Genetic monitoring of the Amazonian fish matrinchã (Brycon cephalus) using RAPD markers: insights into supportive breeding and conservation programmes

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Summary
The importance of genetic evaluations in aquaculture programmes has been increased significantly not only to improve effectiveness of hatchery production but also to maintain genetic diversity. In the present study, wild and captive populations of a commercially important neotropical freshwater fish, Brycon cephalus (Amazonian matrinchã), were analyzed in order to evaluate the levels of genetic diversity in a breeding programme at a Brazilian research institute of tropical fish. Random Amplified Polymorphic DNA fingerprinting was used to access the genetic variability of a wild stock from the Amazon River and of three captive stocks that correspond to consecutive generations from the fishery culture. Although farmed stocks showed considerably lower genetic variation than the wild population, a significantly higher level of polymorphism was detected in the third hatchery generation. The results seem to reflect a common breeding practice on several hatchery fish programmes that use a small number of parents as broodstocks, obtaining reproductive success with few non-identified mating couples. The obtained data were useful for discussing suitable strategies for the genetic management and biodiversity conservation of this species.

Introduction
Freshwater fish farming has been one of the most expanding food producing sectors in Brazil in the last years, especially due to its high hydrographic potential, great number of native species and several favorable areas to this activity. Although one of the migratory fish groups with greater economic importance in the country is represented by the genus Brycon (Müller and Troschel 1844; Pereira Filho, 1994), several species of this genus have been considered endangered due to the growing impact of environmental disturbances (Cecarelli and Senhorini, 1996). Among these species, the Amazonian matrinchã, Brycon cephalus (Günther 1869), stands for an extremely important fishery resource in the Amazonian region (Bittencourt and Cox-Fernandes, 1990) and is also considered as one of the main cultivated fish species in Brazil (Pereira Filho, 1994).

Aquaculture practices may inadvertently decrease the genetic variability present in farmed stocks by the selection and breeding of related individuals or by the use of a small number of parents as broodstock and, unless genetic records are maintained, there is a higher probability of increasing inbreeding. The current aquaculture industry management of the matrinchã is largely based on analyses of stocking density, feeding, nutrition, physiology, behavior, and induced reproduction (Mendonça, 1994). However, information about its genetic structure is still scarce, in spite of its importance in designing appropriate strategies for the management and biodiversity conservation of this neotropical fish species. The synthesis of the data derived from genetic studies, together with those of other disciplines, can be applied directly to species management plans (O’Brien, 1994).

In recent years, several nuclear DNA techniques have been available in accessing genetic variability in fish species (Park and Moran, 1994; O’Reilly and Wright, 1995; Carvalho and Hauser, 1998) and have been employed in aquaculture studies (Harris et al., 1991; Mjolnerod et al., 1997; Coughlan et al., 1998; Norris et al., 1999). One of these approaches is the random amplification of polymorphic DNA [Random Amplified Polymorphic DNA (RAPD) or AP-PCR], which is based on amplification of several random segments of a genome using single short arbitrary primers that can detect polymorphism in the absence of specific DNA nucleotide sequence information (Welsh and McClelland, 1990; Williams et al., 1990). Although one of the drawbacks of using short oligonucleotides as random primers is the low stringency necessary for DNA amplification, which can lead to non-reproducible results, the possibility of working with anonymous polymorphic DNA and the low expense makes it a simple and rapid methodology in the assessment of genetic differences among several groups, including fish species (Bardacki and Skibinski, 1994; BIELAWSKI and PUMO, 1997; CAGIGAS et al., 1999; KOH et al., 1999; PARKER et al., 2002).

