

Phylogeography of a flooded forest specialist fish from central Amazonia based on intron DNA: the cardinal tetra *Paracheirodon axelrodi*

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SUMMARY

1. Historic and extant landscape structures and environmental conditions are known to influence phylogeographic patterns and population histories in organisms from Amazonia. Recent work suggests that events of the Tertiary Period have played a significant role in generating biodiversity in this exceptionally rich but understudied region. However, species distributions and population structures are also affected by recent environmental and physical changes, especially within highly dynamic ecosystems.

2. The cardinal tetra is a small luminous fish native to the dynamic flooded forest ecosystems of the Rio Negro and Orinoco basins of South America. This very popular aquarium fish represents the single most important species in the ornamental fishery of the Rio Negro, an activity of high socio-economic value for local riverine communities. Here we use a fine-scale sampling regime and sequence data from the second intron of the Ribosomal protein S7 (S7 2) to investigate population structure, colonisation history and genealogical relationships in cardinal tetras of the Rio Negro.

3. High levels of S7 2 polymorphisms revealed phylogeographic patterns across several temporal settings that appear associated with the complex dynamics of the region. Our results suggest a long history of isolation and persistence of cardinal tetra populations in the headwaters and upper regions of the Rio Negro and recent events of colonisation within the incipient Rio Negro floodplain. These colonisation events were followed by recent population expansions likely facilitated by the establishment of the extensive Rio Negro floodplain, an anabranching ecosystem of Holocene age.

4. Our reconstruction of the phylogeographic history of cardinal tetras based on S7 2 intron sequence data reflects the complex influence of both geomorphological and climatic events through time in the Rio Negro basin. Our findings also indicate that in order to maintain the cardinal tetra fishery as a sustainable activity for rural Amazonians, the fishery should be restricted to the middle Rio Negro, the region of the basin where population replenishment is more likely to occur.

Keywords: conservation genetics, population genetics, Rio Negro, S7 2, teleosts

Introduction

Historic and extant landscape structures and environmental conditions are known to influence phylo-

geographic patterns and population histories of Amazonian organisms. Recent studies suggest that events of the Tertiary Period have played a significant role in generating biodiversity in this exceptionally rich

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but understudied region (e.g. Hubert & Renno, 2006; Hubert *et al.*, 2007a; Elmer, Davila & Lougheed, 2007). However, species distributions and population structures are also affected by recent environmental and physical changes, especially within highly dynamic ecosystems. One such dynamic region is the Rio Negro basin. Located in central Amazonia, the Rio Negro is a complex aquatic habitat home to over 1000 species of freshwater fish (Chao, 2001). It is characterized by 'black water' (Sioli, 1984) and a spectacular anabranching floodplain system of fluvial archipelagos, *igapós* (flooded forest) and *igarapés* (small streams) (Val & De Almeida-Val, 1995). Draining over 600 000 km² the Rio Negro is the second largest tributary of the Amazon after the Rio Madeira and ranks sixth in the world in terms of discharge (c. 29 000 m³ year⁻¹) (Filizola, 1999; Latrubesse & Franzinelli, 2005).

A large proportion of the ichthyofauna of the Rio Negro comprises small sized fish inhabiting extensive *igapós* and *igarapés*. The diversity of small flooded forest fish supports a thriving ornamental fishery with over 100 fish species frequently traded in the Rio Negro each year (Chao, 2001). The socio-economic value of the ornamental fishery of the Rio Negro region is substantial by local standards representing approximately US\$ 3 million per year with over 30 million live fish exported annually (Chao, 2001). Fortunately, the relatively short life cycles of the many small forest fish (≤ 2 years), high productivity, and the low-impact sampling methods used by the ornamental fishermen have, to date, prevented the over-exploitation of this ornamental resource (Andrews, 1990). However, there is a pressing need to develop management strategies incorporating biological data in conjunction with socio-economic and fishery information that will ensure the sustainability of the Rio Negro ornamental fishery. This is because a stable economic livelihood from the fishery may keep local people from engaging in more ecologically destructive activities (Norris & Chao, 2002).

The cardinal tetra, *Paracheirodon axelrodi* (Schlutz, 1956), is a luminous neon species up to 2.5 cm in length (Axelrod, 1995). It represents over 80% of the ornamental fishery catch and is the single most important species in the ornamental fish trade of the Rio Negro region. The cardinal tetra is native to the 'black water' *igapó* and *igarapés* habitats extending throughout the middle to upper Rio Negro and Orinoco River basins of South America (Harris &

Petry, 2001). During the annual inundation phase of the Rio Negro hydrological cycle, a vast lateral floodplain forms which is largely covered with seasonal *igapó*. Cardinal tetras, like numerous other fish species in this environment, utilize *igapó* for feeding, reproduction and refuge (Goulding, Carvalho & Ferreira, 1988; Marshall, Forsberg & Thome-Souza, 2008). At the beginnings of the inundation cycle, cardinal tetras move upstream and laterally from the *igarapé* habitat to *igapó*, returning only to the *igarapés* during the low water season. Thus the flooded forest environment provides a route for migration and mixing of cardinal tetra populations between shallow streams and adjacent wetlands (Winemiller, 1993; Marshall *et al.*, 2008).

In this study, we used a large sample and sequence data from the second intron of the ribosomal protein S7 (S7 2) to investigate the phylogeographic history of cardinal tetras in the Rio Negro. Phylogeography, the study of genealogical lineages over time and space (Avice, 2000), reveals evolutionary processes driving the relationship between natural genetic pattern and geography (Avice, 2000; Beheregaray, 2008). Phylogeographic studies can reveal population units that have been isolated from conspecific populations for sufficient time to have undergone a distinct evolutionary history. Therefore, these studies can help determine population units with maximum evolutionary potential and thus inform conservation strategies (Ryder, 1986; Moritz, 1994). This study adopted a conservation criterion based on nuclear DNA, in which populations of cardinal tetras that significantly diverge in allele frequencies at S7 2 can be considered as a management unit (MU) with high conservation priority (Moritz, 1994). This is because these population units are little connected by gene flow and are thus compositionally independent (Ryder, 1986; Moritz, 1994).

The S7 2 intron was chosen for this phylogeographic study in cardinal tetras based on the detection of high polymorphism in this species as well as its technical utility for large-scale genotyping (Cooke & Beheregaray, 2007). Nuclear introns are showing an emerging potential for obtaining informative genealogical data in combination with the more popular mitochondrial DNA genome (Zhang & Hewitt, 2003; Beheregaray, 2008). To date, introns of the ribosomal protein gene have been used successfully in phylogeographic and phylogenetic studies across several

groups of teleosts (e.g. BurrIDGE, Melendez & Dyer, 2006; Morrison *et al.*, 2006; Domingues *et al.*, 2007; Hubert *et al.*, 2007b).

