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# BRIEF COMMUNICATION

## Extremely high variability in the S72 intron of the Amazonian cardinal tetra (*Paracheirodon axelrodi*)

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Here, a combination of a DNA screening technique, direct sequencing and cloning was used to carry out a large-scale assessment of intron variability in cardinal tetras – the first to be conducted for an Amazonian forest fish. To the best of the authors' knowledge, the levels of DNA variability reported in this study are the highest ever documented for an intron marker. © 2007 The Authors
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Key words: Amazonia; cardinal tetras; introns; phylogeography; S72.

Animal mtDNA is the most popular marker in phylogeographic studies (Avise, 2000). Data from this small molecule have been used extensively in fish studies to clarify patterns of geographic diversification and population history (Bernatchez & Dodson, 1991; reviewed in Avise, 2000). Nonetheless, analyses based on a single locus (such as mtDNA) suffer from numerous limitations, including the absence of informative mutational events over different time scales and the influence of locus-specific patterns related to the randomness of the coalescence process (Templeton, 2005). As a result, a growing number of phylogeographic studies have used multiple unlinked genes and integrated results of analyses based on mtDNA with those based on nuclear genes (Hare, 2001; Knowles & Maddison, 2002; Templeton, 2005). Introns, the non-coding and theoretically neutral regions of nuclear DNA that interrupt the majority of coding segments (exons), represent a potentially informative class of DNA sequence marker (Friesen, 2000). To date, the utility of introns for phylogeographic studies has presented mixed results. Some studies have found reasonable levels of intron variability, which revealed patterns of genetic structure that parallel and complement results based on other markers (Palumbi & Baker, 1994; Lavoue *et al.*, 2003; Banford *et al.*, 2004; Bernardi *et al.*, 2004; Hickerson & Cunningham, 2005; Bensch *et al.*, 2006; Ruzzante *et al.*, 2006). Other studies have detected low levels of intron variability that precluded the effective use

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of these markers for population level analyses (Smith *et al.*, 2001; Broughton & Harrison, 2003; Hare & Palumbi, 2003; Caccone *et al.*, 2004; Gay *et al.*, 2004; Spinks & Shaffer, 2005; Berrebi *et al.*, 2006).

Here, a combination of single-stranded conformation polymorphism analysis (SSCP), direct sequencing and cloning was used to carry out a large-scale assessment of intron variability in the cardinal tetra (*Paracheirodon axelrodi*) (Schlutz, 1956) – the first to be conducted for an Amazonian fish. The aims were to assess levels of variability in several intron loci, choose an appropriate marker for phylogeographic analysis and use it to screen a large sample of cardinal tetras for levels of polymorphism. Reported here is an unusual finding that probably represents the highest level of variability ever found for an intron marker.

The cardinal tetra is a small forest fish very popular in the international aquarium trade and an important economic resource for the riverine communities of the Rio Negro Floodplain (RNF), central Amazonia, Brazil (Chao, 2001). This study is part of a larger project that combines data from microsatellite DNA (Beheregaray *et al.*, 2004), mtDNA (unpubl. data) and intron markers to investigate phylogeographic history in cardinal tetras and to inform on conservation management strategies for the RNF ornamental fishery.

