Genetics and Molecular Biology, 23, 4, 809-813 (2000)

Genetic evidence for two species of the genus *Pimelodus* Lacépède, 1803 (Siluriformes, Pimelodidae) in the Iguaçu River (Brazil)

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Abstract

The existence of reproductive isolation between two morphs of catfish, endemic to the Iguaçu River (Brazil), was examined by enzyme starch gel electrophoresis. Tissues of 19 catfish (*Pimelodus ortmanni*) and 15 of a similar morph (*Pimelodus* sp.), which differs from *P. ortmanni* by presenting larger and more scattered dusky spots on its skin, were analyzed. A Nei's (1978) genetic identity of 0.551 was determined by the analysis of 22 enzyme loci. The loci *EST*1*, *EST*2*, *GDH*1*, *GPI*1*, *GPI*2*, *IDH*1*, *MDH*1*, *MDH*2*, and *PGM*1* were fixed for different alleles in each morph, that is, no heterozygote was found for these loci. The enzymatic patterns observed for the two morphs indicate both that the taxa are reproductively isolated and that they in fact represent separate species.

INTRODUCTION

The ichthyofauna of the Iguaçu River, a left bank tributary of the Paraná River, is highly endemic (Garavello *et al*., 1997) because of the Iguaçu Falls, a natural barrier limiting fish dispersal that has existed for about 22 million years (Severi and Cordeiro, 1994). The high declivity and velocity of its water makes the Iguaçu River basin useful for hydroelectric purposes, which has resulted in the construction of a series of dams along the river. This fact has led to changes in the characteristics of the river and has threatened some highly endemic fish species (Julio *et al*., 1997).

Until 1995, only one species of the genus *Pimelodus* had been known in the Iguaçu River (*Pimelodus ortmanni* Haseman, 1911). Garavello and Shibatta (1995) reported a new *Pimelodus* species. According to Garavello *et al*. (1997), *Pimelodus* sp. has a larger muzzle and a taller body than *Pimelodus ortmanni*. In addition, *Pimelodus* sp. has larger and more scattered dusky spots on its skin than does *P. ortmanni* (Figure 1). These authors distinguished the two *Pimelodus* species on the basis of these morphological differences. However, morphology is not always a sufficient basis for the recognition of separate species. Morphological polymorphism is observed among populations of polytypic species, whereas cryptic species are sometimes morphologically indistinguishable.

The electrophoresis of enzymes on starch or polyacrylamide gels provides a powerful test of the validity of presumed species. Because this technique allows the measurement of genetic distances among individuals, it can serve as a means for identifying similar species (Shaklee *et al*., 1982). This approach is particularly useful in cases of syntopy. In such situations, genetically differentiated species are easily recognized when fixed allelic differences are detected (Bernardi and Goswami, 1997).

We used enzyme horizontal starch gel electrophoresis to determine the level of genetic identity between the two morphs of *Pimelodus* from the Iguaçu River.

MATERIAL AND METHODS

Thirty-four fishes were collected from the Salto Caxias reservoir of the Iguaçu River using simple gill nets (Figure 2). The liver, white muscle and heart were removed from freshly caught fish and frozen in liquid nitrogen. The tissues were homogenized with plastic pestles in 1.5-ml Eppendorf tubes with four drops of 0.2 M Tris/HCl buffer, pH 7.5. Because of the large quantity of fat present in the liver, 0.5 ml of CCl_4 was added during homogenization (Pasteur *et al*., 1988). The homogenized samples were centrifuged at 44,720 *g* for 30 min at 1-5ºC. The supernatant fractions were analyzed by horizontal electrophoresis on 13% corn starch (Penetrose 30®) covered with an ice pack. Two buffer systems were used: Tris-citrate, pH 7.0 (Shaw and Prasad, 1970) and Tris-borate-EDTA, pH 8.6 (Boyer *et al*., 1963). Electrophoresis was performed for 6 h at 5ºC with voltage gradients of 14 V/cm for the Tris-citrate gels, and 25 V/cm for the Tris-borate-EDTA gels (Table I). The gels were sliced horizontally and then incubated in the staining solution appropriate for each enzyme.