The present study reports the result of a comparative analysis of one wild and three farmed populations of B. cephalus, in order to genetically evaluate an 11-year breeding programme of the species in a Brazilian research institute of tropical fish. The genetic variability within and among the different stocks was evaluated through the use of RAPD. The results were useful not only to characterize each fish stock, but also to highlight future tasks for a better hatchery management and for the biodiversity conservation of the species. Moreover, information about improper management strategies usually applied in hatchery fish programmes suggest that genetic monitoring is extremely necessary for fish culture. The expected outcome is to use molecular methodologies in order to maximize or at least maintain the level of genetic diversity on cultivated stocks of several fish species.
Materials and methods
Sample collection
A wild stock of *B. cephalus* from the Amazon River (municipality of Manaus, Amazonas State, Brazil), which had not been subjected to any supplemental stocking of fish, and three captive stocks of the species that correspond to consecutive generations from a national center of tropical fish research (CEPTA/IBAMA, Centro Nacional de Pesquisa de Peixes Tropicais/Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) (municipality of Pirassununga, São Paulo State, Brazil), were analyzed. The base population used to establish the breeding programme of *B. cephalus* at CEPTA/IBAMA was obtained from the Amazon River. The farming stocks, maintained in isolation in the fishery center research since 1985, had basically the same breeding, management, density stocking, and feeding regimes – the average stocking density was 250 g m⁻², and the annual average temperature and dissolved oxygen was 26°C and 5.5 p.p.m., respectively. A total of 99 individuals were analyzed – 30 samples correspond to the wild stock (W), 11 samples correspond to the first generation (F₁) that was obtained in 1991, 28 samples correspond to the second farmed stock (F₂) obtained in 1995, and 30 samples correspond to the third farmed generation (F₃) obtained in 2000. The F₁ generation hatchery stock was bred from five males and a single female (Mendonça, 1994), and the F₂ cultivated stock bred from a mixture of seven males and just one female of the F₂ hatchery generation (J. A. Senhorini, unpublished data). Unfortunately, there are no precise data about the number of animals of both sexes that were used to compose the F₂ farm stock.

DNA extraction and amplification
DNA was extracted from fish caudal or anal fins using a TNES-urea-digestion buffer [10 mM Tris–HCl pH 8.0; 125 mM NaCl; 10 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0; 0.5% sodium dodecyl sulfate; 4 mM urea], as described in Wasko et al. (unpublished data). Further DNA purification was performed with phenol-chloroform (Sambrook and Russell, 2001). A set of six 10-mer RAPD oligonucleotides (RAPD Analysis Beads; Amersham Pharmacia Biotech, Stockholm, Sweden) (Table 1) were used as single primers to amplify the DNA samples, following the manufacturer’s instructions. Each RAPD-PCR reaction mixture contained 50 ng of target DNA and 25 pmol of single primer in a total volume of 25 μl. Amplifications were carried out in a PTC-100™ (MJ Research, Boston, MA, USA) thermocycler, with the following cycle programme: 45 cycles at 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min. A negative control, without template DNA, was also included to monitor any possible contamination of the reactions with non-target template DNA. Furthermore, amplification of multiple template DNA concentrations of every sample was carried out for all primers surveyed as a means of identifying artifactual variation. DNA amplification fragments were electrophoretically separated on 2% agarose gel in TAE buffer (Tris–Acetic acid–EDTA). The gels were run for 4.5–6.5 h at 110V/150A. DNA bands were visualized using ethidium bromide staining (Sambrook and Russell, 2001) and UV illumination. Molecular weights were estimated using a standard DNA marker. The gel documentation system EDAS (Electrophoresis Documentation and Analysis System 120, Kodak Digital Science 1D, New Haven, CN, USA) was used in order to obtain digital images of the agarose gel and to identify each amplified fragment.

### Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>(G + C)%</th>
<th>Number of loci</th>
<th>Size range of fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GGTGCGGGGAA</td>
<td>70</td>
<td>11</td>
<td>200–1500</td>
</tr>
<tr>
<td>2</td>
<td>GTTTCGCACCTCC</td>
<td>60</td>
<td>17</td>
<td>250–2000</td>
</tr>
<tr>
<td>3</td>
<td>GTAGACCGCGT</td>
<td>60</td>
<td>22</td>
<td>200–1500</td>
</tr>
<tr>
<td>4</td>
<td>AAGAGCGCGG</td>
<td>60</td>
<td>20</td>
<td>150–1500</td>
</tr>
<tr>
<td>5</td>
<td>AAGCAGCACAG</td>
<td>60</td>
<td>20</td>
<td>150–1500</td>
</tr>
<tr>
<td>6</td>
<td>GCCGTACAGCA</td>
<td>70</td>
<td>14</td>
<td>150–1500</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>104</td>
<td>200–1500</td>
</tr>
</tbody>
</table>

Statistical RAPD analysis
Reproducible well-marked amplified fragments were scored and pairwise comparison of banding patterns was evaluated among samples amplified by each primer separately and for the combined data of primers, by calculating an index of genetic similarity using the coefficient method of Jaccard (1901). Mean bandsharing similarity indices between individuals within each stock and among stock samples were computed. For each genotype, the presence and absence of fragments were scored as 1 or 0, respectively, and each DNA fragment or RAPD marker was interpreted to represent a single locus. The percentage of polymorphic loci (P) was also calculated. Statistical analyses were performed using the NTSYS-PC version 1.70 (Numerical Taxonomy and Multivariate Analysis System) computer programme (Rohlf, 1993).

