Comparative study by RAPD analysis of six species of the Pimelodidae family (Osteichthyes, Siluriformes) from the Tibagi River, state of Paraná, Brazil

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ABSTRACT. The genetic similarity of six species from the Pimelodidae family (Osteichthyes, Siluriformes), collected at four sites in the Tibagi River basin, was analyzed using RAPD. RAPD comparative analysis was carried out to observe the degree of genetic similarity among the six species of Pimelodidae. The dendrogram obtained by analysis of the comparative RAPD profiles clearly showed that the individuals investigated from each species clustered together, that is, there was a defined separation among the six species. Two clusters also separated distinctly, one formed by the *P. maculatus*, *P.* cf. *absconditus*, *I. labrosus* and *P. pirinampu* (Pimelodinae subfamily) species and the other by the *P.* aff. *avanhandavae* and *Pimelodella* aff. *meeki* (Heptapterinae subfamily) species. The genetic similarity among these groupings was 0.084.

Key words: Pimelodidae, molecular markers, RAPD.

RESUMO. Estudo comparativo através de análises de RAPD de seis espécies da família Pimelodidae (Osteichthyes, Siluriformes) do rio Tibagi, Estado do Paraná, Brasil. A similaridade genética de seis espécies, pertencentes à família Pimelodidae (Osteichthyes, Siluriformes), coletadas em quatro localidades na bacia do rio Tibagi, foi analisada utilizando-se a técnica de RAPD. A análise comparativa de RAPD foi realizada para observar o grau de similaridade genética entre seis espécies de Pimelodidae. O dendrograma obtido através da análise comparativa dos padrões de RAPD mostrou claramente que os indivíduos estudados de cada uma das espécies se agrupam entre si, ou seja, há uma separação definida entre as seis espécies. Dois agrupamentos foram obtidos, um formado pelas espécies *P. maculatus*, *P.* cf. *absconditus*, *I. labrosus* e *P. pirinampu* (Subfamília Pimelodinae), e outro formado pelas espécies *P.* aff. *avanhandavae* e *Pimelodella* aff. *meeki* (subfamília Heptapterinae). A similaridade genética entre esses agrupamentos foi de 0.084.

Palavras-chave: Pimelodidae, marcadores moleculares, RAPD.

Introduction

The present study is part of the integrated project "Aspects of fauna and flora in the Tibagi River basin" undertaken by Londrina State University, which aims to widen knowledge about the animal and plant communities, their inter-relationships and the impact of environmental factors, to provide a base for environmental education to restore the riverbank vegetation and recompose the food chain and thus to restore the basin.

The Tibagi River basin, located in the state of Paraná, Brazil, is a rich hydrographical net with 65 main branches and hundreds of small tributaries. The area of the river basin is *c.* 26000 km^2 and covers 13% of the surface of the state (cf. Bennemann *et al.,* 1995).

Few studies in Brazil have estimated the genetic variability in freshwater fish, which can provide information on population structure and phylogenetic relationships (Almeida *et al*., 2001; Chiari and Sodré, 2001).

Molecular markers can be used in different types of studies of natural and cultivated fish populations, such as species and hybrid identification, phylogeny establishment, determination of the population structure, genetic variability quantification, estimates of the effective population size, identification of target populations for conservation, genetic determination of the impact of introducing cultivated fish into natural populations, determination of crossing strategies and construction of genetic maps (Ferguson *et al*., 1995).

In common with many areas of contemporary biology, development in molecular genetics has had a significant impact on the fields of taxonomy and systematics, not only in terms of aiding species

recognition, but also through elucidating phylogenetic relationships and mechanisms of speciation (Hillis *et al*., 1996).

The RAPD technique can be used to identify relationships between closely related species, but becomes less effective as evolutionary distance increases, when other techniques are more reliable (Chambers *et al.*, 1998).

The elucidation of phylogenetic relationships among catfish groups is still a major problem in systematic ichthyology. The Pimelodidae family is a heterogeneous assemblage comprising over 300 species in 50-60 genera, and has been historically diagnosed by the lack of specializations seen in other Neotropical catfish families (Pinna, 1998).

The objective of this study was to analyze comparatively six species from the Pimelodidae family, present in greater frequency in the River Tibagi basin to obtain genetic similarity data that could contribute to a better distinction among the genera and species.

Material and methods

Material and Collection Location

Samples of *Iheringichthys labrosus*, *Pimelodus* cf. *absconditus*, *Pimelodus maculatus*, *Pinirampus pirinampu*, *Pimelodella* aff. *avanhandavae* and *Pimelodella* aff. *meeki*, were collected at 4 points in the Tibagi River basin (Sertaneja, Londrina, Sapopema and Tibagi). The Tibagi river is dammed by Getúlio Vargas hydro-electric plant downstream from the Tibagi location (Figure 1).