To visualize specific isozymes the procedures of Aebersold *et al.* (1987) were followed, except for aspartate aminotransferase (AAT) for which the method of Morizot and Schmidt (1990) was used. The nomenclature used is that proposed by Shaklee *et al.* (1990). The data were analyzed for Hardy-Weinberg equilibrium, allele frequency at each locus, frequency of polymorphic loci and Nei's genetic distance using the Biosys 1 software (Swofford and Selander, 1981). The genetic interpretation

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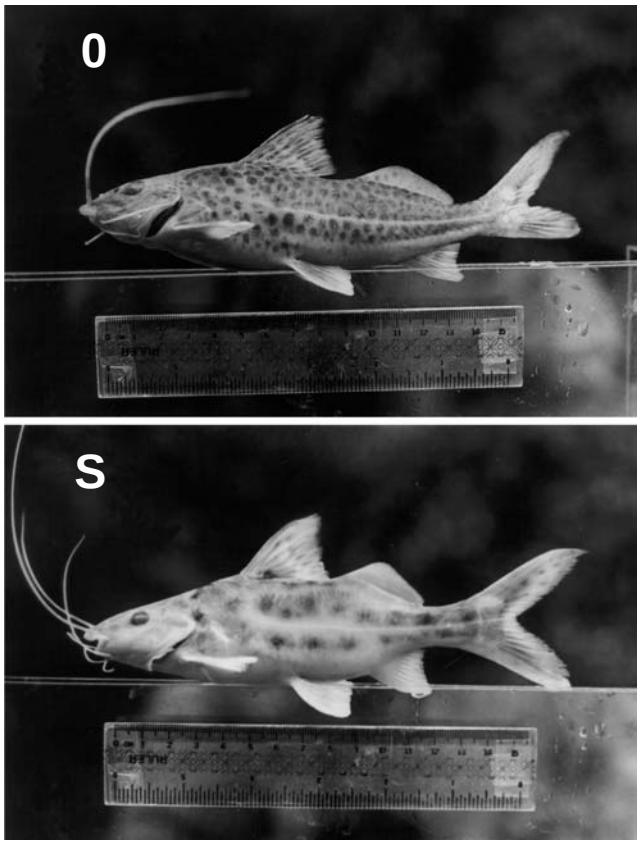

Figure 1 - *Pimelodus ortmanni* (**0**) and *Pimelodus* sp. (**S**) specimens.

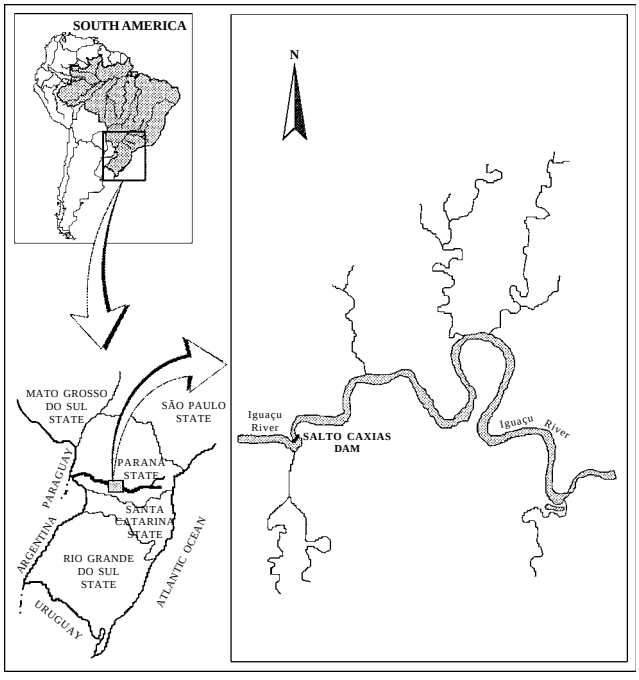

Figure 2 - Geographic location of Salto Caxias on the Iguaçu River.

of enzymatic patterns was based on the quaternary structure of the enzymes as described by Ward *et al.* (1992).