Among cardinal tetra populations, it is likely that phylogeographic patterns in this species may reflect historical events related to riverine dynamics and the geomorphological history of this region (Lundberg, 1998; Benda *et al.*, 2004, Latrubesse & Franzinelli, 2005) since their dispersal potential is coupled with the annual inundation cycle of the Rio Negro floodplain (Winemiller, 1993; Marshall *et al.*, 2008). While tectonic episodes that began during the Cretaceous spawned the major drainage systems in South America (Lundberg, 1998), much of the complex floodplain environment of the Rio Negro evolved recently during the Holocene (Latrubesse & Franzinelli, 2005). During that time the Rio Negro experienced an accumulation of sedimentary deposits. These deposits came as a compound response of the Solimões-Amazon to climatic changes of the mid-Holocene which resulted in an alluvial damming at the mouth of the Rio Negro and the subsequent formation of the Rio Negro floodplain upstream (Latrubesse & Franzinelli, 2005).

The geomorphological history of the Rio Negro (Latrubesse & Franzinelli, 2005) provides a model in which to test phylogeographic predictions in cardinal tetras. These predictions include: (i) older cardinal tetra lineages exist in the geologically ancient and isolated headwater tributaries; (ii) prior to the establishment of the Rio Negro floodplain, multiple colonisation events of the middle Rio Negro tributaries were from the headwaters and (iii) during the Holocene, in association with the establishment of the Rio Negro floodplain, cardinal tetras underwent population

expansions into the new suitable habitat. These predictions are likely to be observed for any floodplain dependant specialists but to date have not been tested for organisms in the Rio Negro (e.g. see Aleixo, 2006 for a study on floodplain forest birds from the Amazon River). We assessed the relative influence of ancient vicariant events, such as habitat fragmentation, compared to more recent events associated with range expansion and gene flow, by adopting an assortment of analytical tools that exploit phylogeographic signals across a wide temporal spectrum (e.g. Avise, 2000; Garrick *et al.*, 2007). Table 1 lists how analytical approaches described in the Methods were used to address each prediction of our study.

Methods

Sample collection

A total of 301 cardinal tetras were collected from 17 tributaries along the Rio Negro (Fig. 1, Table 2), a sampling effort that essentially covers the entire distribution of the cardinal tetra in the Rio Negro. Fish were caught in the flooded forest using hand nets and bait traps. Muscle tissue from behind the dorsal fin was removed and preserved in 95% ethanol. All fish were collected by the senior author (L.B.B.) and no samples from the ornamental fishing trade were used.

Genetic methods

DNA was extracted from the ethanol preserved muscle tissue using a modified salting out method (Sunnucks & Hales, 1996). Polymerase chain reaction (PCR) amplification of the S7 2 was performed with

Table 1 Summary of prediction(s) addressed in this study, temporal scale(s) considered, analytical methods used and associated *a priori* expectations (style based on Garrick *et al.*, 2007)

Prediction	Time scale	Analytical method	Expectations
1	Ancient	ML phylogenetic analysis, haplotype network, nested clade analysis	Headwaters alleles basal and highly divergent, historical fragmentation and evidence of restricted gene flow
2	Ancient–present	Haplotype network, nested clade analysis	Inference chain indicates multiple temporal ‘phases’. Restricted gene flow and long distance colonisation
3	Recent–present	Population parameters, demographic analysis, nested clade analysis	Population structure higher in headwaters than upper to middle Rio Negro. Range expansion and demographic growth detected within floodplain

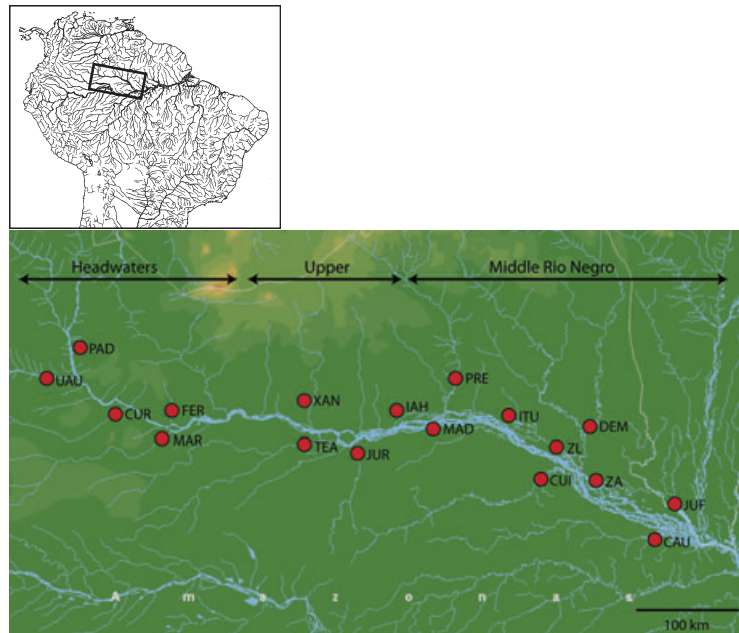


Fig. 1 Sampling localities of cardinal tetras (*Paracheirodon axelrodi*) in the Rio Negro basin, central Amazonia. Inset shows the location of the study area in northern South America.

Table 2 Sampling localities, sample size and measures of genetic diversity for populations of cardinal tetra *Paracheirodon axelrodi* sampled in the Rio Negro, Amazonia (PWD, pairwise difference)

River/igarapé	Abbreviation	Geographic coordinates	Sample size	Mean no. PWD	Nucleotide diversity
Paduá	PAD	00°12'23"N, 67°19'23"W	13	5.11 ± 2.56	0.023 ± 0.013
Vaupés	UAU	00°04'47"N, 67°24'13"W	7	3.27 ± 1.79	0.015 ± 0.009
Curicuriari	CUR	00°13'37"S, 66°48'5"W	6	3.97 ± 2.14	0.018 ± 0.011
Marié	MAR	00°26'37"S, 66°24'58"W	8	2.93 ± 1.62	0.013 ± 0.01
Ferrinha	FER	00°20'24"S, 66°22'8"W	18	0.23 ± 0.28	0.001 ± 0.001
Tea	TEA	00°32'59"S, 65°15'13"W	10	3.52 ± 1.87	0.016 ± 0.009
Arixaná	XAN	00°21'50"S, 62°11'51"W	10	2.62 ± 1.46	0.012 ± 0.007
Jurubaxi	JUR	00°33'07"S, 64°48'06"W	9	2.45 ± 1.39	0.011 ± 0.007
Iahá	IAH	00°25'47"S, 64°36'26"W	9	2.41 ± 1.37	0.01 ± 0.007
Madiquié	MAD	00°25'48"S, 64°24'04"W	7	3.90 ± 2.08	0.17 ± 0.010
Preto	PRE	00°06'40"S, 64°05'03"W	12	2.32 ± 1.31	0.01 ± 0.007
Ítu	ITU	00°26'00"S, 63°07'00"W	10	3.50 ± 1.86	0.016 ± 0.009
Zalala	ZL	00°39'59"S, 63°00'32"W	11	3.05 ± 1.65	0.014 ± 0.008
Demini	DEM	00°23'40"S, 62°51'17"W	11	3.69 ± 1.94	0.017 ± 0.009
Cuiuni	CUI	00°46'09"S, 63°10'40"W	11	1.80 ± 1.08	0.008 ± 0.005
Zamula	ZA	00°51'57"S, 62°46'22"W	6	3.07 ± 1.72	0.014 ± 0.009
Caurés	CAU	01°19'01"S, 62°24'54"W	7	3.62 ± 1.95	0.016 ± 0.009
Jufari	JUF	00°59'40"S, 62°06'10"W	9	2.67 ± 1.49	0.012 ± 0.008