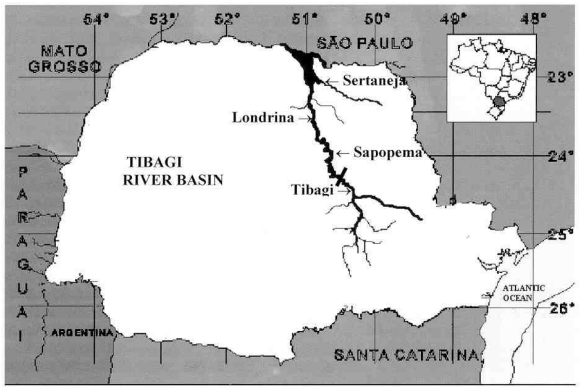

Figure 1. Map of the Tibagi River-basin with designated collection regions

Muscle or blood samples were removed from specimens immediately after capturing and maintained at -20^{0}C until use. The specimens used in this study are preserved and registered in the Zoology Museum at Londrina State University.

DNA extraction and RAPD analysis

DNA was extracted from the muscle or from blood, following the procedure described by Almeida *et al*., (2001).

The 24 different 10-mer oligonucleotides used as random primers in the RAPD screening were purchased from Operon Technologies Ltd, and seven were selected on the basis of the number of bands obtained and on their ability to produce consistent fragment patterns (Table 1). The amplification conditions were based on Almeida *et al.* (2001). Reactions were performed using an MJ (model PTC 100) thermal cycler during 40 cycles of 40s at 92^{0}C, 90s at 40^{0}C and 120s at 72^{0}C, after an initial denaturation of 5 min at 92^{0}C. After 40 cycles, one cycle of 5 min at 72^{0}C was performed.

Table 1. Codes and sequences of the Operon Technologies random primers used in the present study

Primer codes	Sequence (5' to 3')
OPW 1	CTCAGTGTCC
OPW 7	CTGGACGTCA
OPW 8	GACTGCCTCT
OPW 9	GTGACCGAGT
OPW 13	CACAGCGACA
OPAM 7	AACCGCGGCA
OPAM 9	TGCCGGTTCA

The RAPD products were resolved by electrophoresis in 1,4% agarose gels run with TBE buffer (0.89 M Tris, 0.89 M boric acid and 2 mM EDTA pH 8.3) diluted 1:10. Electrophoresis was conducted at 3V cm^{-1}. Gels were stained with ethidium bromide and photographed under ultraviolet light using black and white Kodak film.

Data analysis

Comparative analysis among species (interspecific), was performed by using one selected primer in each gel with 5 randomly selected individuals of each species. Each individual was scored for the presence (1) or absence (0) of amplification products. These data were entered into a binary matrix and a pairwise similarity matrix was constructed using the Jaccard similarity (J) index (Sneath and Sokal, 1973). J values were calculated for the number of shared bands between 2 individuals divided by the sum of all the bands. A UPGMA cluster based on J values was generated using the NTSYS (numerical taxonomy system, Applied Biostatistics, Setauket, NY) computer application software (Rohlf, 1988).

Technical problems from the application of the RAPD technique, in the field of genetic population research have been reported by many authors (Hadrys *et al*., 1992; Naish *et al*., 1995; Liu *et al*.,

1999). The same DNA, magnesium, dNTP and primer concentrations were used in the reactions to avoid reproducibility problems. All the samples were amplified in the same thermal cycler using the same program.

Results and discussion

RAPD comparative analysis (Figure 2) was carried out to observe the degree of genetic similarity among the six Pimelodidae species. The dendrogram obtained by analysis of the 132 loci obtained with 7 primers, in the comparative RAPD profiles (Figure 3) clearly showed that the individuals investigated from each species clustered together, that is, there is a defined separation among the six species. There was also separation of two distinct clusters, one formed by the *P. maculatus*, *P.* cf. *absconditus*, *I. labrosus* and *P. pirinampu* species and the other by the *P.* aff. *avanhandavae* and *Pimelodella* aff. *meeki* species. The genetic similarity of these two clusters was 0.084.

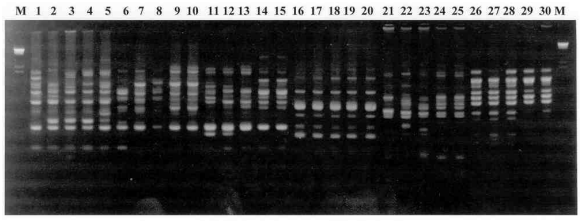

Figure 2. Comparative analysis of RAPD markers among individuals of six Pimelodidae species, amplified by primer OPW 13. Columns: M = molecular weight markers DNA λ *Hind*III; 1-5 = *Pimelodus maculatus*; 6-10 = *Pimelodus* cf. *absconditus*; 11-15 = *Iheringichthys labrosus*; 16-20 = *Pimelodella* aff. *avanhandavae*; 21-25 = *Pimelodella* aff. *meeki* and 26-30 = *Pinirampus pirinampu*

