geographically adjacent. Similar to BRE, the two westernmost OTUs representing the ARE major-lineage are found on both sides of the continental divide: ARE 1 in the El Valle volcano region in Central Panama, and ARE 2 in the area of the Panama Canal (Fig. 1). The second of the two exceptions to our OTU criterion was observed in ARE 2, with a maximum intra-OTU K_s of 2.1%, marginally greater than our 2.0% cut-off value.

We increased our sample size of the ARE mtDNA major–lineage to 97 individuals, through the addition of 60 fish that were genotyped using PCR-RFLP analysis of the *ATPase 6* & *8* genes.

BRYCONAMERICUS WESTERN MTDNA MAJOR-LINEAGE (ARW)

Complete *ATPase 6* & *8* genes were sequenced for 18 individuals representing the *Bryconamericus* '*scleroparius*' species group collected from five drainages, each represented by an endemic OTU. The five *Bryconamericus* OTUs in this major-lineage derive from a common polytomy for which no basal lineages have been sampled, beyond those representing the ARE major-lineage (Table 1). With the exception of ARW ↑bayano, all OTUs are distributed to the west of the *Bryconamericus* ARE mtDNA major-lineage (Fig. 1E, F). ARW ↑bayano represents the monotypic genus *Eretmobrycon* and like BRE ↑bayano, is restricted to, and numerically dominant, in the same two adjacent tributaries of the Bayano drainage ($n = 13$). The congeneric ARE bayano ($n = 16$) was collected from five well-separated locations in the Rio Bayano, including two individuals collected syntopically with ARW ↑bayano. From a mtDNA perspective, the phylogenetic position of *Eretmobrycon* renders *Bryconamericus* paraphyletic (Fig. 2, Table 1). Consequently, *Eretmobrycon* is regarded as a synonym of *Bryconamericus* for analytical purposes in the present study.

In addition to the 18 ARW individuals sequenced directly, *ATPase 6* & *8* genotypes were determined for an additional eight fish by PCR-RFLP.

CYPHOCHARAX MTDNA MAJOR-LINEAGE (CYP)

We sequenced 12 *C. magdalenae* individuals representing seven distinct drainages across the Mesoamerican range of this species. The single *Cyphocharax* OTU (CYP 1) is distributed along the 900 km Pacific slope of Panama and Costa Rica, and the Rio Chagres on Panama's Atlantic slope (Fig. 1F). The maximum observed divergence between *C. magdalenae* individuals is three substitutions, equaling 1.4% K_s (Fig. 2). Meek & Hildebrand (1916) did not report *Cyphocharax* in the Rio Chagres in their ichthyological survey of this drainage, completed prior to the construction of the Panama Canal (1904–14). Thus, we consider it likely that *C. magdalenae* reached the Atlantic slope of Panama via the Panama Canal. In any event, our collection of *C. magdalenae* in Lago Gatun represents the first reported record of this species from the Atlantic slope of Mesoamerica. Although no *Cyphocharax* outgroups were sampled, the systematic revision of the genus by Vari (1992) posits that lineages basal to CYP 1 (*C. magdalenae*) would be encountered in the Lago Maracaibo basin of Venezuela.

MESOAMERICAN OTUS ALMOST EXCLUSIVELY DERIVE FROM POLYTOMIES.

A striking feature of the trees presented in Figure 2 is that 24 of the 26 OTUs with distributions exclusively west of the Panama/Colombia border stem from polytomies. The exceptions are BRE p.chico and BRE acla from the San Blas region of eastern Panama. The areas of the phylograms shaded grey in Figure 2 encompass nodes connected by uniformly short branches. Polytomies were also a feature of the phylogenies published by Bermingham & Martin (1998) for the Mesoamerican representatives of the genera *Roeboides* and *Pimelodella*, and these species are included in the analysis presented in Figure 3. Polytomies were also a conspicuous feature in both the two Mesomerican lineages of genus *Rhamdia* analysed by Perdices *et al*. (2002); these are not reanalysed here for reasons of space.

