REVUE SUISSE DE ZOOLOGIE 106 (1): 91-105; mars 1999

Genetic and morphometric analysis of three species of the genus *Hypostomus* Lacépède, 1803 (Osteichthyes: Loricariidae) from the Rio Iguaçu basin (Brazil)[1]

Cláudio Henrique ZAWADZKI*, Erasmo RENESTO* & Luís Maurício BINI**

* Universidade Estadual de Maringá, Nupélia, Av. Colombo 5790, 87020-900 Maringá-PR: Brazil, e-mail: chzawadzki@nupelia.uem.br

** Universidade Federal de Goiás, Departamento de Biologia Geral, 74001-970 Goiânia-GO: Brazil.

Genetic and morphometric analysis of three species of the genus *Hypostomus* Lacépède, 1803 (Osteichthyes: Loricariidae) from the rio Iguaçu basin (Brazil). - Three species of *Hypostomus* from the Rio Iguaçu basin, *H.* aff. *commersonii*, *H. derbyi* and *H. myersi*, were examinated by multivariate analysis, PCA and size-free discriminant canonical analysis, by the external characters truss network method and starch gel isoenzyme electrophoresis in heart, liver and muscle tissues. Twenty-six gene loci of 14 isoenzyme systems (AAT, ACP, ADH, GDH, G3PDH, G6PDH, GPI, IDHP, LDH, MDH, ME, PGM, PER and SOD) were scored for the first time for these species. The percentage of polymorphic loci (P0.99) varied from 11.54% for *H. derbyi* and *H. myersi* to 19.23% in *H.* aff. *commersonii*. Expected mean heterozygosity (H_e) varied from 0.011 in *H. derbyi* to 0.044 in *H.* aff. *commersonii*. Nei's genetic distance (D) between *H.* aff. *commersonii* and *H. derbyi* was 0.142, between *H.* aff. *commersonii* and *H. myersi* 0.621, and between *H. derbyi* and *H. myersi* 0.776. We discuss hypotheses for the maintenance of the low mean heterozygosity observed, and for the presence of these species in this drainage basin.

Key-words: Loricariidae – *Hypostomus* – Rio Iguaçu – enzyme electrophoresis – size-free canonical discriminant analysis - morphometry

INTRODUCTION

Because of the large number of species in the genus *Hypostomus* Lacépède and the great intraspecific variation in morphology and pigmentation pattern in the species of this genus, systematists have encountered much difficulty in correctly identifying many of these species. Moreover, summary and incomplete descriptions, in conjunction with limited knowledge of the distribution patterns of the South American ichthyofauna, have resulted in proliferation of identification errors in the literature (REIS *et al.* 1990a).

[1] This paper is a part of the Master thesis of Cláudio Henrique Zawadzki, at the State University of Maringá, granted by CAPES.

Manuscript accepted 27.07.1998

The drainage basin of the Rio Iguaçu is characterized by a high degree of endemism (about 75% of its ichthyofauna), by a low number of fish species and the absence of many fish families very common in the Paraná basin (JULIO JR *et al.* 1997). The Iguaçu basin became separated from the Rio Paraná about 22 million years ago by the formation of Iguaçu Falls, which are about 70 meters high (SEVERI & CORDEIRO 1994). Its isolation makes the basin an excellent natural laboratory for studies of the *Hypostomus* species, which requires studies at the regional level because of its complexity (REIS *et al.* 1990a).

The differences and similarities between groups of organisms must be quantified in terms of morphological, physiological, ecological and/or molecular characters. With the development of the enzyme electrophoresis technique on a porous support medium by HUNTER & MARKERT (1957), it became possible to obtain information about the structural enzymes of living organisms. Additionally, the subsequent development of calculations of allelic frequencies and indices of distance and similarity to evaluate the genotypic variation between intra- or interspecific populations, made possible the use of enzyme electrophoresis in systematic studies.