RESULTS

Twenty-two putative loci were detected based on 12 enzymatic systems analyzed for the 15 *Pimelodus* sp. and 19 *P. ortmanni* specimens (Table II).

The expression of the loci *MDH*1* and *PGM*1* (see Table I for abbreviations) was limited to *P. ortmanni* (Figure 3).

AAT: This dimeric enzyme showed greater activity in the liver than in heart or muscle. All the specimens gave two well-separated bands. This phenotype was interpreted as two loci without heterodimer formation.

ADH: This dimeric enzyme showed activity only in the liver. In *P. ortmanni* liver three phenotypes were seen: a single cathodic band, a single anodic band, and a mixture of three bands (one cathodic, one anodic and one at the origin). All the *Pimelodus* sp. specimens had only one cathodic band which migrated farther from the origin than the cathodic band of *P. ortmanni*. This pattern may be explained as a single locus with three alleles; *Pimelodus* sp. was monomorphic at this locus.

EST: This monomeric enzyme was restricted to the liver tissue and showed two anodic bands in both morphotypes, although those of *Pimelodus* sp. were less anodic than those of *P. ortmanni*. These phenotypes were assumed to result from two loci monomorphic for different alleles.

GDH: *Pimelodus* sp. had a single band which was more anodic than that of *P. ortmanni*. Both were considered monomorphic for different alleles at a single locus.

G3PDH: All but one of the specimens showed three bands for this enzyme in muscle. Since this enzyme has a dimeric structure, the three bands must result from two loci with heterodimer formation. Only one fish (*Pimelodus* sp.) gave five bands; it was likely heterozygous for two alleles at a single locus.

GPI: All of the specimens showed three bands, but the three bands of *Pimelodus* sp. were less anodic than those of *P. ortmanni*. This dimeric enzyme must be codified by two loci with heterodimer formation. The two morphotypes were monomorphic for different alleles.

IDHP: Both liver and heart gave a single band for this activity. The heart enzymes in the two morphotypes were coincident, but those of the liver differed. The liver enzyme of *Pimelodus* sp. migrated faster than the heart enzymes and that from *P. ortmanni* liver. Only one specimen of *P. ortmanni* gave three bands in heart tissues. The monomorphic active locus in the liver is therefore different from that in the heart. The three-band phenotype in heart samples is indicative of heterozygosity for two alleles.

LDH: All of the specimens gave five bands. Since this enzyme has a tetrameric structure, the phenotype must result from two loci which are monomorphic in both morphotypes.

Table I - Names, abbreviations, enzyme commission numbers (E.C. No.), tissues, buffers, quaternary structure (Q.S.) and number of loci for each enzyme assayed in tissues of *Pimelodus ortmanni* and *Pimelodus* sp. from the Iguaçu River. L = Liver; M = muscle; H = heart; TBE = Tris/borate/EDTA; TC = Tris/citrate.

Enzyme (abbreviation)	E.C. No.	Tissue	Buffer	Q.S.	Loci
Aspartate aminotransferase (AAT)	2.6.1.1	L	TBE	Dimeric	2
Alcohol dehydrogenase (ADH)	1.1.1.1	L	TBE	Dimeric	1
Esterase (EST)	3.1.1.1	L	TBE	Monomeric	2
Glucose dehydrogenase (GDH)	1.1.1.47	L	TBE	Dimeric	1
Glycerol-3-phosphate dehydrogenase (G3PDH)	1.1.1.8	M	TC	Dimeric	2
Isocitrate dehydrogenase (IDHP)	1.1.1.42	L, H	TC	Dimeric	2
Glucose-6-phosphate isomerase (GPI)	5.3.1.9	L, H	TC	Dimeric	2
Lactate dehydrogenase (LDH)	1.1.1.27	H	TC	Tetrameric	2
Malate dehydrogenase (MDH)	1.1.1.37	L, H	TC	Dimeric	3
Malic enzyme (MEP)	1.1.1.40	L, H	TC	Tetrameric	2
Phosphoglucomutase (PGM)	5.4.2.2	L, H	TC	Monomeric	3
Superoxide dismutase (SOD)	1.15.1.1	L	TBE	Dimeric	1

Table II - Allele frequency estimates for *Pimelodus ortmanni* and *Pimelodus* sp. from the Iguaçu River.