The genus *Pimelodella* is subdivided into groups A and B (Bermingham & Martin, 1998), each of which represents an independent colonization from South America. The possibility that the polytomies are an artifact of nucleotide saturation can be confidently discounted (data not shown), which is not surprising given that the maximum divergence between OTUs stemming from shaded polytomy nodes is in all instances less than 8.0% HKY85. Figure 3 graphically represents pairwise K_s distance matrices (used to con-

struct phylogenies of the type shown in Fig. 2), between exemplar individuals of OTUs of the same major-lineage, as histograms. If moderate rate constancy within major-lineages is assumed (maximum-likelihood clock tests applied to third codon position sites, where synonymous substitutions predominate, fail to reject the existence of clocks in each major-lineage, data not shown), then high frequency histogram categories evidence historical periods of extensive bifurcation (or low extinction rates) between ancestors of OTUs. Conversely, low frequency categories, which generally exist to the left and right of the black polytomy bars, indicate periods of relatively low bifurcation (or high extinction rates) among ancestors of the sampled OTUs. The CYP major-lineage and *Pimelodella* A are represented in Mesoamerica by fewer than three OTUs and consequently could not exhibit a polytomy in the same sense as all the other groupings shown in Figure 3. Figure 3 also illustrates that the divergence observed between Mesoamerican OTUs representing major-lineages is broadly similar to or less than that observed between pairs of marine fishes (termed geminates in Jordan, 1908) hypothesized to have been separated by the final rise of the Isthmus of Panama (Bermingham *et al*., 1997b).

DISCUSSION

Mitochondrial data for the Characiform genera *Brycon*, *Bryconamericus, Eretmobrycon*, and *Cyphocharax* were used to investigate the colonization of Mesoamerica by primary freshwater fishes. In the Results, we presented evidence for the phylogenetic relationships and geographical distribution of the different mtDNA major-lineages. Here, we look across groups to provide a more general assessment of the history of Mesoamerica's colonization by primary freshwater fishes, and their subsequent diversification. We briefly describe the geographical partitioning of genetic diversity and explore: (1) the relationship between sympatry and genetic divergence; (2) the timing of colonization of Mesoamerica (in light of previous hypotheses); (3) the tempo and spatial scale of expansion events; and (4) develop a model to reconcile the contemporary rarity of sympatry between closely related OTUs with the historically high levels of interdrainage exchange evidenced by phylogeographical analysis.

Based on previously described ranges of species and species groups (see Systematic Background), it can be reasonably assumed that with minor omissions all five major-lineages have been sampled across their entire Mesoamerican range. With the exception of ARW, all have nearly continuous Mesoamerican distributions extending west of the Panama/Colombia border. Where basal lineages have been sampled, they have South American and eastern San Blas distributions (locations are shown in Fig. 1A), which is consistent with a South American source for the extant Mesoamerican primary freshwater fishes. Although the eastern San Blas area is west of the Panama/Colombia border and is not geographically speaking part of South America, it is immediately adjacent to the mouth of the Rio Atrato drainage, which drains a large area of the Colombian Choco. Bathymetric data indicate that a significant portion of this area became part of the Atrato drainage during low sea level stands (Loftin, 1965). Consequently, the basal position of taxa sampled in eastern San Blas is not surprising and, for the remainder of the discussion, reference to Mesoamerica does not generally include this region.

Absence of sympatry among closely related Mesoamerican OTUs

The previously described ranges of *Brycon* and *Bryconamericus* species indicate significant sympatry throughout Mesoamerica among members of the same species group (see Systematic Background). By contrast, our mtDNA results identify only one instance of sympatry (west of the Panama/Colombia border) between OTUs representing the same major-lineage (each of which corresponds to a named species group, see Systematic Background). Thus, accepting that species represent monophyletic groups at some level, most previous observations of sympatry probably resulted from incorrect species identification using keys based on overlapping meristic counts. Because of the unambiguous nature of DNA sequence and PCR-RFLP differences among OTUs, it was possible to consistently type moderate numbers of individuals and, by doing so, more confidently define the geographical ranges of OTUs. Such an approach resulted in a much more parsimonious geographical distribution of lineages than was implied by previously described species ranges. The nearly complete absence of sympatry among OTUs within any of the five major-lineages (BRW, BRE, ARW, ARE, CYP) across their entire Mesoamerican distributions represents one of the most striking features of the entire data set. Thus, it appears that divergence in allopatry occurred among the primary freshwater fish OTUs described here, with the limits of river drainages representing isolation barriers.

The single exception is the sympatric distribution of the closely related BRE ↑bayano and BRE 3 in the Rio Bayano drainage (Figs 1, 2). In our collections, BRE ↑bayano is restricted to, and numerically dominant in, a small group of high gradient tributaries, whereas BRE 3 is widespread throughout the remainder of the sampled Bayano drainage. However, the two OTUs are syntopic in at least one tributary of the Bayano, a

finding relevant to specific status under most species concepts (Templeton, 1989).