AVISE (1974) mentions among other advantages of enzyme electrophoresis, the possibility of acquiring adequate data for the description of a species for systematic purposes, even for a sample with a relatively small number of individuals, and that phylogenetically proximate species can be arranged according to the percentages of alleles or distinct genotypes. However, THORPE & SOLÉ-CAVA (1994) maintain that isoenzyme electrophoresis should not be used as a substitute for morphological analyses, but as complementary to these. For this purpose, isoenzyme electrophoresis, combined with techniques of multivariate morphometry, has for a long time been cited and used in evolutionary and systematic biology (SHAKLEE & TAMARU 1981; O'MAOILEIDIGH *et al.* 1988; HEDGECOCK *et al.* 1989; ROBY *et al.* 1991; LOPEZ-LEMUS 1991; THORPE & SOLÉ-CAVA 1994). Nevertheless, such combined techniques have not yet been used to investigate the taxonomic status or phylogenetic relationships of most groups of neotropical fishes. Therefore it is necessary to test and refine these techniques, in order that they can assist in clarifying relationships between groups of fishes where traditional systematics has encountered great difficulties in obtaining precise results.

Accordingly, the present work aimed to investigate the genetic relationships of *H.* aff. *commersonii* Valenciennes, 1840, *H. derbyi* (Haseman, 1911), and *H. myersi* (Gosline, 1947), to analyze the morphological variation between these species by means of morphometric techniques and to address questions about the presence of the species in the Rio Iguaçu basin.

MATERIALS AND METHODS

Morphometric analyses. For the morphometric analyses, 22 specimens of *H.* aff. *commersonii*, 24 of *H. derbyi*, and 26 of *H. myersi* were collected from the Rio Iguaçu basin (Fig. 1), from March 1993 through February 1996. The specimens were fixed in 10% formalin and they are alcohol preserved in the synoptic collection of Nupélia

(Limnology, Ichthyology and Aquaculture Research Center) of the State University of Maringá voucher specimens are also deposited in the collections of the Muséum d'histoire naturelle, Geneva (Switzerland). A list of specimens was arranged in the text as follow: locality, town, collecting date, number of specimens (if there are more than one) between parenthesis and the standard length in centimeters (the smallest and biggest if there are more than one in the lot).

LIST OF SPECIMENS STUDIED: BRAZIL, PARANA.

H. aff. *commersonii*. Morphometric analyses. Downstream Segredo dam, Pinhão, 04.ii. 1994, 21.5; 04.ii.1995, 18.6; 02.ii.1995, 23.0; Upstream Segredo dam, Pinhão, 06.i.1995, 18.4; 03.viii.1995, (2) 23.7-25.2; Rio São Pedro, Pinhão, 12.i.1994, 26.5; Rio Iratim, Palmas, 09.viii.1993, 21.5; 08.x.1995, (2) 24.1-30.3; 09.x.1995, (2) 27.4-28.0; 07.xi.1994, 26.1; 07.ii. 1995, 15.8; 05.xi.1995, (2) 31.0-32.7; Rio Verde, Pinhão, 30.0; Downstream Areia dam, Bituruna, 10.ii.1994, 22.5; Upstream Areia dam, Bituruna, 07.viii.1993, (3) 16.5-22.5; 27.v.1994, 17.5. Electrophoretic analysis. Downstream Segredo dam, Pinhão, 03.xi.1995, 18.0; 02.ii.1996, 23.0; Rio Iratim, Palmas, 04.xi.1995, (2) 19.9-32.5; 06.xi.1995, 31.0.

H. derbyi. Morphometric analyses. Downstream Segredo dam, Pinhão, 03.viii.1994, 12.0; Upstream Segredo dam, Pinhão, 03.ii.1995, (2) 20.8-23.0; 04.ii.1995, (2) 18.5-24.2; 05.ii.1995, 19.1; Rio Iratim, Palmas, 09.vii.1993, (2) 22.5-26.5; 07.xi.1993, (2) 25.0-27.0; 07.xi.1995, (2) 27.7-29.4; Rio Butiá, Palmas, 04.xi.1994, 24.7; Rio Covó, Mangueirinha, 04.ii.1994, 16.5; 04.iii.1994, 19.4; 04.xi.1994, (2) 21.5-22.1; Downstream Areia dam, Bituruna, 13.xi.1994, (2) 21.0-23.5; 09.viii.1995, 20.7; Upstream Areia dam, Bituruna, 10.viii.1993, (4) 16.0-22.5. Electrophoretic analysis. Downstream Segredo dam, Pinhão, 03.xi.1995, (8) 14.2-18.3; Upstream Segredo Dam, Pinhão, 02.xi.1995, (3) 17.1-22.9; Rio Iratim, Palmas, 08.xi.1995, 21.6; Downstream Areia dam, 06.xi.1995, (2) 17.3-19.4.