A total of 301 cardinal tetras were collected from 17 Rio Negro tributaries, a sampling effort that covers the entire distribution of the species in the RNF. Fish were caught in the flooded forest using hand nets and bait traps. DNA was extracted from ethanol preserved muscle tissue using a modified salting out method (Sunnucks & Hales, 1996). In order to identify an appropriate intron for large-scale screening, exon primed intron-crossing primers were used to amplify the following introns: tropomyosin (Trop), lactate dehydrogenase b (LDHb), ribosomal protein 40 (RP40) (primers in Friesen *et al.*, 1999) and the first and second introns of the S7 ribosomal protein gene (S71 and S72, respectively) (primers in Chow & Hazama, 1998). Polymerase chain reaction (PCR) amplifications for introns Trop, S71 and S72 were successfully optimized for cardinal tetras. For these introns, a 10 μl PCR containing *c.*150 ng of template DNA, 1·2 pmol of each primer, 2 units of *Taq* Polymerase (Qiagen, Valencia, CA, USA), 200 μM of dCTP, dGTP, dATP and dTTP, 2 mM MgCl2 and 1·2 μl of supplied buffer was used. PCR conditions were 94° C for 4 min, 30 cycles at 94° C for 30 s, 53° C for 30 s and 72° C for 45 s, and an extension at 72° C for 3 min. Levels of variability and divergence were assessed in these three introns by initially sequencing one individual from each of the sampled tributaries. This strategy was used because it covers the entire distribution of the species in the RNF and the range of genetic distinctiveness detected in cardinal tetras by mtDNA and microsatellite analyses (unpublished). Fresh 20 μl PCR products were prepared, purified with Ultra Clean DNA Purification Kit (MO BIO Laboratories, Carlsbad, CA, USA) and sequenced using an Automatic Sequencer 3730xl following the manufacturer's directions. Sequences were aligned and edited using SEQUENCHER 4.1 (Gene Codes Corporation). Levels of divergence between sequences were calculated in PAUP*4.0b10 (Swofford, 2003) based on a Tamura-Nei model of sequence evolution chosen in ModelTest 3.7 (Posada & Crandall, 1998).

The sequence data for Trop (217 bp), S71 (655 bp) and S72 (221 bp) (GenBank numbers EF176069–EF176131) indicated that intron S72 was the most

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appropriate marker for population level analysis. This was due to three reasons. First, S72 showed the highest percentage of polymorphic sites (10·4%), followed by S71 (9·6%) and Trop (0·5%) in the initial set of 17 sequences. The marked difference between Trop and the other introns is probably due to differing levels of constraints since this same pattern has been observed in two species of Amazonian forest fishes, *Carnegiella marthae* (Myers, 1927) (G. M. Cooke, N. L. Chao & L. B. Beheregaray, unpubl. obs.) and *Nannostomus unifasciatus* (Steindachner, 1876) (M. J. Sistrom, N. L. Chao & L. B. Beheregaray, unpubl. obs.). Second, S72 alleles showed the highest sequence divergence (up to 5·61%), consistent with levels of mtDNA and microsatellite divergence detected for the same set of samples (unpubl.). Finally, S72 is amenable to SSCP analysis, a technique that minimizes the expense and labour of phylogeographic projects that use large sample sizes. SSCP is a simple and inexpensive screening technique that offers a precise method for detecting whether or not DNA fragments amplified by PCR are identical in sequence (Sunnucks *et al.*, 2000). Given that the sensitivity of SSCP is inversely proportional to the size of the amplified fragment (Orti *et al.*, 1997; Sunnucks *et al.*, 2000), it is expected that its resolving power will be higher for S72 (221 bp) than for the other variable intron S71 (665 bp).

SSCP was then used to screen all 301 cardinal tetras for polymorphism in the S72 intron following Sunnucks *et al.* (2000). PCR and cycling conditions were as described above but with the addition of 0·07 μl of α-33P at 10mM Ci $mmol^{-1}$. The proportion of unique gel phenotypes and observed heterozygosity per population was calculated directly from SSCP gels (Table I). A total of 10 individuals per population representing different SSCP phenotypes, both homozygous and heterozygous, were sequenced. Given the high allelic variability (below and Fig. 1), it was not possible to unambiguously ascertain the phase of heterozygous polymorphisms from sequence data alone. Heterozygotes were cloned using TOPO TA Cloning vectors (Invitrogen, Carlsbad, CA, USA), transformed into chemically competent *Escherichia coli* cells and plated on Luria-Bertani agar. Multiple colonies from each cloning reaction were sequenced until the phase of polymorphisms was discerned. These data were used in ARLEQUIN 3.1 (Schneider *et al.*, 2000) to calculate nucleotide diversity (π) and to perform Fu's (1997) test for selective neutrality.

The intron S72 showed remarkably high levels of variability in cardinal tetras (Fig. 1 and Table I). From the SSCP gel autoradiographs, 65·31% of the 301 screened individuals represented a unique phenotype (population values

TABLE I. Parameters of S72 variability in cardinal tetras (*Paracheirodon axelrodi*)

| S72 variability | Range (average) |
|---|---|
| Per cent unique SSCP phenotypes* | 44·40–94·1 (65·31) |
| Observed heterozygosity* | 0·41–1·00 (0·73) |
| Allelic diversity** | 3–7 (4·6) |
| Nucleotide diversity** | 0·008–0·170 (0·023) |

*Based on all SSCP data ($n = 301$).