Mesoamerican patterns of OTU distribution and divergence within major-lineages

Overall, the genetic data presented in the results for Mesoamerican primary freshwater fish can be said to describe two contrasting patterns. The first pattern, exemplified by BRW and CYP, is one of low genetic divergence among populations distributed over geographically widespread areas. The second pattern, representing the remaining major mtDNA lineages (BRE, ARE, and ARW), is characterized by restricted distributions and high levels of genetic divergence between geographically separated populations. The contrast between the two patterns is clearly evident in the maximum K_s genetic distances (as well as mean $K_s \pm$ SD observed among OTUs) within major-lineages representing the two distribution types (excluding those basal taxa representing South American and eastern San Blas). For BRW, the maximum K_s genetic distance is 3.3% (2.76% ± 0.3) across 2000 km whereas, for CYP, divergence between mtDNA haplotypes does not exceed 1.4% across the 900 km range of the single OTU. By contrast, the major mtDNA lineages ARW, ARE, or BRE exhibit much higher maximum divergences of 12.9% (9.1 ± 1.9%), 7.7% (4.9 ± 1.6%), and 13.3% (9.1 ± 3.8%), respectively, although none have a distribution extending more than 900 km (Figs 2, 3). Despite strong differences in the relationship between geographical distance and genetic divergence, the observed genetic diversity was partitioned geographically at all hierarchical levels within the five major-lineages. For example, in cases where variation was observed within a designated OTU, drainages or groups of adjacent drainages were reciprocally monophyletic. Furthermore, when the sister relationships between OTUs could be confidently determined, they are always geographically adjacent (or potentially so allowing for unsampled areas).

Excepting BRE ↑bayano and BRE 3, the maximum Mesoamerican intradrainage divergence observed within any major-lineage was 1.0% K_s, or three *ATPase 8* & *6* nucleotide substitutions. Thus, it appears that there are no barriers to intradrainage exchange (whether physical, ecological or behavioural) operating over the time scale resolvable by the nucleotide substitution rate of the mitochondrial gene regions examined here. Moreover, the very low levels of intradrainage nucleotide diversity across Mesoamerican primary fishes ($\leq$ 1.0% K_s) implies that rapid mtDNA lineage sorting, probably resulting from population bottlenecks and/or high variation in female reproductive success, is a pervasive feature of fish populations in these drainages.

The colonization of Mesoamerica from South American

There is overwhelming consensus that South America represents the source of primary division fishes in Mesoamerica (Darlington, 1957; Loftin, 1965; Miller, 1966; Myers, 1966), but several hypotheses have been proposed regarding the number and timing of dispersion events (Myers, 1966; Bussing, 1976; Bussing, 1985; Bermingham & Martin, 1998; Bussing, 1998). The importance of the Pliocene rise of the Isthmus of Panama as a colonization route for South American primary fishes entering Mesoamerica is common to all models, and Myers (1966) emphatically stated that Mesoamerica 'was and always had been devoid of primary freshwater fish prior to the very late Tertiary'. Bussing (1976, 1985), on the other hand, argued that the putatively slow pace of primary fish expansion indicated that taxa with extensive Mesoamerican distributions reaching as far north as Mexico must have arrived earlier. Thus, he posited two waves of colonization: the first, approximately Cretaceous in age (~60 Mya) indicated by his 'Old-Southern' distribution pattern, and the second, indicated by his 'New-Southern' pattern, corresponding to the emergence of the Panama landbridge (~3.1 Mya). The long time interval between the New-Southern and Old-Southern colonization waves resulted from the separation of South and nuclear Mesoamerica by the Bolivar Seaway (Coates & Obando, 1996; Collins *et al*., 1996). Bussing (1976, 1985) and Myers (1966) used essentially the same data on contemporary species distributions to arrive at their different conclusions regarding the development of Mesoamerica's ichthyofauna, whereas Bermingham & Martin (1998) based their Mesoamerican colonization model on mtDNA-based phylogeographical analyses of three genera of primary division fishes. Similar to Bussing, they hypothesized more than a single wave of colonization, but suggested that the first wave of primary freshwater fish dispersion to Mesoamerica was late Miocene (4–7 Mya) at the earliest.

Our phylogenetic analyses of Mesoamerican *Brycon*, *Bryconamericus*, and *Cyphocharax* identify five major mtDNA lineages (BRE, BRW, ARE, ARW, CYP) that permit additional assessments of the chronological history of Mesoamerican colonization. Two of these taxa (BRW and ARW) fall within Bussing's Old-Southern element and the remaining three lineages are included in his New-Southern element. On the basis of the South American location of basal mitochondrial lineages, and previously proposed taxonomic affinities with Colombian taxa (see Systematic Background section), it can be reasonably assumed, with the possible exception of ARW, that the major-lineages represent independent dispersal events from South America.