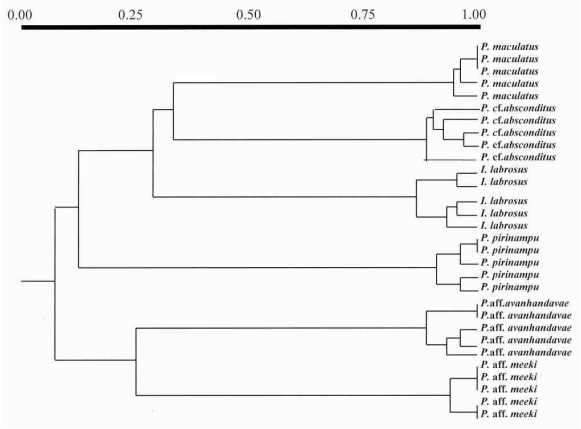

Figure 3. Dendrogram obtained by the Jaccard similarity index and method UPGMA for six species of Pimelodidae

Several authors have demonstrated that the RAPD-PCR method is a powerful tool in the assessment of genetic markers which are capable of discriminating between species or subspecies in a wide range of organisms, including fishes (Welsh and McClelland, 1990; Williams *et al*., 1990; Bardakci and Skibinski, 1994; Naish *et al*., 1995).

The RAPD technique might not be ideal for genetic studies, but the approach seems useful for identification and phylogenetic studies. The RAPD method is the best suited for identification of species, and for differentiating among conspecific populations, particularly in cases where the morphological characters do not permit an unambiguous or a rapid identification of species (Dahle *et al*., 1997). Almeida *et al*. (2001), sucessfully used the RAPD technique to distinguish between the two Pimelodidae species (*Pimelodus* cf. *absconditus* and *Iheringichthys labrosus*) that presented very similar external morphology.

According to Pinna (1993), three major well-defined monophyletic groups, belonging to the Pimelodidae family, currently ranked as subfamilies, are now recognized: Pimelodinae, Heptapterinae, and Pseudopimelodinae. These are the largest monophyletic groups of pimelodids so far demonstrated. Based on previous results (Pinna, 1993) each of the three clade is more closely related to other catfifh than to each other and therefore each must be raised to family in a phylogenetic classification.

Although the monophyly and subfamilial rank of the Pimelodinae, Pseudopimelodinae, and Heptapterinae are well-established, and generally accepted, the nomenclatural aspects of classification still need to be worked out in more detail (Pinna, 1998).

The Pimelodinae is a vast assemblage of medium-to-large-sized catfishes, composed mostly of predatory species and including forms as distinct as the pelagic filter-feeding. New species of the Pimelodinae are regularly discovered, which demonstrates how little is yet known about Neotropical freshwater fish diversity. The Heptapterinae (until recently known as Rhamdiinae) includes small-to-medium-sized fishes forming one of the largest radiations of Neotropical catfishes. Some Heptapterinae genera (eg. *Pimelodella* and *Rhamdia*) seem to be among the most common siluriforms in South American freshwater (Pinna, 1998).

Three of the genera studied belonged to the Pimelodinae (*Pimelodus, Iheringichthys* and *Pirinampus*) subfamily and one genus belonged to the Heptapterinae subfamily (*Pimelodella*). Of the species of the Pimelodinae subfamily studied, there

was a greater genetic similarity (0.335) between the *Pimelodus* species (*P. maculatus* and *P.* cf. *absconditus*), the clustering similarity of the *Pimelodus* genus with *I. labrosus* was 0.296, and these three species with *P. pirinampu* it was 0.131. The genetic similarity was 0.260 in the species of the Heptapterinae subfamily studied, *P.* aff *avanhandavae* and *Pimelodella* aff. m*eeki* (Table 2).

Table 2. Jaccard similarity between the six species of Pimelodidae

*P. maculatus/P.*cf. *absconditus*	0.335
*P.*cf. *absconditus/I. labrosus*	0.296
I. labrosus/P. pirinampus	0.131
P. pirinampus/P. aff. *avanhandavae*	0.084
P. aff. *Avanhandavae/P.* aff. *meeki*	0.260

RAPD comparisons are based on the assumption that bands of the same molecular weight, amplified with the same primer, represent homologous DNA sequences, but the more distant the two specimens, the less likely this assumption will be met. Nevertheless, RAPD markers remain a powerful tool to detect genetic polymorphism in closely-related organisms, as shown in numerous studies. These observations suggest that comparisons up to the family level are more likely to be reliable (Kump and Javornik, 1996; Landry and Lapointe, 1998).

According to Chambers *et al.* (1998) and Landry and Lapointe (1998), the results show that RAPD should not be used to study phylogenetic relationships at higher taxonomic levels; comparisons among species, genera and families were in most cases connected and the results should always be validated with a resampling procedure.

In spite of the limitations of the RAPD technique regarding the analyses at family level previously mentioned by the authors, in the present study there was a separation obtained between the subfamilies, is in line with the classification proposed by Pinna (1998).

It is therefore inappropriate to assume that molecular markers can provide the final answer to species identification; they are instead an additional marker system, which can be used to increase the resolution in taxonomic research (Carvalho and Hauser, 1999), as the data in the present study indicate.

Acknowledgements

The authors thank Dr Oscar Akio Shibatta, Mário Luís Orsi and Edson Santana da Silva from the Departamento de Biologia Animal e Vegetal of Universidade Estadual de Londrina for identification and collection of the specimens. We also thank CAPES and Universidade Estadual de Londrina for financial support received for the execution of this study.

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Received on February 05, 2002.
Accepted on March 18, 2002.