Locus	Allele	P. ortmanni N = 19	*Pimelodus* sp. N = 15
**AAT-1*	a	1.000	1.000
AAT-2	a	1.000	1.000
ADH-1	a	0.000	1.000
	b	0.562	0.000
	c	0.438	0.000
EST-1	a	1.000	0.000
	b	0.000	1.000
EST-2	a	1.000	0.000
	b	0.000	1.000
G3P-1	a	0.000	0.033
	b	1.000	0.967
G3P-2	a	1.000	1.000
GDH-1	a	1.000	0.000
	b	0.000	1.000
GPI-1	a	0.000	1.000
	b	1.000	0.000
GPI-2	a	0.000	1.000
	b	1.000	0.000
IDH-1	a	1.000	0.000
	b	0.000	1.000
IDH-2	a	1.000	1.000
LDH-1	a	1.000	1.000
LDH-2	a	1.000	1.000
MDH-1	a	1.000	0.000
MDH-2	a	1.000	0.000
	b	0.000	1.000
MDH-3	a	1.000	0.967
	b	0.000	0.033
MEP-1	a	1.000	1.000
MEP-2	a	1.000	1.000
PGM-1	a	1.000	0.000
PGM-2	a	1.000	1.000
SOD-1	a	1.000	1.000

*See Table I for abbreviations.

MDH: For this enzyme, *Pimelodus* sp. exhibited two bands and *P. ortmanni* had four. These phenotypes may be explained by two loci without heterodimer formation in the former morphotype and by three loci with heterodimer formation between the more anodic forms in the latter morphotype. Only locus *MDH*3* of *Pimelodus* sp. was polymorphic.

MEP: The expression of this enzyme in liver was different from that in the heart in both morphotypes. Both of the tissues gave four bands. In the heart the most intense band was the most anodic and in liver the most intense band was the least anodic. These phenotypes were interpreted as resulting from two monomorphic loci.

PGM: A single band in *Pimelodus* sp. and two bands in *P. ortmanni* were detected for this enzyme. In both morphotypes there were secondary bands. These patterns indicate a single monomorphic locus in the former morphotype and two loci in the latter.

SOD: All specimens gave a single band indicative of a single locus.

P. ortmanni was polymorphic only at *ADH*1* and *IDH*2*, and *Pimelodus* sp. only at *G3P*1* and *MDH*3*. Moreover, the two species were fixed for different alleles at nine of the 22 loci (Table II). The unbiased estimated mean heterozygosity per locus (Nei, 1978) was 0.006 for *Pimelodus* sp. and 0.024 for *P. ortmanni*. All polymorphic loci assayed were in Hardy-Weinberg equilibrium. Nei's unbiased genetic identity and unbiased genetic distance (Nei, 1978) between the two species was 0.551 and 0.597, respectively.

DISCUSSION

The biological species concept is based on the reproductive isolation of groups of true-breeding populations from other such groups (Mayr, 1970). From conventional definitions under the biological species concept, two syntopic, conspecific morphs should have the same gene frequencies at each locus (Thorpe and Solé-Cava, 1994).