The initial direction of these five waves of colonization must have been westerly, although back migration is not precluded.

Figure 3 graphically presents the pairwise estimates of divergence among congeneric Mesoamerican OTUs and between Mesoamerican and basal South American OTUs for eight major-lineages of primary freshwater fish (including data from Bermingham & Martin, 1998) considered to represent independent colonizations from South America. Also provided in Figure 3 are the genetic divergence estimates between seven geminate pairs of shallow water marine fishes putatively formed as a consequence of the division of their ancestors' range by the rise of the isthmus of Panama (Jordan, 1908; Bermingham *et al*., 1997b). If it is assumed that: (1) the severance of the shallow water connection between the Atlantic and Pacific closely preceded the establishment of a continuous terrestrial land bridge between South America and nuclear Mesoamerica; (2) that there is an approximate similarity in the rate of accumulation of synonymous mitochondrial nucleotide substitutions across all Actinopterygii; (3) that the geminate pairs identified through the approach of 'concordant measures' (Bermingham *et al*., 1997b) were simultaneously isolated (or nearly so) by the rise of the isthmus; and (4) that mtDNA polymorphism present in ancestral species was similar to that observed in contemporary species, then it would be expected that levels of mtDNA divergence within freshwater fish lineages that colonized the Isthmus of Panama subsequent to the establishment of the land bridge should be equal to or less than mtDNA divergence observed between marine geminate species. Examination of Figure 3 clearly illustrates that there exists considerable variation in the divergence between the putative marine geminate pairs, which may reflect differences in the timing of the cessation of gene flow due to life history differences (Bermingham & Lessios, 1993; Knowlton *et al*., 1993; Jackson, Budd & Coates, 1996), variation in the rate of synonymous substitution between the seven pairs of geminates, and/or variation in the level of polymorphism within the ancestral species (Edwards & Beerli, 2000).

Figure 3 also demonstrates that mtDNA divergences among OTUs representing each of the major-lineages of Mesoamerican freshwater fish are similar to or less than the genetic divergences observed between marine geminates (Fig. 3, bottom panel). Thus, the molecular data are consistent with models indicating that Mesoamerica was exclusively colonized by primary freshwater fishes following the late Tertiary rise of the Isthmus of Panama (athough see also the discussion of a synonymous molecular clock below). Consequently, the molecular data appear to confirm the hypothesis of Myers (1938) that the families of freshwater fish he defined as being primary do indeed represent sensitive indicators of direct historical connectivity between terrestrial bodies (in this case, nuclear Mesoamerica and South America). Additionally, the fact that the genetic divergence between basal South American and derived Mesoamerican *Roeboides* and BRW OTUs (both included in Bussing's Old-Southern element) is comparable to that observed in BRE, ARE, and *Pimelodella* A & B (New-Southern elements, considered to have crossed the Pliocene isthmian land bridge in all models) argues against the existence of an Old-Southern element dating to the early Cenozoic.

The remarkable temporal correspondence between historical scenarios derived from a basic appraisal of synonymous divergence (Fig. 3) and those previously proposed based on the geological development of the isthmus may not, as is argued here, be indicative of a degree equivalence in the rate of synonymous substitution in the recent history of the Actinopterygii lineages sampled here. However, given the rate of synonymous substitution (estimated at 3.6% per million years; Bermingham *et al*., 1997b) and the fact that many of the genera sampled in Figure 3 diverged in excess of 100 million years ago, it is difficult to conceive a valid statistical test for universal clock-like properties of synonymous substitutions that is not profoundly compromised by saturation. In this light, a maximum-likelihood clock test applied to third codon position sites (note that 82–100% of all substitutions within major-lineages are synonymous and the vast majority of these occur at third positions), which failed to reject the existence of a clock for the sampled Actinopterygii ($P \geq 0.75$, d.f. = 21), should not be regarded as even suggestive evidence that such a 'universal' clock exists.

In summary, even allowing for a high degree of unclocklike behaviour in the synonymous substitution rate of the fish considered here, the magnitude of the observed mtDNA divergence within major-lineages and their divergence from basal South American taxa provides no evidence that primary freshwater fishes colonized Mesoamerica prior to the formation of the Panama landbridge at the end of the Tertiary.

Were the colonizations and expansions of Mesoamerican primary freshwater fishes contemporaneous?