primers S7RPEX2F (5'-AGCGCCAAAATAGTGAA-GCC-3') and S7RPEX3R (5'-GCCTTCAGGTCAGAG-TTCAT-3') (Chow & Hazama, 1998). All samples were screened for sequence polymorphism in S7 2 using single-stranded conformation polymorphism analysis (SSCP) as described in Sunnucks *et al.* (2000). We used 10 μ L radiolabelled PCR for the SSCP containing: c.150 ng of template DNA, 1.2 pmol of each primer,

2 units of *Taq* polymerase (Qiagen, Doncaster, Vic., Australia), 200 μ M of dCTP, dGTP, dATP and dTTP, 2 mM of MgCl₂, 1.2 μ L of supplied buffer and 0.07 μ L [α ³³P] at 10 mM Ci mmol⁻¹ overlaid with mineral oil. PCR cycling conditions were 94 °C for 4 min, 30 cycles at 94 °C/30 s, 53 °C/30 s, 72 °C/45 s, and an extension at 72 °C for 3 min. Up to 10 unique SSCP gel phenotypes were sequenced per population.

Homozygote gel phenotypes were sequenced preferentially to reduce cloning effort, followed by the highest frequency heterozygote gel phenotype (Table 2). This strategy was consistently followed to ensure a conservative estimate of the allelic composition of each population. Where gel phenotypes were identical, sequences were inferred for those individuals. To insure that gels were scored correctly, identical gel phenotypes on an SSCP gel were sequenced for comparison. Fresh PCR products were prepared, purified with Ultra Clean DNA purification Kit (MO BIO Laboratories, Carlsbad, CA, U.S.A.) and sequenced using an Automatic Sequencer 3730 xl following manufacturers directions. Heterozygous individuals were cloned using TOPO TA Cloning vectors (Invitrogen, Carlsbad, CA, U.S.A.), transformed into chemically competent *Escherichia coli* cells and plated on Luria-Bertani agar. Multiple colonies from each cloning reaction (up to 5) were sequenced until the phase of polymorphisms could be discerned.

Data analysis

All sequences were aligned and edited using SEQUENCHER™ 4.1 (Gene Codes Corporation, Ann Arbor, MI, U.S.A.). Nucleotide diversity (π) (the probability that two randomly chosen homologous nucleotides are different) (Nei, 1987) and the mean number of pairwise differences (PWD) between each allele was calculated for each population using ARLEQUIN 3.01 (Excoffier, Laval & Schneider, 2005).

Analysis of genealogical relationships

Maximum likelihood (ML) values for different models of sequence evolution were obtained for S7 2 using MODELTEST version 3.06 (Posada & Crandall, 1998). The Akaike information criterion used by ModelTest found the transversion model (TVM) to be the most likely model of sequence evolution in S7 2. This model assumes variable bp frequencies, variable transversion frequencies and equal transition frequencies. Using TVM, a ML tree was constructed in PAUP* 4.0b10 (Swofford, 2003) using a heuristic search based strategy. Support to internal branches was based on a neighbour-joining (NJ) bootstrap analysis with 100 replicates. Incorporated in the ML tree was a cardinal tetra sampled from the Orinoco River. The headwaters of the Orinoco lie

adjacent to the headwaters of the Rio Negro, and we hypothesize that ancient cardinal tetra lineages originated during the formation of these black water river systems. Therefore, placement of the Orinoco sample in ML analysis was useful for testing hypothesis 1 and making inferences about population ancestry. A specimen of the neon tetra *Paracheirodon innesi* (Myers, 1936) collected from the Rio Demini (Rio Negro basin) was employed as out-group for phylogenetic analysis. Allele 41 was excluded due to a large deletion (56 bp) that caused inconsistencies due to the alignment of the data.

Nested clade phylogeographic analysis

Nested clade phylogeographic analysis (NCPA) (Templeton, Routman & Phillips, 1995) was implemented to approximate the temporal chronology of recurrent population processes versus historical events without assuming a molecular clock (Templeton, 2004). First genealogical relationships within and among tributary samples were investigated by constructing a network in TCS (Clement, Posada & Crandall, 2000). The TCS program estimates gene genealogies from DNA sequences using the statistical parsimony method defined by a 95% confidence interval. Since the treatment of indels in sequence alignment can interfere with positional homology (Hills, Moritz & Mable, 1996), the network was constructed excluding deletions as a fifth character. This enabled a more conservative and reliable estimation of genealogical relationships between S7 2 alleles. The Orinoco allele was included in the network, however it was not incorporated into the subsequent NCPA.

Using the network estimated in TCS (Clement *et al.*, 2000) hierarchical nesting categories were assigned following Templeton & Sing (1993) and Templeton, B & Sing (1987). Geographical distances between sites along river margins/main floodplain areas were estimated from geographic positioning system coordinates using Google Earth (2005) and implemented in the analysis as a distance matrix. The null hypothesis was tested following a contingency chi-squared test in GEODIS 2.2 (Posada, Crandall & Templeton, 2000) with 1000 permutations. Templeton (2005) inference key was implemented when the null hypothesis was rejected (<http://darwin.uvigo.es/software/geodis.html>). The inference key interprets relationships between D_c (clade distance) and D_n (nested clade

distance) and I-T (interior-tip) contrasts enabling biological interpretations of the nesting design.