H. myersi. Morphometric analyses. Upstream Segredo Dam, Pinhão, 23.iii.1993, 17.4; 05.ii.1995, (5) 9.0-14.5; Rio Iratim, Palmas, 06.xi.1993, 11.4; 09.ii.1995, (2) 12.7-14.9; 05.viii.1995, (2) 12.4-12.5; Rio Butiá, Palmas, 19.v.1993, (2) 8.5-11.2; Downstream Areia dam, Bituruna, 12.i.1995, (2) 13.2-17.4; 08.viii.1995, (4) 14.6-15.8; 09.xi.1995, (7) 10.1-17.8. Electrophoretic analysis. Downstream Segredo dam, Pinhão, 02.xi.1995, (4) 10.1-14.3; Rio Iratim, Palmas, 05.xi.1995, (6) 12.5-13.8; Downstream Areia dam, 08.xi.1995, (5) 11.4-14.3.

Using ten landmarks, a group of 21 measurements (interlandmark distances) were made, based on the truss network method (Fig. 2), and used in the studies of multivariate morphometry. This method consists of a series of measurements calculated between anatomical points (landmarks), considered homologous for the groups under study, which form a regular pattern of continuous cells along the body (STRAUSS & BOOKSTEIN 1982). The measurements were taken with a caliper to a precision of 0.05 mm.

The multivariate morphometry techniques used were principal components analysis (PCA) and size-free canonical discriminant analysis (SF-CDA), according to the procedure of REIS *et al.* (1990b). This technique basically consists of removing the effect of within-group ontogenetic variation, regressing each character separately on the within-group first principal component (PC1) which is a multivariate estimate of size. PC1 is obtained from an analysis performed on a covariance matrix of the measurements centered by the mean group and then applying canonical discriminant analysis to the residuals obtained from the regressions (STRAUSS 1985). These analyses were performed using the software Statistica for Windows®, version 5.1, 1996.