A striking feature of the trees shown in Figure 2 is the radiation of Mesoamerican OTUs which, for each mtDNA major-lineage, derive from a single polytomy. We interpret these polytomies as evidence of rapid expansion across the Mesoamerica landscape. The lack of phylogenetic resolution is not due to nucleotide saturation, and thus the polytomies indicate that the

rate of OTU diversification has been rapid in comparison to nucleotide substitution in the mtDNA *ATPase 6* & *8* genes.

Pairwise divergence estimates calculated across the boxed nodes in Figure 2, and indicated graphically in Figure 3 (black bars) establish clear differences in the frequency distribution of *ATPase* K_s divergence across eight Mesoamerican major-lineages of primary freshwater fish. For example, there is almost no overlap between the frequency distributions of pairwise OTU distances derived from the ARW polytomy compared with those of ARE (Fig. 3). Thus, under the assumption of nucleotide substitutional rate constancy within genera (note the modest level of mtDNA divergence between ARE and ARW in Table 1), it would follow that ARW spread across Mesoamerica prior to ARE. To more generally compare the relative chronology of Mesoamerican expansion by the eight major-lineages, we estimated the time-line presented in Figure 4. The relative expansion times were calculated from clock-constrained trees, by estimating the genetic divergence across each polytomy, or by using the maximum observed Mesoamerican divergence where no polytomy was observed. Although our interpretation assumes an approximate mtDNA *ATPase* clock for the class Actinopterygii, lineage specific clocks would have to tick at very different rates to support a model of simultaneous expansion by all major-lineages (e.g. CYP compared with BRE).

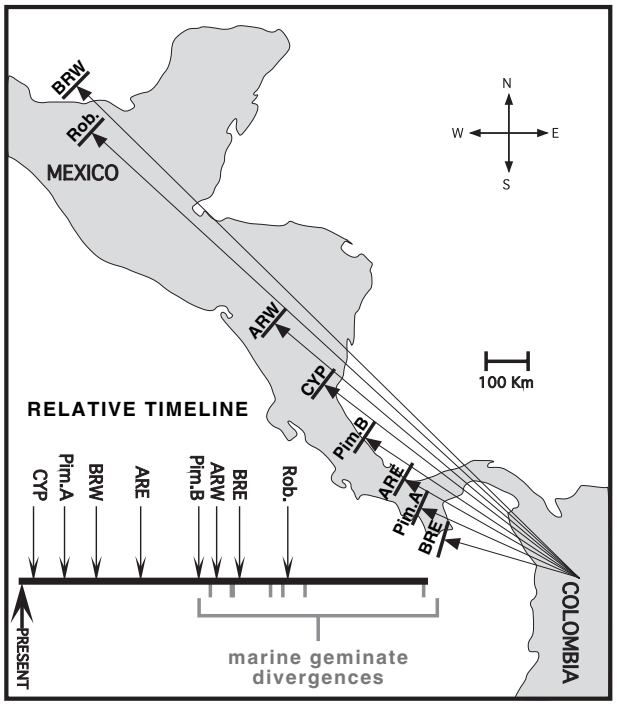

Figure 4. Inferred timing and geographical limit of initial waves of Mesoamerican primary freshwater fish expansion out of South America. Arrows mark the most westerly extent of waves of expansion, estimated from contemporary species distributions. The timeline represents an approximate reconstruction of the relative temporal sequence of waves of expansion based on the relative phylogenetic depth of observed polytomies for each lineage (see Results for details). Below the line are divergence estimates of marine fish geminate pairs (unlabelled lines). For the lineages *Pimelodella* A and CYP in which no polytomy between operational taxonomic units was observed the maximum observed divergence estimate among Mesoamerican taxa was used to estimate the relative timing of their expansion. Major-lineage abbreviations are as described in the text.

What factors limit the spatial expansion of mtDNA major-lineages?

Polytomies permit strong inference that the major-lineages established their respective geographical distributions across Mesoamerica in single expansionary waves (Figs 1, 2). The major-lineage CYP represents only a single OTU and cannot be considered to be derived from a polytomy in the same sense as the other four. However, CYP appears to have achieved its current distribution across the Pacific slope of Panama and Costa Rica in a process identical to that we hypothesized to have occurred in the various historical periods represented by the polytomies in the other four major-lineages. In the case of CYP, only three nucleotide substitutions have accumulated between populations representing the extremes of CYP's distribution, indicating a very recent and rapid expansion. Assuming a South American colonization source and ignoring the impact of any historical extinction events at the distribution extremes of major-lineages, Figure 4 establishes the geographical extent of the expansion waves for eight major-lineages of primary freshwater fishes. Regarding the taxa presented in the present study, the expansion of BRE and ARE progressed less than 500 km, whereas CYP, BRW, and ARW major-lineages spread approximately 900, 2000, and 1100 km, respectively (Fig. 4). If correct, this would suggest that, contrary to expectations based on the inferred dispersal capacity of individuals, primary freshwater fish do (at least in some circumstances) possess the potential to disperse extremely rapidly (relative to the rate of synonymous mitochondrial substitutions).