Analysis of population structure

Genetic differentiation between populations was assessed by comparing tributary samples using Weir & Cockerham's (1984) pairwise fixation index estimated in ARLEQUIN 3.01 (Excoffier *et al.*, 2005). A Tamura correction, which allows for unequal nucleotide frequencies, and unequal frequencies of transversions and transitions (Tamura, 1992) was implemented in the pairwise distance (θ_{ST}) analysis. The Tamura correction is the most similar model of sequence evolution to TVM offered by ARLEQUIN 3.01 (Excoffier *et al.*, 2005). A hierarchical analysis of molecular variance (AMOVA) using a Tamura correction with 10 000 permutations was conducted in ARLEQUIN 3.01 (Excoffier *et al.*, 2005). AMOVA partitions total genetic variance into covariance components to compute fixation indexes as a measurement of genetic differentiation (Excoffier, Smouse & Quattro, 1992). AMOVA groupings were assigned to quantify the amount of genetic variance imputable to *a priori* population and geographic subdivisions. An AMOVA was conducted for all populations as a single group, and for all populations on the left river margin versus the right river margin. To test for a correlation between θ_{ST} and geographical distance, Mantel tests (Mantel, 1967; Smouse & Long, 1986) were employed using ARLEQUIN 3.01 (Excoffier *et al.*, 2005). The Mantel test implements a permutation procedure to test the significance of any correlation between two distance matrices. The same geographical distance matrix used for NCPA was employed in our Mantel test.

Analysis of demographic history

Demographic history was assessed by computing pairwise mismatch distributions using ARLEQUIN 3.01 (Excoffier *et al.*, 2005). Mismatch analysis computes the distribution of the observed number of PWD between alleles, testing for the agreement of the data set with a distribution expected under a model of demographic expansion (Rogers & Harpending, 1992; Excoffier *et al.*, 2005). A unimodal distribution is expected for populations experiencing demographic expansion, while multimodal distributions are

expected for populations at demographic equilibrium. Mismatch analysis were performed on (i) all populations; (ii) headwater populations; (iii) all sampled populations excluding the headwater populations, including the upper and middle Rio Negro and (iv) populations sampled of the mid-floodplain region (JUR, MAD, IAH, ITU, CUI, ZL and ZA) (Fig. 1). These populations occupy the most recently formed floodplain region of the Rio Negro according to the geomorphological reconstruction of the Rio Negro by Latrubesse & Franzinelli (2005). All mismatch analyses were conducted excluding deletions as the fifth character.

Results

Characterisation of SSCPs and subsequent sequencing of unique gel phenotypes revealed remarkable levels of variation (see Cooke & Beheregaray, 2007). The aligned S7 2 gene sequence in *P. axelrodi* is 221 base pairs (bp) in length, of which 93 bp were variable. Forty-six alleles were detected in our sample of 174 fully genotyped individuals (Table 3). Nucleotide sequences were deposited in GeneBank under accession numbers EF176114–EE176069. Levels of genetic diversity differed considerably between populations (Table 2). Comparatively, headwater populations had high mean PWD ($\hat{u} = 3.84$) relative to the upper (PWD $\hat{u} = 2.98$) and middle Rio Negro regions (PWD $\hat{u} = 2.97$). Further, nucleotide diversity was also high in the headwaters relative to downstream sites, with the exception of MAD (0.17 ± 0.010).

Marked population structure was detected based on θ_{ST} analysis, with 80% of population comparisons being significantly different ($P \leq 0.05$) (Table 4). High levels of differentiation were mostly observed in comparisons involving the headwater populations CUR, FER, MAR, PAD and UAU. In general, these samples represent highly distinct populations ($P \leq 0.01$) when compared to each other and to the remaining populations of the Rio Negro. In contrast, genetic differentiation was generally reduced in the upper and middle Rio Negro populations in which gene flow was evident between tributary samples (Table 4). The AMOVA identified that 28.2% of the genetic variation was accounted for by differences between populations ($P < 0.0001$). On the other hand, no significant genetic differentiation was found when grouping populations by river margin ($P = 0.309$).

Table 3 The frequency of each allele at each sampling location

	Allele																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
UAU	–	8	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PAD	2	–	–	–	–	–	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MAR	–	–	–	–	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CUR	–	–	–	–	–	2	–	–	–	–	4	2	2	2	2	2	–	–	–	–	–	–	–
FER	1	–	–	34	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–
TEA	6	–	–	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
XANA	2	–	8	–	–	–	–	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
JUR	6	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MAD	8	–	3	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IAH	4	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PRE	9	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	4	–	–	–
ITU	–	–	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	3	8
CUI	–	10	–	5	–	–	–	5	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–
ZL	2	–	–	6	–	–	–	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DEM	3	–	2	3	–	–	–	–	–	12	–	–	–	–	–	–	2	–	–	–	–	–	–
ZA	2	2	1	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–
CAU	–	–	4	–	–	–	–	7	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
JUF	7	6	–	2	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

	Allele																						
	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
UAU	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	6	6	–	–	–	–	–
PAD	–	–	–	–	–	–	–	–	1	1	2	2	1	–	–	–	–	–	–	–	–	–	–
MAR	–	–	–	–	2	2	2	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CUR	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
FER	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TEA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7	–	–	–	–	–	–	–	–
XANA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–
JUR	4	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MAD	–	–	2	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IAH	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PRE	–	–	–	–	–	–	–	–	–	–	–	–	–	5	–	–	–	–	–	–	–	–	–
ITU	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CUI	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ZL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5
DEM	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ZA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	2	2	–
CAU	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
JUF	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Similarly, the Mantel test revealed no correlation between genetic and geographic distance ($r = 0.16$, $P = 0.122$). These two non-significant results suggest a more complex scenario of differentiation than that expected if the main channel of the Rio Negro has acted as a major barrier for dispersal, or under a simple model of isolation by distance, respectively.

The S7 2 network linked all alleles based on a criterion of 95% statistical parsimony (Fig. 2). Alleles that were different due to deletions alone were

grouped (1 and 10, 2 and 3, 7 and 24, 8 and 17, 15 and 16). For sampling locations of each allele see Table 3. Allele 1 was identified as ancestral and is found in populations throughout the entire Rio Negro. The signal of ancestry in networks can be confounded by demographic growth and sampling bias (Posada & Crandall, 2001); for this reason, a second network was constructed excluding allele frequencies (figure not shown). For this network allele 7, which is closely related to allele 1 and found largely

Table 4 Pairwise θ_{ST} comparisons for the 18 sampled populations of cardinal tetra *Parachanna axelrodi* in the Rio Negro