**Based on sequence data ($n = 156$).

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1 2 3 4 5 6 7 8 9 10 9 7 2 2 7 11 12

FIG. 1. SSCP gel autoradiograph showing the high levels of S72 variation detected in a single population of cardinal tetras (*Paracheirodon axelrodi*). Each number represents a different S72 genotype.

ranged from 44·40 to 94·10%). Mean observed heterozygosity was 0·73 (population values ranged from 0·41 to 1·00). From the S72 sequence data ($n = 156$ individuals), 93 of the 221 bp region examined were variable. These sites characterized 46 distinct alleles and had 31 parsimoniously informative positions

TABLE II. Polymorphic sites for the 46 S72 alleles found in cardinal tetras (*Paracheirodon axelrodi*). A dash (–) symbolizes a deletion compared to allele 1, whereas sequence identity is denoted by a dot (.)

Nucleotide position

| Haplotype | | | | | | | | | | | | | | | | | | | | | | | 11 | 111 | 111 | 111 | 111 | 111 | 111 | 111 | 112 | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 222 | 23 | 333 | 444 | 444 | 444 | 455 | 555 | 555 | 556 | 666 | 666 | 666 | 777 | 777 | 777 | 788 | 888 | 888 | 889 | 999 | 900 | 111 | 222 | 233 | 333 | 445 | 556 | 667 | 770 | 1 |
| | 276 | 147 | 82 | 789 | 012 | 345 | 678 | 901 | 234 | 567 | 890 | 123 | 456 | 789 | 012 | 345 | 678 | 901 | 234 | 567 | 890 | 123 | 689 | 159 | 013 | 913 | 567 | 385 | 793 | 453 | 462 | 0 |
| 1 | TAA | AAA | AT | ACA | CAG | GTG | TTC | TG- | --C | --- | ATC | A-- | -TC | A-T | TTT | CAG | TTT | GTT | TAA | ATA | TAA | AAG | GGA | CCA | ATT | TGA | GTA | TAA | AC- | AAA | AAA | T |
| 2 | ... | ... | .. | ... | ... | ... | ... | ..- | --. | --- | ... | .TT | T.. | -G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... | .C. | ... | ..- | ... | ... | . |
| 3 | ... | ... | .. | ... | ... | ... | ... | ..- | --. | --- | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... | .C. | ... | ..- | ... | ... | . |
| 4 | ... | ... | .. | ... | ... | ... | ... | ..C | AT. | ATC | ..T | TT- | --. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 5 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | .G. | ... | ... | ... | ... | ..A | ... | ... | . |
| 6 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | .C. | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 7 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 8 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | T.. | ... | ... | ... | ... | ..- | ... | ... | . |
| 9 | ... | ... | .. | T.. | ... | ... | ... | ..- | --. | ATC | ..- | --- | --. | .T. | ..- | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | .G. | ... | A |
| 10 | ... | ... | .. | ... | ... | ... | ... | ..- | --. | --- | ... | .-- | -.. | --. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 11 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ..- | --- | --. | -T. | .G- | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ..- | ... | ... | . |
| 12 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | TG. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 13 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ..- | --- | --. | .T. | ..- | ... | ... | ... | ... | ... | ... | ... | ... | .G- | .-. | ... | ..T | ... | ..- | ... | ... | . |
| 14 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ..- | --- | --. | -T. | ..- | ... | ... | ... | ... | ... | ... | ... | ... | .G. | ... | ... | A.. | ... | ..- | ... | ... | . |
| 15 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | .A. | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 16 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | -G. | ..G | ... | ... | ... | ... | ... | ... | ... | .A. | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 17 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | -G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | T.. | ... | ... | ... | ... | ..- | ... | ... | . |
| 18 | ... | ... | .. | ... | ... | ... | ... | ..C | AT. | ATC | ..T | TT- | --. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ..T | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 19 | ... | ... | .. | ... | ... | ... | ... | C.- | --. | --- | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... | .C. | ... | T.- | ... | ... | . |