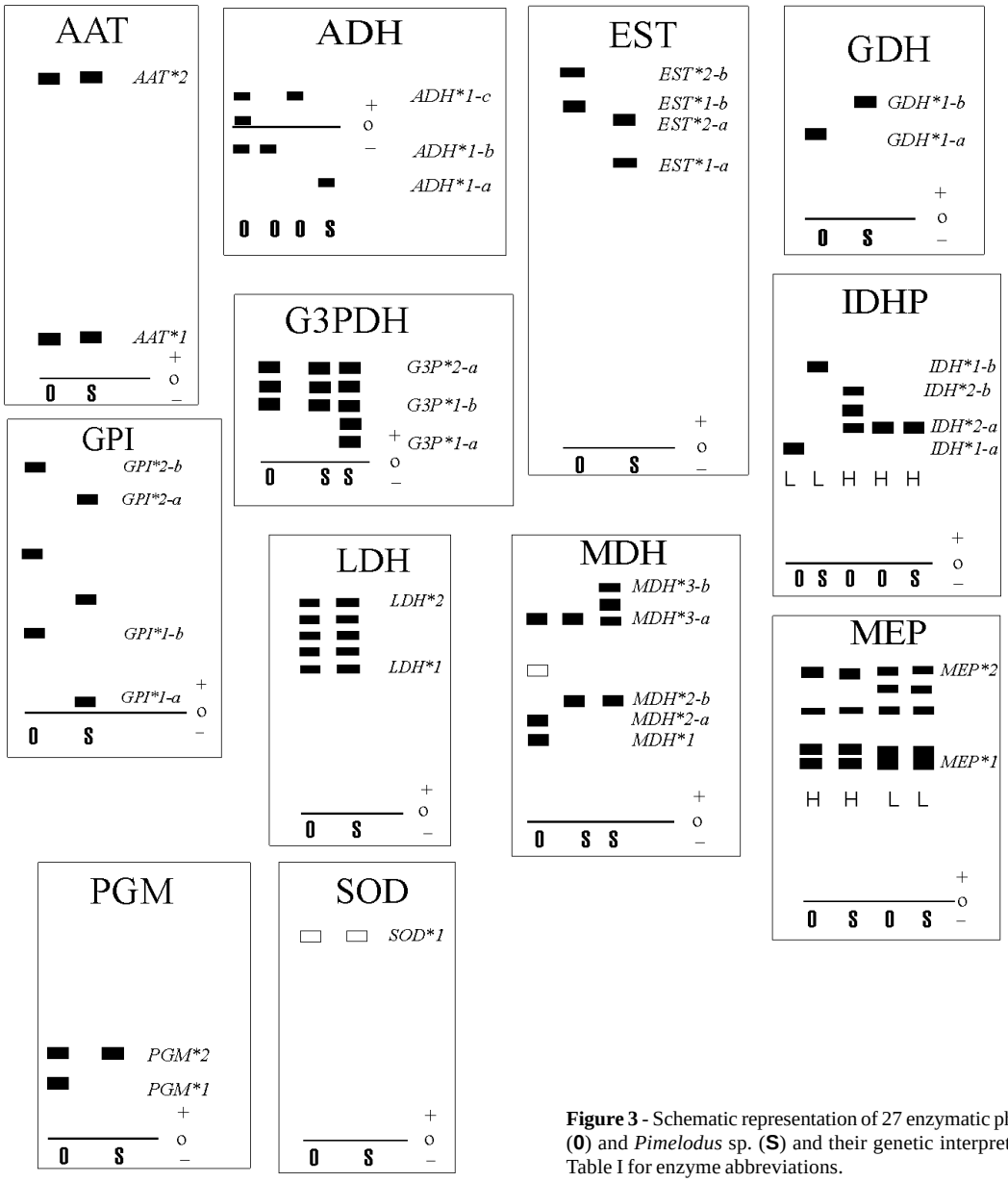

Figure 3 - Schematic representation of 27 enzymatic phenotypes of *Pimelodus ortmanni* (**0**) and *Pimelodus* sp. (**S**) and their genetic interpretation. H = Heart; L = liver. See Table I for enzyme abbreviations.

If two morphs or populations share no common allele at a locus, they must be reproductively isolated and considered true biological species.

The electrophoresis data revealed a low genetic diversity in the two morphs of *Pimelodus*. The heterozygosities estimated for *Pimelodus* sp. (0.006) and for *P. ortmanni* (0.024) were both below that estimated for *Pimelodus maculatus* (0.06) from the Tibagi River (another river in the Paraná River basin) by Almeida and Sodré (1998) and below the overall mean of 0.051 estimated for fishes by Ward *et al.* (1992).