	Upper Rio Negro									Middle Rio Negro								
	UAU	PAD	MAR	CUR	FER	TEA	XANA	JUR	MAD	IAH	PRE	ITU	CUI	ZL	DEM	ZA	CAU	
UAU	0																	
PAD	0.1045	0																
MAR	0.1806*	0.0867	0															
CUR	0.2477**	0.1372*	0.2042**	0														
FER	0.5993**	0.7938**	0.8324**	0.8141**	0													
TEA	0.1835**	0.1704**	0.3072**	0.2929**	0.5451**	0												
XANA	0.0973	0.1452	0.2263*	0.3233**	0.7792**	0.2036**	0											
JUR	0.1113	0.0646	0.2492**	0.2537**	0.7686**	0.1253**	0.0479	0										
MAD	0.1737**	0.1564*	0.3545**	0.3199**	0.7532**	0.1218*	0.1738*	0.0302	0									
IAH	0.1038*	0.1692*	0.2720**	0.3236**	0.7346**	0.1739**	0.0475	0.0647	0.1254*	0								
PRE	0.1842**	0.3172**	0.4606**	0.4570**	0.7270**	0.2598**	0.1836*	0.1466*	0.1498*	0.1237*	0							
ITU	0.1535*	0.1225	0.2835**	0.2888**	0.6978**	0.1361**	0.1341	0.0519	0.0482	0.1127	0.1704*	0						
CUI	0.1216*	0.2428**	0.3535**	0.3814**	0.6107**	0.1296*	0.0756	0.0999	0.1589*	0.0663	0.0844	0.1489*	0					
ZL	0.1199*	0.1601*	0.2804**	0.2934**	0.5445**	0.03762	0.0866	0.0584	0.0931	0.0792	0.1433*	0.0997	0.0092	0				
DEM	0.2257**	0.2510**	0.4552**	0.3907**	0.7196**	0.1065*	0.2898**	0.1164	0.0098	0.2121	0.1986**	0.1026	0.1927*	0.1074	0			
ZA	0.1555*	0.3144**	0.4186**	0.4471**	0.7720**	0.2730**	0.1684	0.1788*	0.2072*	0.1057	0.0436	0.1938*	0.0671	0.1470*	0.2776**	0		
CAU	0.1409	0.1064	0.1845**	0.2639**	0.7437**	0.1183**	0.0207	0.0499	0.1416*	0.0693	0.2236**	0.1105	0.0895	0.050	0.2335**	0.2071*	0	
JUF	0.1048	0.1859*	0.3597**	0.3342**	0.6860**	0.1213*	0.1227	0.0363	0.04621	0.0758	0.0438	0.0843	0.0268	0.0362	0.0660	0.0751	0.1253	

* $P \leq 0.05$; ** $P \leq 0.01$.

in the headwaters, was identified as ancestral based on 95% statistical parsimony. Allele 7 is a likely ancestral candidate as it is placed internal to many alleles.

The haplotype network (Fig. 2) revealed deeper levels of sequence divergence between headwater alleles compared to alleles found in both the upper and middle region of the Rio Negro. Of the 25 nested clades within the haplotype network, 16 had significant phylogeographic structure, and eight yielded biological inferences by NCPA (Table 5). The partial spatial and temporal scattering of headwater alleles throughout both nesting clades 3-1 and 3-2 indicates several events of genetic interchange between headwater populations and the remainder of the Rio Negro (Templeton, 1998). The oldest inferred scenario for the Rio Negro involved restricted gene flow in the presence of isolation by distance (total cladogram). This seemed to be followed by a range expansion and restricted gene flow with some long distance dispersal. Continual range expansion proceeded the following cycles of restricted gene flow with limited long distance dispersal, isolation by distance and population fragmentation. Thus, at most timescales, restricted gene flow and isolation by distance were the processes inferred. This is consistent with expectations of a low mobility fish found across a relatively large spatial scale. On three cases (clades 1-6, 2-2 and 3-2) restricted gene flow was coupled with rare long distance dispersal over large spatial scales (Table 5).

The ML phylogenetic analysis (Fig. 3) revealed that the most basal alleles were sampled in the headwaters, whereas more recently derived alleles were sampled throughout the entire Rio Negro study area (Table 3). Allele 28, 33 and 39 were sampled in MAR, PAD and UAU respectively. The allele found in the Orinoco basin was also basal and most closely related to headwater alleles.

For headwater populations, there was a significant deviation from a unimodal distribution expected under demographic expansion ($P = 0.023$ and 0.004 based on SSD and r tests, respectively). This result is typical of old populations, including those isolated for

a relatively long time (Rogers & Harpending, 1992). Mismatch analysis (Fig. 4) performed on populations from both the upper and middle Rio Negro also showed a lack of support for demographic expansion ($P = 0.002$ and 0.002 based on SSD and r tests, respectively). On the other hand, evidence for population expansion was only found in mid-floodplain populations (JUR, MAD, IAH, ITU, CUI, ZL and ZA) with a non-significant deviation of the distribution expected under a model of demographic expansion based on the r index ($P = 0.043$; SSD test was only marginally significant $P = 0.043$).