| 20 | ... | .G. | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | T.. | ... | ... | ... | ... | ..- | ... | ... | . |
| 21 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | G.. | ... | T.. | ... | ... | ... | ... | ..- | ... | .G. | . |
| 22 | ... | ... | .. | ... | ... | ... | ... | ..- | --. | --- | ... | .-- | -.. | .-. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | .T- | ... | ... | . |
| 23 | ... | ... | .. | ... | ... | ... | ..G | ..- | --. | --- | ... | .-- | -.. | .-. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 24 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | -G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 25 | C.. | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | T.. | ... | ... | ... | ... | ..- | ... | ... | . |
| 26 | ... | ... | .. | ..G | ... | ... | ... | ..- | --. | --- | ... | .-- | -.. | .-. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... | ... | ..- | ... | ... | . |
| 27 | ..G | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | T.. | ... | ... | ... | ... | ..- | ... | ... | . |
| 28 | ... | G.. | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.A | .G. | ..G | ... | ... | ... | ... | ... | G.. | ... | ... | .T. | ... | .A. | ... | ... | ..- | ... | ... | . |
| 29 | ... | ... | .. | ... | ... | ... | ..T | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | C.. | ..- | ... | ... | . |
| 30 | ... | ..T | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | T.. | ... | ... | ... | .G. | ..- | ... | ... | . |
| 31 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | T.. | ... | ... | ... | ..G | ..- | ... | ... | . |
| 32 | .G. | ... | .. | ... | ... | .C. | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | T.. | ... | C.. | ... | ... | ..- | ... | ... | . |
| 33 | ... | G.. | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.A | .G. | ..G | ... | ... | ... | ..G | ... | G.. | ... | A.. | .T. | ... | .A. | ... | ... | ..- | ... | ... | . |
| 34 | ... | ... | .. | ... | ... | ... | ..T | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 35 | ... | ... | .C | ... | ... | ... | ... | ..- | --. | --- | ... | .-- | -.. | .-. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 36 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | G.. | ... | ... | ... | ... | ... | ... | ..- | ... | ... | . |
| 37 | ... | ... | G. | ... | ... | ... | ... | ..- | --. | --- | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... | .C. | ... | ..- | ..G | ... | . |
| 38 | ... | ... | .. | ... | ... | .C. | ... | .TC | TG. | ATC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | T.. | ... | ... | ... | ... | ..- | ... | ... | . |
| 39 | ... | G.. | .. | ... | ... | ... | ... | .TC | TG. | ATC | ... | .TT | T.A | .G. | ..G | ... | ... | ... | ... | ... | G.. | ... | A.. | .T. | ... | .A. | ... | ... | ..- | ... | ... | . |
| 40 | ... | ... | .. | ... | ... | ... | .C. | ..- | --. | --- | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... | .C. | ... | ..- | T.. | ... | . |
| 41 | ... | .C. | .. | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --A | ... | ... | ... | ... | ... | ... | ..- | ... | G.. | . |
| 42 | ... | ... | .. | ... | ... | ... | ... | .TC | TG. | TTC | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | .-- | -C. | ... | ... | ... | ..- | ... | ... | . |
| 43 | ... | ... | .A | ... | ... | ... | ... | ..- | --. | --- | ... | .-- | -.. | .-. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..- | ... | ..T | . |
| 44 | ... | ... | .. | ... | ... | ... | ... | ..- | --. | --- | ... | .TT | T.. | .G. | ..G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... | .CG | ..G | ..- | ... | ... | . |
| 45 | ... | ... | .. | ... | ... | ... | ... | ..- | --. | --- | ... | .TT | T.. | .G. | ..G | ... | ..C | ... | ... | ... | ... | ... | ... | ... | ..G | ... | .C. | ... | ..- | ... | ... | . |
| 46 | ... | ... | .. | ... | ... | ... | ... | ..- | --. | --- | ... | .TT | T.. | .G. | C.G | ... | ... | ... | ... | ... | ... | ... | ... | ... | ..G | ... | .C. | ... | ..- | ... | ... | . |