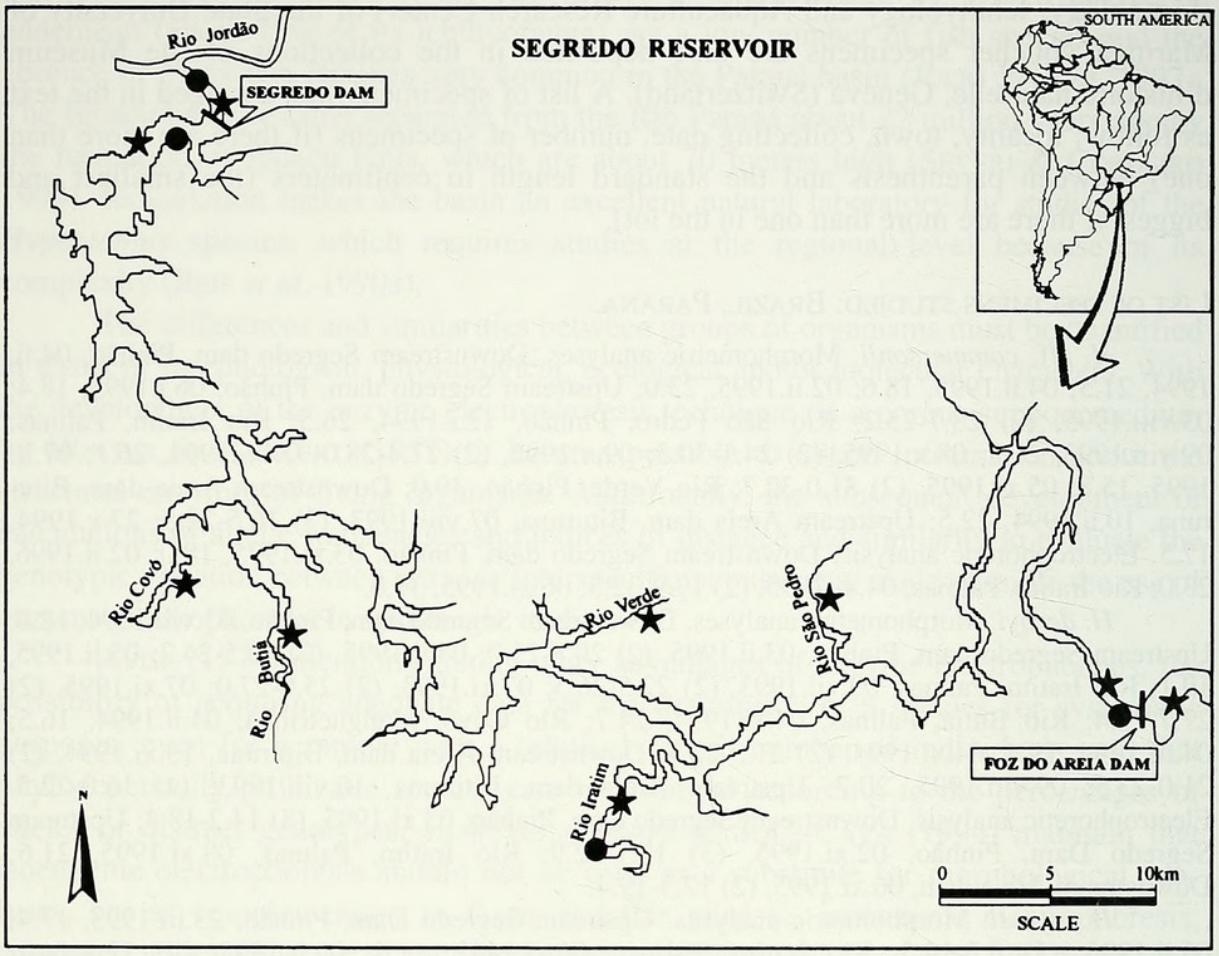

FIG. 1

The sampling sites in the Rio Iguaçu basin. The symbols ★ and ● means the locations of the specimens collected for the morphometric and electrophoretic analyses respectively.

Electrophoretic analyses. The specimens used for the enzymatic electrophoresis were collected in the Rio Iguaçu using simple gill nets. The collections were carried out from November 1995 through February 1996, in conjunction with the same project mentioned previously. In total, 5 specimens of *H.* aff. *commersonii*, 14 of *H. derbyi*, and 15 of *H. myersi* were analyzed. The liver, muscle and heart were removed from live fish and frozen in liquid nitrogen. For analysis, the tissues were homogenized by macerating with plastic pestles in 1.5 ml Eppendorf tubes with 0.02 M Tris/HCl buffer, pH 7.5 in 1:1 proportion. In macerating the liver it was necessary to add carbon tetrachlorate in a 1:2 concentration (tissue: tetrachlorate), because of the large quantity of fat present in this material (PASTEUR *et al.* 1988). The homogenized samples were centrifuged at 5500 rpm for 30 minutes at 5° C. The supernatant fractions were submitted to horizontal electrophoresis in 13% starch gel (Penetrose-30®) at 5° C for six hours. The enzyme systems and buffers used are shown in Table 1.