Our phylogeographical analyses permit us to draw two interesting conclusions that apply to primary freshwater fishes in Mesoamerica and potentially to freshwater taxa in general. First, broadly speaking, the rapid rate and extent of range expansion indicated by the molecular data suggest that the frequency and spatial distribution of interdrainage

exchange events did not restrict the extent or rate of range expansion. Therefore, there is little if any discernible positive relationship between the distance lineages have dispersed from their assumed centre of origin (in this instance, South America) and the chronology of the expansionary waves that led to their contemporary distributions (Fig. 4). Second, it would appear that the westerly limits of freshwater fish distributions in Mesoamerica (with the possible exception of *Cyphocharax*) are defined largely by ecological factors rather than limited opportunities for dispersal via interdrainage exchange events, as had previously been assumed by some authors (Bussing, 1998). This conjecture assumes that the likelihood of dispersal via river capture or anastomosis is broadly similar across all major-lineages, whereas their ecologies are not.

Can demography and niche-overlap explain the pattern of rapid colonization and genetic diversification of Mesoamerican primary freshwater fishes?

The observed lack of sympatry between OTUs representing the same major-lineage is difficult to reconcile with the substantial connectivity among Central American drainages demonstrated by the rapid expansion of mtDNA major-lineages. Significant interdrainage exchange among primary freshwater fish is also reflected in the distribution of single OTUs, which are often distributed across multiple drainages but show no or little genetic variation (particularly when measured against inter-OTU divergence). In our view, the resolution of the paradox lies in recognizing that the establishment probability of immigrants is much higher at the leading edge of colonizing waves than behind it. As discussed by Hewitt (1993) in the context of postglacial re-colonization of Europe, the probability that the offspring and alleles of immigrants will be represented in succeeding generations is greatly affected by the extent to which requisite resources are exploited in the areas to which they disperse. The probability that immigrant alleles become established in habitable areas entirely unoccupied by taxa sharing the same fundamental niche (most likely conspecifics or congenerics) is high, regardless of the immigrant propagule size (Zaret & Paine, 1973; Van Den Bosch, Hengeveld & Metz, 1992). However, immigrant alleles are much less likely to become established in habitats or regions already at the carrying capacity (K) for a particular taxon, unless the immigrant propagule size is large relative to K and/or the frequency of immigration is high. Thus, a priority effect comes into play, whereby high frequency alleles characterizing established populations are more likely to persist than low frequency alleles representing small groups of immigrants (unless the latter have a significant selective advantage). Such a priority effect could result solely from genetic drift, thus indicating that the level of mtDNA divergence (and phenotypic differentiation) observed among OTUs representing the same major-lineage is not associated with significant impediments to reproduction between them. Alternatively, the lack of sympatry between closely related OTUs could signal competitive exclusion, given that a destabilizing effect on coexistence has been empirically and theoretically described for species for which a high degree of niche-overlap leads to intense competition for at least one limiting resource (Abrams, 1983; MacArthur & Levins, 1967; Heller & Gates, 1971; MacArthur, 1972; Hutchinson, 1978; Bowers & Brown, 1982; Bernatchez & Wilson, 1998; Galis & Metz, 1998). Thus, unless an immigrant lineage has a strong selective advantage over a resident occupying the same fundamental niche, the persistence of the resident is simply a function of its numbers relative to the immigrant.

Consequently, it can be seen how the very different processes of drift and interlineage competition could act in concert or separately to generate priority effects as taxa expand their ranges. Such priority effects will only be manifest when the niches of resident and immigrant individuals are sufficiently similar, as will generally be the case between recently separated allopatric or parapatric sister taxa. The anticipated absence of genetic similarity among sympatric Mesoamerican freshwater fishes is strongly reflected in the data presented in the present study. Excepting the single example of BRE ↑bayano and BRE 3, which are 3.3% K_s divergent, all other cases of sympatry (on a per drainage basis) west of the Panama/Colombia border involve OTUs drawn from different major-lineages with levels of genetic divergence exceeding 24.2% K_s. It is worth emphasizing that the absence of sympatry among phylogenetic neighbours stands in stark contrast to the 12 cases of parapatry (or putative parapatry) among OTUs of the same major-lineage (represented in all cases by mtDNA distances less than 10% K_s). In summary, it can be seen how an assumption of a high degree of niche-overlap between closely related OTUs explains the near absence of sympatry among them in the face of evidence for high levels of interdrainage connectivity and extensive areas of sympatry between more highly divergent OTUs.