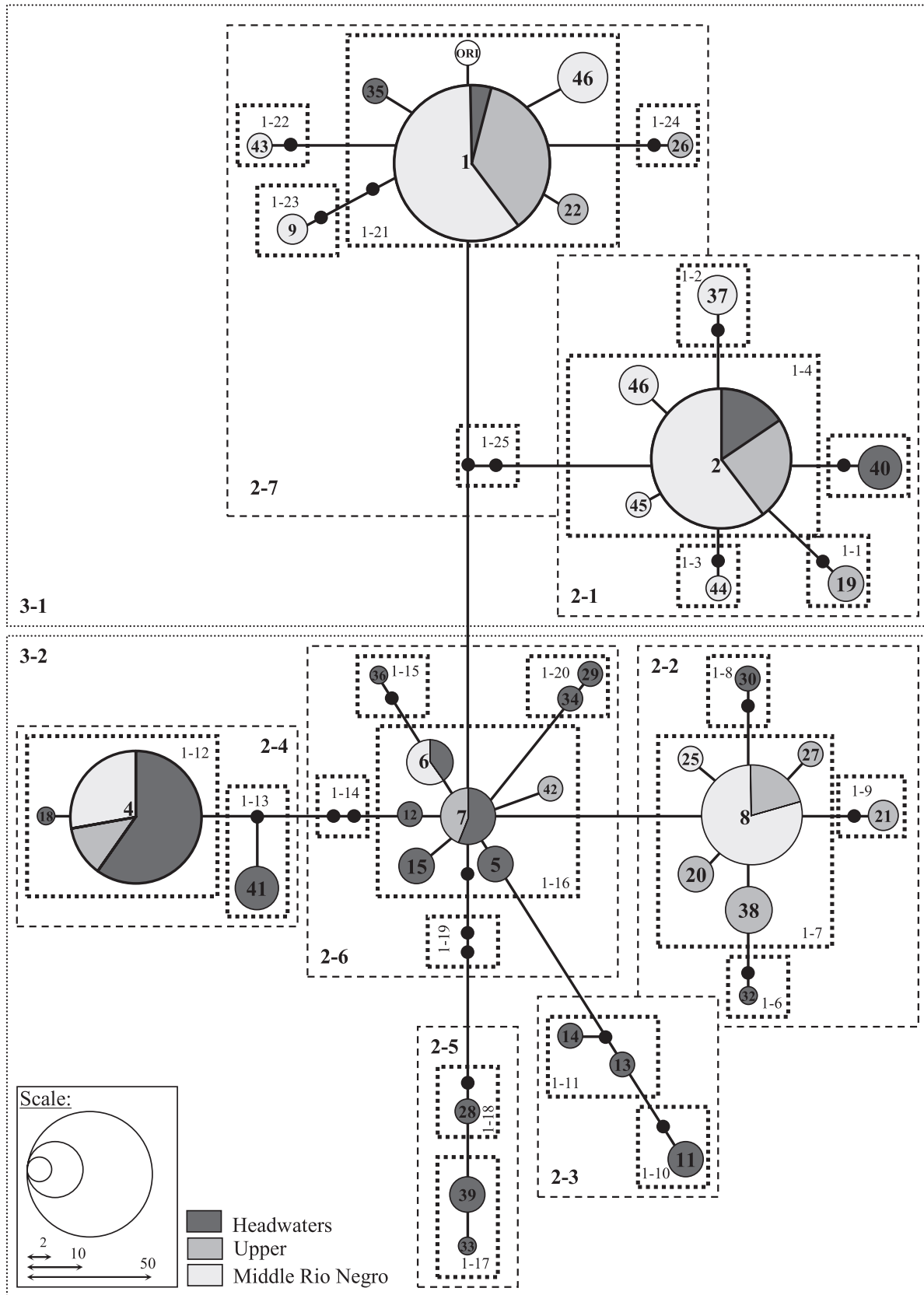
Discussion

In this study, we used the intron S7 2 to investigate population structure, colonisation history and genealogical relationships of cardinal tetras sampled along the Rio Negro in central Amazonia. We found high levels of polymorphism in the S7 2 data set, consistent with high genetic variability observed for cardinal tetras based on studies using microsatellite markers and mitochondrial DNA (Beheregaray *et al.*, 2004; L.B. Beheregaray, unpubl. data). The S7 2 polymorphism detected here was informative to detect geographic and genealogical associations in cardinal tetras, validating its usefulness as a molecular marker for intraspecific studies. This information is used below to propose a two-phase scenario that accounts for the phylogeographic structure of cardinal tetras in the region and assist in the development of management strategies for this socio-economically important species.

Population histories derived from intron DNA data

Large and complex river systems like the Rio Negro provide a dynamic setting in which to study the phylogeography of aquatic organisms. Typically, freshwater fish display greater genetic structuring than their estuarine or marine counterparts (Ward, Woodwark & Skibinski, 1994). Yet, understanding the events that have shaped population structure in large

Fig. 2 Statistical parsimony network and nesting design of S72 alleles across cardinal tetra populations of the Rio Negro. Relationships among alleles are estimated using the parsimony method of Templeton *et al.*, (1992). Each circle represents a single allele and the area of the circle is proportional to the frequency of that allele. The shade of the circle defines the region of the Rio Negro in which that allele was sampled. Circle labelled ORI represents allele sample from the Orinoco basin. Lines between alleles indicate one mutational change or step, black dots on lines represent alleles that were not sampled or are extinct. Hierarchical nesting categories were assigned following Templeton & Sing (1993) and Templeton *et al.* (1987).



Significant nested clades	χ^2 permutation <i>P</i> -value	Chain of inference	Inferred process or event	<i>c.</i> Spatial scale (km)
Clade 1-4	<0.001	1-2-3-4-9	Allopatric FRAG.	606
Clade 1-7	<0.001	1-2-3-5-15	Past FRAG. and/or LDC	457
Clade 1-16	<0.001	1-2-3-5-6-7	RGF/disp. With some LDD	596
Clade 1-21	<0.001	1-2-3-4	RGF with IBD	596
Clade 2-1	<0.001	1-2-3-4	RGF with IBD	606
Clade 2-2	<0.001	1-2-3-5-6-7	RGF/disp. With some LDD	571
Clade 2-7	<0.001	1-2-3-4	RGF with IBD	596
Clade 3-1	<0.001	1-2-11-RE-12	CRE	606
Clade 3-2	<0.001	1-2-3-5-6-7	RGF/disp. with some LDD	606
Total cladogram	<0.001	1-2-3-4	RGF with IBD	606

FRAG, fragmentation; CRE, continuous range expansion; RE, range expansion; RGF, restricted gene flow; LDC, long-distance colonisation; disp., dispersal; LDD, long-distance dispersal; IBD, isolation by distance.

Spatial scale refers to the maximum distance between sampling sites. *P*-value represents the frequency with which 1000 chi-squared statistics generated by random permutation were greater than or equal to the observed chi-squared.

river systems is hampered by their environmental complexity compared to other aquatic environments (e.g. Leclerc *et al.*, 2008). The aquatic systems of Amazonia are no exception. Over one-quarter of all known fish species inhabit the freshwaters of the Neotropics (Schaefer, 1998), yet the underlying mechanisms that have shaped genetic structure and generated diversification are poorly understood (Montoya-Burgos, 2003). To date, most phylogeographic studies in the Neotropics have focused on ancient diversifying events of the Tertiary (e.g. Lovejoy & De Araujo, 2000; Sivasundar, Bermingham & Orti, 2001; Montoya-Burgos, 2003; Hubert & Renno, 2006). In contrast, intraspecific studies focusing on more recent demographic events in Amazonia are rare (for a notable exception see Hubert *et al.*, 2007b). However, species distributions and population structure are affected by recent environmental changes, especially within a highly dynamic ecosystem. Environmental disturbances can produce a rapid change of niche availability leading to range expansions and contractions (Hewitt, 2000, 2004). Furthermore, extant landscape structures and environmental conditions are known to influence contemporary gene flow and promote recent divergences (Castric, Bonney & Bernatchez, 2001; Beheregaray, Sunnucks & Briscoe, 2002). Therefore, more recent environmental changes, such as the formation of the Rio Negro floodplain during the Holocene, are expected to leave genetic signatures in extant populations.