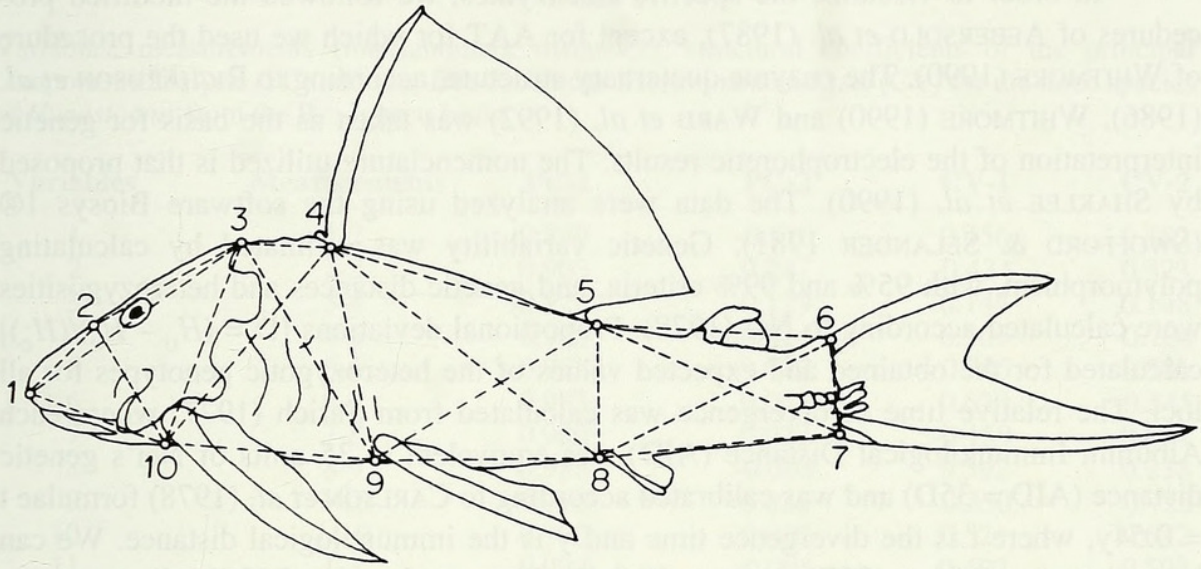

FIG. 2

Illustration of the 21 measurements used in the morphometric analyses. The locations of the 10 landmarks for the truss network data are shown as open circles, and the measurements of morphometric distances between the circles as dotted lines. The landmarks refer to the (1) anterior end of the snout; (2) median line of nasal pores; (3) posterior occipital margin; (4) anterior origin of dorsal fin; (5) posterior origin of dorsal fin; (6) origin of base of upper spine of caudal fin; (7) origin of base of lower spine of caudal fin; (8) anterior origin of anal fin; (9) anterior origin of pelvic fin; (10) end of operculum.

TABLE 1

Enzyme systems, tissues and buffers used to investigate the *Hypostomus* species. * H, heart; L, liver; M, muscle. I - Tris-citrate buffer (pH 7.0) (Shaw & Prasad 1970). II - Tris-borate-EDTA buffer (pH 8.7) (BOYER *et al.* 1963).

Enzyme	**E. C. Number**	**Structure**	**Tissue***	**Buffer**
Acid phosphatase	ACP 3.1.3.2	Monomeric	L	I
Alcohol dehydrogenase	ADH 1.1.1.1	Dimeric	L	I and II
Aspartate aminotransferase	AAT 2.6.1.1	Dimeric	H, L and M	II
Glucose dehydrogenase	GDH 1.1.1.47	Monomeric	L	II
Glucose-6-phosphate dehydrogenase	G6PDH 1.1.1.49	Tetrameric	L	II
Glucose-6-phosphate isomerase	GPI 5.3.1.9	Dimeric	H, L and M	I
Glycerol-3-phosphate dehydrogenase	G3PDH 1.1.1.8	Dimeric	L and M	I
Isocitrate dehydrogenase	IDHP 1.1.1.42	Dimeric	H, L and M	I
Lactate dehydrogenase	LDH 1.1.1.27	Tetrameric	H and M	I
Malate dehydrogenase	MDH 1.1.1.37	Dimeric	H, L and M	I
Malic enzyme	MEP 1.1.1.40	Monomeric	H, L and M	I
Peroxidase	PER 1.11.1.6	Tetrameric	H and L	I and II
Phosphoglucomutase	PGM 5.4.2.2	Monomeric	H, L and M	I
Superoxide dismutase	SOD 1.15.1.1	Tetrameric	L	II

In order to visualize the specific isoenzymes, we followed the modified procedures of AEBERSOLD *et al.* (1987), except for AAT for which we used the procedure of WHITMORE (1990). The enzyme quaternary structure, according to RICHARDSON *et al.* (1986), WHITMORE (1990) and WARD *et al.* (1992) was taken as the basis for genetic interpretation of the electrophoretic results. The nomenclature utilized is that proposed by SHAKLEE *et al.* (1990). The data were analyzed using the software Biosys 1® (SWOFFORD & SELANDER 1981). Genetic variability was estimated by calculating polymorphism, with 95% and 99% criteria, and genetic distances and heterozygosities were calculated according to NEI (1978). Proportional deviations [$D = (H_o - H_e)/(H_e)$] calculated for the obtained and expected values of the heterozygotic genotypes for all loci. The relative time of divergence was calculated from Sarich (1977) where each Albumin Immunological Distance (AID) was equivalent to 35 units of Nei's genetic distance (AID = 35D) and was calibrated according to CARLSON *et al.* (1978) formulae t = 0.54y, where t is the divergence time and y is the immunological distance. We can have then t = 0.54 x (35D) --> t = 18.9 D. Thus, each unit of Nei's distance was equivalent to 18.9 millions of years.