The two morphs were fixed for different alleles at nine loci. No heterozygote was found for these loci. If the morphs were conspecific, a few heterozygotes should have been encountered in the population. Since 15 *Pimelodus* sp. and 19 *P. ortmanni* were analyzed, 68 alleles for each locus were screened. Thus, the expected allele frequencies would be p = 30/68 = 0.44 for the alleles of *Pimelodus* sp. and q = 38/68 = 0.56 for the alleles of *P. ortmanni*. The expected proportion of heterozygotes would therefore be 2pq = 2 x 0.44 x 56 = 0.493 for each locus. The probability of not obtaining a heterozygote for one of the seven loci would be 1 - 0.493 = 0.507. The probability of not obtaining any heterozygotes in a sample of 34 individuals would be $(0.507)^{34}$. For nine loci, this probability would be $(0.507)^{34x9} = (0.507)^{306}$. In other words, it would be impossible not to obtain any heterozygous individual for the nine loci. The null hypothesis of a single random breeding population is therefore rejected.

Based on published data on the frequency of differ-

ent values of Nei's (1972) *I* for different levels of systematic divergence, Thorpe (1982) calculated empirical curves for probability against *I* values between confamilial genera, congeneric species and conspecific populations and concluded that if populations of uncertain status have genetic identities between 0.35 and 0.85, they should not be considered conspecific. The genetic identity between *Pimelodus* sp. and *P. ortmanni* was estimated to be 0.551, which is below the critical value of 0.85 established by Thorpe (1982). Thus, *Pimelodus* sp. could be considered a species that is actually distinct from *P. ortmanni*.

Taxonomically and ecologically, the Iguaçu River is still a poorly studied fluvial system. As a result, little is known of how these species were formed and why they are endemic to this basin. However, recent studies have provided some information on this subject. While working on the Iguaçu River around the Segredo reservoir (250 km upstream the Caxias dam, not shown in Figure 2), Suzuki and Agostinho (1997) noted intensive reproductive activity of *Pimelodus* sp. downstream from the Segredo dam, as well as massive reproductive activity of *P. ortmanni* in the Iratim River (a tributary of the Segredo reservoir, 60 km upstream the Segredo dam). This could mean that the two species require different reproductive sites, which could have led to the extensive genetic differentiation found between them. The recognition of the two populations and their presumed different reproductive sites needs to be taken into account by the Brazilian authorities when constructing new hydroelectric dams in the Iguaçu River basin to guarantee the genetic diversity of these fishes and their ecological importance.

ACKNOWLEDGMENTS

The authors thank COPEL (Companhia Paranaense de Energia Elétrica) for financial support, Dr. Oscar Shibata and Carla Pavanelli for the suggestions to the manuscript, Celso Ikeda for taking the photos, and Harumi I. Suzuki for collecting the fish samples.

RESUMO

Eletroforese de enzimas em gel de amido foi utilizada para verificar a existência de isolamento reprodutivo entre duas formas de mandi endêmicas do Rio Iguaçu (Brasil). Foram analisados tecidos de 19 mandis (*Pimelodus ortmanni*) e 15 de uma forma semelhante (*Pimelodus* sp.), que difere de *P. ortmanni* por apresentar manchas escuras maiores e mais espaçadas sobre a pele. A análise de 22 locos enzimáticos exibiram uma identidade genética de 0,551. Os locos *EST*1*, *EST*2*, *GDH*1*, *GPI*1*, *GPI*2*, *IDH*1*, *MDH*1*, *MDH*2 e PGM*1* se mostraram fixos para alelos alternativos em cada forma, isto é, nenhum heterozigoto para estes locos foi encontrado. Os padrões enzimáticos das duas formas indicaram que elas são reprodutivamente isoladas e que elas representam, de fato, duas espécies separadas.

REFERENCES

Aebersold, P.B., Winans, G.A., Teel, D.J., Milner, G.B. and **Utter, F.M.** (1987). Manual for starch electrophoresis: a method for detection of genetic variation. *NOAA Technical Report NMFS 61*: 1-17.

Almeida, F.S. and **Sodré, L.M.K.** (1998). Analysis of genetic variability in three species of Pimelodidae (Ostariophysi - Siluriformes). *Genet. Mol. Biol. 21*: 487-492.