The discrete nature of river drainages and our molecular phylogenetic approach provide an opportunity to estimate the mean time between interdrainage dispersal events. Utilizing a molecular clock calibration of 3.6% K_s per million years (based on the average marine geminate fish mtDNA divergence and an isthmus completion date of 3.1 million years), we have

crudely estimated that the duration of the expansionary waves represented by the polytomies for the two *Brycon* and two *Bryconamericus* major-lineages ranged between 90 000 and 442 000 years. Dividing these estimates of duration time by the minimum number of interdrainage movements required to span the contemporary Mesoamerican distribution of a given major-lineage gives us $\bar{f}_{edge}$, which is the mean frequency of dispersal events at the leading edge of the colonizing wave. The probability that mtDNA of an immigrant propagule will be represented in subsequent generations after entering a 'vacant' drainage is very high, and here is assumed to be effectively 1. By contrast, within an 'saturated' drainage already at carrying capacity for a particular major-lineage, the mean frequency of events resulting in the replacement of a resident mtDNA with that of an immigrant's ($\bar{f}_{saturated}$) will be low (and approximately proportional to the number of females in the immigrant propagule divided by the total number of established females in the drainage, ignoring the impact of any fitness differences between immigrant and resident females).

Taking BRW as an illustrative example of a recent colonizer that probably expanded across a Mesoamerican riverine network broadly similar to the present, the average interval between interdrainage colonization events at the leading edge of the expansionary wave $\bar{f}_{edge}$, can be estimated as 3600 years (90 000 years per 25 drainages). Next, if we assume a mean historical immigrant propagule size of 300 females ($\overline{P}_f$), and a mean drainage carrying capacity for BRW of 25 000 females ($\overline{K}_f$), it is possible to estimate the frequency of interdrainage replacement events between 'saturated' drainages:

$$(K_f/P_f)\bar{f}_{edge} = \bar{f}_{saturated}$$

In this example, the calculated value of $\bar{f}_{saturated}$ equals 300 000 years, which is the mean time interval between the turnover of occupant lineages by immigrant ones.

Clearly, our example ignores differences in historical population sizes and drainage connectivity, as well as the impact of competitive interactions with distantly-related taxa (diffuse competition). However, the example serves to illustrate how demographic factors not present at the leading-edge of expansionary waves could limit the spread of immigrant alleles between drainages within contiguous distributions, and thus provides an explanation for the significant phylogeographical structure observed in Mesoamerican freshwater fishes in spite of the opportunity for movement between drainages implied by the relatively rapid historical expansion of each major-lineage.

Obviously, under our simple model, the turnover time between immigrant and resident mtDNA lineages in geographically adjacent drainages is completely explained by $\overline{K}_f$ to $\overline{P}_f$ ratios. In turn, variation in the number of drainages occupied by OTUs, and the geographical location of phylogeographical breaks between OTUs, would indicate that $\overline{K}_f$ to $\overline{P}_f$ ratios have varied dramatically, probably as a function of both taxon-specific ecology and drainage history. The natural history of Mesoamerican fishes is not known well enough to speculate on ecological differences among the taxa considered here (e.g. elevational distribution within drainages, relative abundance, dispersal characteristics, etc.). However, phylogenetic inference regarding the relative levels of connectivity between drainages can be combined with information on the physical features of drainages, both contemporary and historical, to help identify general features that may influence $\overline{K}_f$ to $\overline{P}_f$ ratios.

For example, the blocks of colour in Figure 1 identify groups of river drainages that have recently undergone interdrainage exchange events resulting in the establishment of immigrant haplotypes, and it appears likely that at least two processes have contributed to the relative mtDNA homogeneity across these regions. The first is large-scale immigration (large $\overline{P}_f$), such as might occur when rivers anastomose near their mouths. Particularly during low sea-level stands, rivers braid as they drain across exposed coastal plains, thus providing increased opportunity for the movement of immigrants between adjacent rivers along a coastline. The second process is local population reduction (resulting in unsaturated drainages) or extinction followed by recolonization, such as might occur as a result of disease or volcanic eruptions. In these cases $\bar{f}_{saturated}$ approaches or equals $\bar{f}_{edge}$, and the probability of successful immigration between drainages increases dramatically. For example, in the case of BRW 3, the recent coalescence of mtDNA haplotypes is well explained by reference to bathymetric data and regional volcanic activity along the Pacific slope of western Panama and Costa Rica, suggesting roles for river anastomosis and local population extinction or reduction in the demographic history of *Brycon* populations in this region.