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TABLE III. Intraspecific variability detected in a range of intron studies (measured as percentage of polymorphic sites)

| Group | Species and common name | Intron | Polymorphic sites (%) | Source |
|---|---|---|---|---|
| Fish | *Paracheirodon axelrodi*, cardinal tetra | S72 | 42·08 | This study |
| | *Crystallaria asprella*, crystal darter | S71 | 7·75 | Morrison *et al.* (2006) |
| | *Percichthys trucha*, creole perch | GnH3-2 | 4·95 | Ruzzante *et al.* (2006) |
| | *Percilia gillissi*, perch | GnH3-2 | 3·77 | Ruzzante *et al.* (2006) |
| | *Prochilodus mariae* and *Prochilodus rubrotaeniatus*, coporo | EF1α-6 | 9·3 | Moyer *et al.* (2005) |
| | *Gillichthys seta*, shortjaw mudsucker | Creatine kinase M7 | 0·00 | Huang & Bernardi (2001) |
| Birds | *Brachyramphus marmoratus*, marbled murrelet | Aldolase | 0·02 | Friesen *et al.* (1997) |
| | *Brachyramphus marmoratus*, marbled murrelet | Gapdh | 0·05 | Friesen *et al.* (1997) |
| Mammals | *Megaptera novaeangliae*, humpback whale | Actin | 1·21 | Palumbi & Baker (1994) |
| | *Lagenorhynchus acutus*, Atlantic white-sided dolphin | Actin | 0·018 | Hare *et al.* (2002) |
| Invertebrates | *Ceratitis capitata*, medfly | Vitellogenin | 14·21 | Villablanca *et al.* (1998) |
| | *Acropora cervicornis*, stony coral | Pax-C | 1·12 | Van Oppen *et al.* (2000) |
| | *Gryllus firmus*, field cricket | Cyt-c | 0·05 | Broughton & Harrison (2003) |
| | *Spisula solidissima*, Atlantic surfclam | Calmodulin – A | 10·2 | Hare & Weinberg (2005) |

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(Table II). If indels are excluded from the data, the number of variable sites, distinct alleles and parsimoniously informative sites are reduced to 56, 42 and 21, respectively. The mean nucleotide diversity across the samples was 0·023 (population values ranged from 0·008 to 0·17) (Table I).

The levels of S72 variability detected for cardinal tetras are likely to be the highest ever reported for an intron DNA marker, with 42·08% of the sites of the fragment being polymorphic (25·34% if the indels are removed). This contrasts to results from other intraspecific intron surveys, such as those shown in Table III, of which the highest number of variable sites for a single fish species was 7·7%. Although the large size of the cardinal tetra sample screened in this study partially accounts for the genetic variation observed, the amount of variability detected is nonetheless noteworthy and deserves consideration. Fu's (Fu, 1997) test of selective neutrality was used to assess if the high variability could be due to positive selection. Although neutrality tests are considered to have limited power (Neilsen, 2001; Vasemagi & Primmer, 2005), the results suggested that the S72 did not deviate from a neutral model of evolution ($P = 0{\cdot}391$). The amplification of a pseudogene of S72 is another unlikely reason to account for the high variation observed. A single and strong PCR band that showed high sequence homology was obtained for all population samples. In addition, there were no size differences in any replicate PCR and sequence reactions ($n = 15$ replicates). Furthermore, the S72 primers used have also been shown to amplify only single copies of this gene in several other teleost groups (Chow *et al.*, 2001; Lavoue *et al.*, 2003).

It appears that the best explanation for the high genetic variability is that cardinal tetras from the RNF are comprised of populations with very large effective sizes. The Rio Negro drains an area of around 0·75 million $km^2$, which includes a vast and pristine floodplain home to one of the world's largest ornamental fisheries (Chao *et al.*, 2001). Cardinal tetras represent around 80% of the total catch of the RNF ornamental fishery, with over 30 million fish caught and exported every year (Chao, 2001). The high variability in S72 is also consistent with a preliminary assessment of microsatellite variation in cardinal tetras, in which up to 22 alleles per locus have been detected in a sample of only 30 individuals (Beheregaray *et al.*, 2004). The potential of drawing phylogeographic inferences from intron markers has depended both on technical feasibility and on the resolution of the data (Hare, 2001). This study reported that the S72 intron is not only highly variable, but it is also technically robust. This marker proved amenable for large-scale screening by the SSCP technique and cloning of heterozygotes, resulting in an extensive and potentially informative data set for phylogeographic and population genetic studies.

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