Our study revealed complex population dynamics across multiple temporal settings. Ancestral alleles with deeper population divergences were detected in the headwaters, and to a much lesser degree, in the upper Rio Negro. These results were based both on genealogical and phylogenetic analyses and are consistent with mitochondrial DNA data (L.B. Beheregaray, unpubl. data), suggesting long-term population persistences of cardinal tetras in that region. These findings are also corroborated by the close phylogenetic relationship of the Orinoco allele with several basal Rio Negro alleles (allele ORI). Our small Orinoco sample does not allow us to infer whether the Rio Negro acted as the source of colonisation of cardinal tetras into the Orinoco or vice-versa. Although historical connections between these two drainages via the Casiquiare River are feasible (see Winemiller *et al.*, 2008) and could account for the close relationship of Orinoco and headwater alleles, the data overall strongly support our first prediction that populations in the headwaters and upper regions of the Rio Negro represent ancient lineages.

The separation of the Orinoco and Rio Negro systems began during the Tertiary (*c.* 11 Ma) and was associated with the uplift of the Vaupes arch (Lundberg *et al.*, 1998). This was well before the formation of the Rio Negro floodplain environment during the Holocene (Latrubesse & Franzinelli, 2005). Our results are also in concert with the temporal setting and directionality of the second prediction that

Table 5 Biological inferences for nested clades with significant phylogeographical structure in the cardinal tetra *Paracheirodon axelrodi*, as determined by a χ^2 nested contingency test

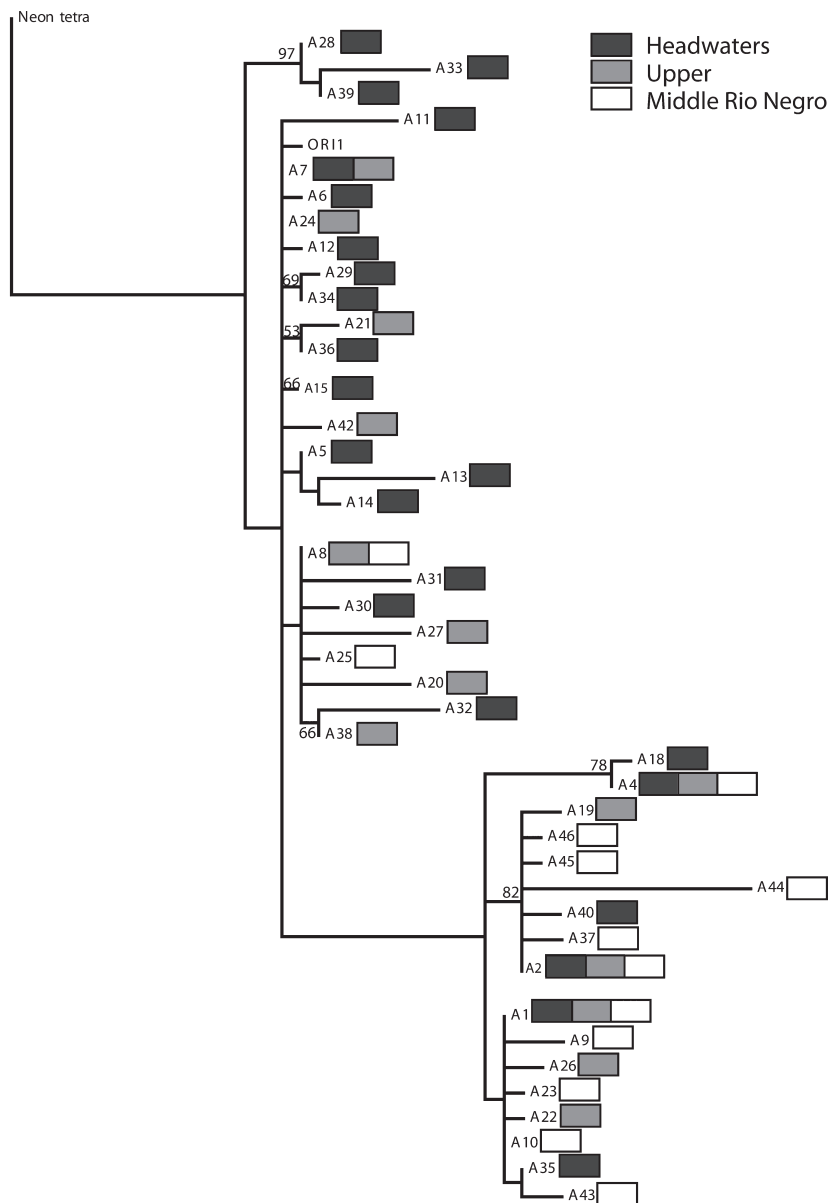


Fig. 3 Maximum likelihood phylogenetic tree of S72 alleles including bootstrap values (on branches). Numbers correspond to alleles (as in Fig. 2). The shade beside each allele defines the region in which that allele was sampled. A sample from the Orinoco basin is included (ORI).

colonisation of the middle tributaries of the Rio Negro was from headwater populations via long distance dispersal episodes. Alleles from the upper and middle Rio Negro were generally derived from headwater alleles, indicating a more recent origin. Ecological factors that would have contributed to the marked genetic structure observed in the headwaters compared to the remaining Rio Negro include poor habitat connectivity and ancient fragmentation (e.g. uplift of the Vaupes arch). High dispersal between headwater tributaries is unlikely since there is less

igapó habitat there compared to the lower reaches of the river and the floodplain. Additionally, the headwaters contain numerous waterfalls and rapids (Latrubesse & Franzinelli, 2005) that can potentially reduce connectivity between tributary populations.

In contrast, some evidence of gene flow was detected between tributary samples from middle and upper Rio Negro. Human-mediated translocation is a very unlikely explanation since a relatively small area of the floodplain is used for the ornamental fishery compared to the large region for which we