The low $\overline{K}_f$ to $\overline{P}_f$ ratios that we suggest characterize drainages within OTU ranges stand in contrast to the high $\overline{K}_f$ to $\overline{P}_f$ ratios that we posit must explain the phylogeographical breaks between the colour blocks shown in Figure 1. For example, it seems highly probable that immigration will be low (low $\overline{P}_f$) between drainages that can only interconnect via highland stream captures. This expectation is met by the fact that OTUs tend to be distributed on one side or the other of the continental divide (Bermingham & Martin, 1998; Perdices *et al*., 2002). A notable exception is the cross-cordillera relationship of ARE and BRE OTUs in the region of the El Valle volcano in Central Panama, which we attribute to drainage rearrange-

ments caused by volcanic activity which continued up until the last few centuries (Coates, 1997).

Thus, priority effects have effectively stabilized mtDNA lineage distributions, leading to the increased temporal persistence of some lineages. This temporal persistence is demonstrated by the polytomies observed in even the earliest colonizers of the isthmus, a phylogenetic pattern that would not occur if rates of mtDNA lineage turnover were uniformly high. Furthermore priority effects also explain why only a single wave of colonization is apparent in each major-lineage, rather than a number of temporally and spatially staggered waves.

Future directions

As noted by previous authors, the impact of demography (Hewitt, 2000) and niche-overlap (Letcher *et al.*, 1994; Galis & Metz, 1998; Barraclough & Vogler, 2000) can affect the fate of immigrant lineages, and the present study describes how how this can have profound implications for local levels of biodiversity, rate of lineage turnover and the nature and location of range boundaries. Consequently, it is surprising that the explicit consideration of these factors in molecular phylogeographical studies, often within species or between closely related species, is so rare (athough see Galis & Metz, 1998; Mattern & McLennan, 2000). In his seminal book, MacArthur (1972) compellingly argued that the impact of niche-overlap on coexistence probabilities was most likely to be inferred from historical biogeographical analysis, rather than through ecological field observations. However, historical biogeographers have rarely reversed his logic to consider the role that niche-overlap may play in maintaining parapatry. The increasing number of parapatric and sympatric distributions elucidated through molecular phylogeographical studies, in combination with the temporal framework that they can afford, provides an excellent opportunity to rigorously examine any relationship between genetic divergence and the nature of geographical distributions in a wide variety of organisms. It should be noted, however, that the demographic and ecological constraints on colonization that lead to priority effects are likely to be taxon and locality specific to some extent, being greatest within taxonomic groups and locations where populations sizes are consistently large relative to the number of exchanged migrants, and between taxa where even a low degree of niche-overlap destabilizes their coexistence. Phylogeographical patterns that can usefully be considered in terms of niche-overlap priority effects are not likely to be confined to primary freshwater fishes (although the unitary nature of their populations may make their impact more strikingly apparent) and consistent examples can readily be found in a wide diversity of taxonomic groups; for example, mammals (Sage, Atchley & Capanna, 1993), birds (Mayr & Short, 1970), and insects (Buckley, Simon & Chambers, 2001). In this vein, a potentially rich focus of investigation is the parapatric distributions commonly observed between phylogroups representing members of the European flora and fauna, which have expanded since the last glacial maxima (Hewitt, 1996; Hewitt, 2000). It is plausible that priority effects can provide an explanation for both the relative temporal stability of barrier-free parapatric distributions and why highly divergent monophyletic refugial populations are predominantly observed as opposed to closely related polyphyletic ones. Future work developing the priority-effect model presented here will be necessary to rigorously test the predictions of the theory against both simulated and observed phylogeographical data (integrating relevant ecological evidence when available) and to fully assess the role that niche-overlap plays in explaining the spatial and temporal distributions of biological organisms.

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SUPPLEMENTARY MATERIAL

The following material is available for this article online:

Table S1: Taxonomy and collections details
Table S2: *ATPase 6* & *8* PCR-RFLP restriction sites

This material is available as part of the online article from http://www.blackwell-synergy.com