Results
The six decamer oligonucleotides (Table 1), used as single primers to amplify DNA samples of *B. cephalus*, produced fragments ranging from approximately 150 to 2000 base pairs (bp) and each of them generated an unique pattern of amplification products. The total number of reliable amplified bands, generated by each primer separately and that were selected for further analyses, ranged from 11 (primer 1) to 22 (primer 3) with an average of 9.7 bands per individual and primer (Table 1). From a total of 104 fragments that were amplified by the six RAPD primers, 16 loci (15.4%) could be visualized in all the DNA samples of *B. cephalus*, while 88 loci (84.6%) were polymorphic (Table 1). Although some of the observed variations could be due to the RAPD technique itself and, therefore, would not be real polymorphisms, this seems not to be probable as control procedures were utilized in the PCR amplifications.

Primers were also evaluated based on intensity of bands, consistency within individuals, and presence of smearing. Repeated amplifications with the primers 1, 2, 4, 5, and 6 were reproducible, and generated good quality banding patterns, with intense stained fragments well separated from other bands, and of sufficient variability. Therefore, the bands produced by these five primers were selected for the genetic statistical analysis. From a total of 82 selected bands, 12 (14.6%) were conserved among all analyzed individuals, while 70 (85.4%) amplification products were polymorphic.
Distinct levels of genetic variability were detected among the four analyzed stocks of *B. cephalus* (Tables 2 and 3). The three cultivated stocks of the species evidenced a lower genetic diversity than the wild stock, as a lower number of bands/alleles and a higher genetic similarity index were detected in the cultivated samples (Tables 2 and 3). While the percentage of polymorphic loci (*P*) observed for the individuals of the wild stock was 85.36%, the cultivated stocks *F*₁, *F*₂, and *F*₃ evidenced a percentage of variable bands of 81.81, 72, and 79.74%, respectively (Table 2). Moreover, despite the fact that any of the tested primers evidenced a diagnostic band (present in all and exclusively the members of a determined stock), the frequency of several amplified fragments differed substantially among the analyzed stocks. As an example, a conspicuous band of approximately 650 bp, identified in *B. cephalus* using the primer 4, was detected in 53.33% of the animals from the wild stock, and in 81.81, 93.33, and 53.57% of the individuals from the cultivated generations *F*₁, *F*₂, and *F*₃, respectively (Fig. 1).

The values of mean similarity indices (*J*) within and among the four stocks, obtained using data of the primers 1, 2, 4, 5, and 6 separately and of a combination of these primers, are given in Table 3. A mean genetic similarity index of 0.58 was observed for the wild stock of *B. cephalus*. The cultivated stocks *F*₁, *F*₂, and *F*₃ of the species showed mean genetic values of 0.62, 0.71, and 0.63, respectively. Although each individual primer had differed in the overall level of genetic variability detected, it was possible to verify that the animals of the cultivated stocks presented a higher genetic similarity index and, therefore, lower variability, when compared with the wild stock samples from the Amazon River.

![Fig. 1. RAPD patterns of individuals of *Brycon cephalus* (1–12) using primer 4. Arrow denotes a band of approximately 650 bp](image)