Bernardi, G. and **Goswami, U.** (1997). Molecular evidence for cryptic species among Antarctic fish *Trematomus bernachii* and *Trematomus hansoni*. *Antarct. Sci. 9*: 381-385.

Boyer, S.H., Fainer, D.C. and **Naughton, M.A.** (1963). Myoglobin inherited structural variation in man. *Science 140*: 1228-1231.

Garavello, J.C. and **Shibatta, O.A.** (1995). Duas novas espécies para o gênero *Pimelodus* Lacépede, 1803, das bacias do rio Iguaçu e Guaíba (Ostariophysi, Pimelodidae). *In: 11 Encontro Brasileiro de Ictiologia,* Pontifícia Universidade Católica de Campinas, Sociedade Brasileira de Ictiologia, Campinas, 1995, p. B4.

Garavello, J.C., Pavanelli, C.S. and **Suzuki, H.I.** (1997). Caracterização da ictiofauna do rio Iguaçu. *In: Reservatório de Segredo - Bases Ecológicas para o Manejo* (Agostinho, A.A. and Gomes, L.C., eds.). Editora da Universidade Estadual de Maringá, Maringá, pp. 61-84.

Julio, H.F., Bonecker, C.C. and **Agostinho, A.A.** (1997). Reservatório de Segredo e sua inserção na bacia do rio Iguaçu. *In: Reservatório de Segredo - Bases Ecológicas para o Manejo* (Agostinho, A.A. and Gomes, L.C., eds.). Editora da Universidade Estadual de Maringá, Maringá, pp. 1-17.

Mayr, E. (1970). *Populations, Species and Evolution*. Harvard University Press, Cambridge.

Morizot, D.C. and **Schmidt, M.E.** (1990). Starch gel electrophoresis and histochemical visualization of proteins. *In*: *Electrophoretic and Isoelectric Focusing Techniques in Fisheries Management* (Whitmore, D.H., ed.). CRC Press, Boca Raton, pp. 23-80.

Nei, M. (1972). Genetic distance between populations. *Am. Nat. 106:* 283-292.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics 89*: 583-590.

Pasteur, N., Pasteur, G., Bonhomme, F., Catalan, J. and **Britton-Davidian, J.** (1988). *Practical Isozyme Genetics*. Ellis Horwood Limited, Chichester.

Severi, W. and **Cordeiro, A.A.M.** (1994). *Catálogo de Peixes da Bacia do Rio Iguaçu*. Intituto Ambiental do Paraná (IAP), Curitiba.

Shaklee, J.B., Tamaru, C.S. and **Waples, R.S.** (1982). Speciation and evolution of marine fishes studied by the electrophoretic analysis of proteins. *Pac. Sci. 36*: 141-157.

Shaklee, J.B., Allendorf, F.W., Morizot, D.C. and **Whitt, G.S.** (1990). Gene nomenclature in protein-coding loci in fish. *Trans. Am. Fish. Soc. 119*: 2-15.

Shaw, C.R. and **Prasad, R.** (1970). Starch gel electrophoresis - a compilation of recipes. *Biochem. Genet. 4*: 297-320.

Suzuki, H.I. and **Agostinho, A.A.** (1997). Reprodução de peixes do Reservatório de Segredo. *In: Reservatório de Segredo - Bases Ecológicas para o Manejo* (Agostinho, A.A. and Gomes, L.C., eds.). Editora da Universidade Estadual de Maringá, Maringá, pp. 163-182.

Swofford, D.L. and **Selander, R.B.** (1981). BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *J. Hered. 72*: 281-283.

Thorpe, J.P. (1982). The molecular clock hypothesis: biochemical evolution, genetic differentiation and systematics. *Annu. Rev. Ecol. Syst. 13*: 139-168.

Thorpe, J.P. and **Solé-Cava, A.M.** (1994). The use of allozyme electrophoresis in invertebrate systematics. *Zool. Scr. 23*: 3-18.

Ward, R.D., Skibinski, D.O.F. and **Woodward, M.** (1992). Protein heterozygosity, protein structure, and taxonomic differentiation. *Evol. Biol. 26*: 73-157.

(Received January 3, 2000)