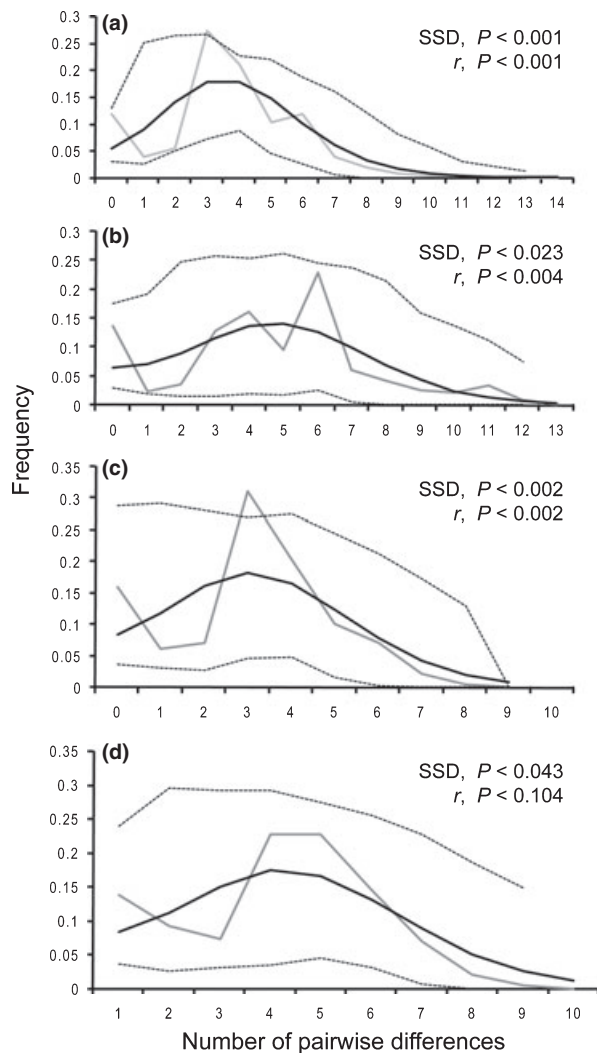


Fig. 4 Mismatch distributions and summary statistics of S72 alleles in cardinal tetras sampled from (a) all populations, (b) headwater populations, (c) all populations excluding headwaters and (d) middle floodplain populations. The grey solid line represents the observed relative frequencies of nucleotide differences between pairs of individuals, the black solid line represents the distribution fitted to the data under a model of demographic expansion and the dashed lines represent the 95% confidence interval values of 1000 simulations. SSD, sum of squared deviations; r , Harpending's Raggedness index.

obtained samples from. On the other hand, seasonal flooding in middle Rio Negro may account for this result. Cardinal tetras utilize different feeding habitats, and depending on seasonal water level changes, flooded environments may provide a potential conduit for genetic interchange between populations (Geisler & Annibal, 1986; Marshall *et al.*, 2008). However, it does not necessarily follow that the annual

hydrological cycle and floodplain environment homogenize population genetic structure. On the contrary, Hubert *et al.* (2007b) observed high genetic structure in *Serrasalmus rhombeus* (Linnaeus, 1766) (white piranha) even at the small geographic scale of a floodplain in the Madeira Basin, Amazonia. In agreement with this, a few comparisons between floodplain samples in our study showed significant population differentiation (e.g. PRE versus DEM, PRE versus CAU, ITU versus ZA, DEM versus CAU). So it is likely that molecular markers with greater sensitivity than introns to infer population genetic structure (e.g. microsatellite DNA; Sunnucks, 2000) are expected to prove useful to test for fine-scale population differentiation in flooded forest specialist in the Rio Negro (Beheregaray *et al.*, 2004).

We found no support for a simple model of contemporary isolation by distance between tributary populations (as assessed by Mantel tests). If downstream gene flow was prevalent, with each passing generation there would be a higher likelihood that a persisting lineage would spread to nearby locations downstream (Templeton, 1998). However, the only signals of isolation by distance detected were based on NCPA, and these were mostly inferred across temporal phases interspersed by restricted gene flow, long distance dispersal and fragmentation. We propose that patterns of structure derived from the intron data set are more likely to have been shaped by historical events (e.g. isolation in headwaters) than simply by the distance currently separating tributary populations.

Our final prediction was that during the Holocene cardinal tetras underwent population expansions due to the establishment of extensive floodplain habitat in the Rio Negro (Latrubesse & Franzinelli, 2005). Despite the potential complications that a single-locus analysis might show due to coalescent stochasticity (e.g. Garrick *et al.*, 2008), our analyses generally revealed results consistent with our predictions about historical demography. Evidence of range expansion and continual range expansion was detected by the NCPA for alleles predominantly sampled in the upper to middle Rio Negro. During a range expansion, alleles from ancestral populations are expected to become geographically widespread, and some alleles sampled from expanding populations may be quite distinct from those restricted to the ancestral, pre-expansion locations (Templeton, 1998). These

expectations are illustrated in our network. Headwater alleles (presumed to be ancestral) are geographically widespread, while some of the recent alleles sampled from lower reaches of the Rio Negro are genetically divergent. Evidence of population expansion was also provided by mismatch analysis (Rogers & Harpending, 1992) of alleles sampled in the most geomorphologically recent portion of the Rio Negro floodplain. In contrast, no evidence of demographic growth was detected in either the headwaters, or in upper Rio Negro populations. Thus, the generation of new *igapó* environment in the mid-floodplain region during the Holocene is likely to have prompted rapid range expansion and demographic growth in cardinal tetras. Demographic expansion coinciding with the establishment of Holocene aged floodplain environment in central and eastern Amazonia has also been recorded in two floodplain dependant bird species (Aleixo, 2006).

Thus, we can infer a two phase scenario accounting for patterns of colonisation and differentiation in cardinal tetras of the Rio Negro. Phase one involved the down stream long distance dispersal of individuals with ancient and divergent headwater alleles. These fish colonized the incipient floodplain environment in the upper and middle Rio Negro. Phase two probably occurred during the Holocene in association with the final formation of the Rio Negro floodplain (Latrubesse & Franzinelli, 2005). During this phase, an increase in flooded forest habitat is thought to have facilitated historical population expansions in cardinal tetras. The phylogeography of cardinal tetras likely reflects the intensely dynamic and multifaceted nature of the Rio Negro basin, and mirrors the influence of geomorphological and climatic events through several timescales. Hypothesis-driven comparative phylogeographic studies based on multi-locus DNA data are needed within this region to decipher the chronology of evolutionary processes that have shaped population histories and speciation of Rio Negro's incredible ichthyological diversity.

Implications for conservation management

Populations of cardinal tetras from the headwaters appear highly distinct and generally isolated from each other. Our data also suggest that these populations have been evolving independently for relatively long periods. Adopting the criterion of MU,

which is based on nuclear data (Moritz, 1994), our S7 2 data suggest six headwater populations to be considered as a conservation priority: (i) Vaupés; (ii) Paduá; (iii) Marié; (iv) Curicuriari; (v) Ferrinha and (vi) Tea. Generally, these populations appear as genetically unique, and therefore represent potential sources of genetic diversity that should be of adaptive value.

Importantly, our S7 2 intron data also suggest that in order to maintain the ornamental fishery as a sustainable activity for rural Amazonians, the fishery should be restricted to the middle Rio Negro region. Evidence for greater gene flow in these populations, compared to those upstream, implies a greater likelihood of population replenishment. Therefore, the middle Rio Negro region may represent an important resource for a sustainable fishery if managed carefully. Since the ornamental fishery rarely permeates the headwaters region, the sustainability of both the fishery and the Rio Negro environment is promising providing that more damaging activities (e.g. deforestation associated with road constructions) do not become economic opportunities within the region. Further studies based on multi-locus nuclear markers are required to infer levels of variability and connectivity between cardinal tetra populations and provide fine-scale management considerations for this important socio-economic activity.

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