**Table 2**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Wild (W)</th>
<th>Cultivated (F₁)</th>
<th>Cultivated (F₂)</th>
<th>Cultivated (F₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90.90</td>
<td>81.81</td>
<td>72.72</td>
<td>90.90</td>
</tr>
<tr>
<td>2</td>
<td>88.23</td>
<td>82.35</td>
<td>82.35</td>
<td>82.35</td>
</tr>
<tr>
<td>4</td>
<td>90.00</td>
<td>88.23</td>
<td>82.35</td>
<td>88.88</td>
</tr>
<tr>
<td>5</td>
<td>90.00</td>
<td>88.88</td>
<td>68.75</td>
<td>78.94</td>
</tr>
<tr>
<td>6</td>
<td>64.28</td>
<td>64.28</td>
<td>50.00</td>
<td>57.14</td>
</tr>
<tr>
<td>c.d.</td>
<td>85.36</td>
<td>81.81</td>
<td>72.00</td>
<td>79.74</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Wild (W)</th>
<th>Cultivated (F₁)</th>
<th>Cultivated (F₂)</th>
<th>Cultivated (F₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.58</td>
<td>0.69</td>
<td>0.77</td>
<td>0.60</td>
</tr>
<tr>
<td>2</td>
<td>0.54</td>
<td>0.58</td>
<td>0.68</td>
<td>0.62</td>
</tr>
<tr>
<td>4</td>
<td>0.53</td>
<td>0.58</td>
<td>0.63</td>
<td>0.55</td>
</tr>
<tr>
<td>5</td>
<td>0.51</td>
<td>0.56</td>
<td>0.76</td>
<td>0.64</td>
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<tr>
<td>6</td>
<td>0.72</td>
<td>0.74</td>
<td>0.78</td>
<td>0.75</td>
</tr>
<tr>
<td>c.d.</td>
<td>0.58</td>
<td>0.62</td>
<td>0.71</td>
<td>0.63</td>
</tr>
</tbody>
</table>

**Discussion**

The actual hatchery programme of *B. cephalus* at CEPTA/IBAMA can be considered well established pertaining to stocking density, feeding, nutrition, physiology, behavior, and induced reproduction (Mendonça, 1994; Senhorini et al., 1998). However, there is still a lack of genetic data of the potential breeders, and only a low number of couples have been used in the induced reproductions that occur annually. Molecular markers are currently used in order to identify the levels of genetic variation within and between stocks of *B. cephalus* and also for individual genetic characterization. Therefore, the present study intended to contribute to a practical and theoretical framework to the fishery management and genetic conservation of the species.

Even with the limited survey of primers and animal samples used in the analyses, it was possible to identify a considerable amount of genetic variability within the wild and the three cultivated stocks of *B. cephalus* and to detect significantly distinct band sharing-based similarity values and different polymorphic loci percent among the stocks. Although each stock could be characterized by a different level of genetic diversity, an overall variability decrease was observed in the successive cultivated stocks (generations *F*₁, *F*₂, and *F*₃) of the species, which seems to reflect a common practice in hatchery fish programmes that generally use a low number of genetically non-identified breeders in induced spawning, which can lead to the occurrence of possible consanguineous mating. Moreover, polymorphism seemed to be lost in the hatchery generations. A reduction in the number of polymorphic loci can also be correlated to the limited number of wild specimens and/or populations used to settle hatchery stocks (Winkler et al., 1999), which occurred in the fishery establishment of *B. cephalus* at CEPTA/IBAMA as only one wild population from the Amazon River represented the first breeding stock of the species. A genetic variability decrease was also observed in hatchery stocks of another species of the genus *Brycon* – *B. cf. reinhardtii* (Galhardo and Toledo Filho, 1987).

Numerous studies using genetic markers correlated the domestication process in fish species with a decrease in genetic variability (Vespoor, 1988; Fergusson et al., 1991; Youngson et al., 1991; Taniguchi et al., 1993; Mjolnerod et al., 1997;...
minimum increase in inbreeding rate in each cultivated generation. Moreover, it would be important for continuing the genetic characterization and monitoring of their natural and captive stocks in the coming years. To pursue this issue we are currently continuing the genetic diversity analyses using RAPD and microsatellite markers. Appropriate results will be of great value not only for fish farmers concerned with commercial production of the species but also to meet the needs of the conservation programme, i.e. the development of an integrated strategy that conserves as much genetic diversity within a species as possible and ensures the presence of utilizable fish resources.

Acknowledgements

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Conclusion

The careful selection of natural stocks and potential breeders, based on genetic criteria, can determine the success and sustainability of hatchery and conservation programmes (Quattro and Vrijenhoek, 1989; Crozier, 1994). The suggested approaches for B. cephalus fishery management could ensure a...


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