RESULTS

Multivariate morphometric analyses

A preliminary principal components analysis discriminated the species in three nearly distinct groups. No sexual dimorphism was evident, and therefore this source of variation was not considered. The first principal component explained 85% of the total variation, and was considered as an axis of size, as indicated by the positive and approximately equal coefficients of the variables (Table 2).

When the data were treated by size-free discriminant canonical analysis, projection of individual scores along the canonical variables (CV) 1 and 2 for the three species studied (Fig. 3) showed that *H. myersi* was discriminated from *H.* aff. *commersonii* and *H. derbyi* along CV-1, while *H.* aff. *commersonii* and *H. derbyi* were discriminated from each other along CV-2.

The correlation vectors between the original characters and the canonical variables 1 and 2 are shown in Table 2. These coefficients demonstrated that *H. myersi* differed from the other species principally in measurements (6-7), (8-9), (1-10), (5-9) and (5-7). Measurements (4-5), (7-8), (9-10), (3-9) and (6-8) most influenced the separation of *H. derbyi* from *H.* aff. *commersonii*.

Electrophoretic analysis

We detected 48 alleles distributed in 26 isoenzyme loci. The frequencies of alleles for the loci studied are shown in Table 3.

A summary of the statistical analyses of genetic variation is presented in Table 4. The proportion of polymorphic loci was low in all species (P0.95 = 12% on average). *Hypostomus* aff. *commersonii* showed the highest variation, notably in relation to *H. derbyi* (P0.95 = 20% vs. 4%). The mean number of alleles per locus (AN) was higher in *H.* aff. *commersonii* than in *H. myersi* and *H. derbyi*. The mean H_e for the three species was 0.024.

TABLE 2

Variables, measurements (interlandmark distances), structural coefficients of the principal components analysis (PC) and size-free canonical discriminant analysis (CV) for the three species of *Hypostomus* from the Rio Iguaçu basin.

Variables	Measurements	PC-1	PC-2	CV-1	CV-2
1	1-2	0.849	-0.516	0.456	0.182
2	2-3	0.903	0.247	-0.434	0.323
3	3-4	0.920	0.129	0.144	0.148
4	4-5	0.986	0.024	0.509	-0.704
5	5-6	0.975	0.022	-0.745	0.586
6	6-7	0.985	0.008	0.620	-0.345
7	7-8	0.980	0.027	-0.159	0.684
8	8-9	0.988	-0.008	0.592	0.238
9	9-10	0.970	0.026	-0.350	-0.725
10	1-10	0.990	0.029	0.521	0.632
11	1-3	0.992	-0.005	0.407	0.593
12	2-10	0.976	-0.107	0.264	0.264
13	3-10	0.994	0.005	0.251	0.018
14	4-10	0.990	0.034	0.182	-0.202
15	3-9	0.978	0.029	-0.157	-0.784
16	4-9	0.974	-0.004	-0.312	-0.655
17	5-9	0.989	0.021	0.860	-0.264
18	4-8	0.992	0.001	-0.506	-0.625
19	5-8	0.963	0.059	0.245	-0.233
20	6-8	0.984	0.042	-0.391	0.705
21	5-7	0.982	0.028	0.660	0.626

The three species showed a tendency in favor of homozygotes, for most of the genes analyzed. Proportional deviations (D), varied from 0.000 in *H. derbyi* to -0.529 in *H. myersi*. However, the different number of individuals sampled among the groups must be taken into account. Except *MDH-1* (P = 0.007) all loci are in the Hardy-Weinberg equilibrium.

The three species of *Hypostomus* analyzed showed clear genetic differences. The loci *AAT-2* and *3*, *ACP-1*, *GDH-1*, *G6PDH-1* and *2*, *IDHP-1* and *2*, *MDH-3*, *ME-1* and *GPI-1* are diagnostic for *H. myersi*, and *GPI-2* is diagnostic for *H. derbyi*. The calculations for Nei's genetic distance (Nei 1978) between species also showed substantial separation (Table 5).

DISCUSSION

Morphometric analysis

The 21 variables examined by principal components analysis showed a clear tendency for separation among the three species studied. Nevertheless, some superposition of the species was observed. This superposition is due more to a limitation of the technique itself in the presence of ontogenetic variations, than to sample similarity, since in PCA the size may be confounded with the form of the organisms. Generally, the first principal component shows the coefficients of structure having the same sign

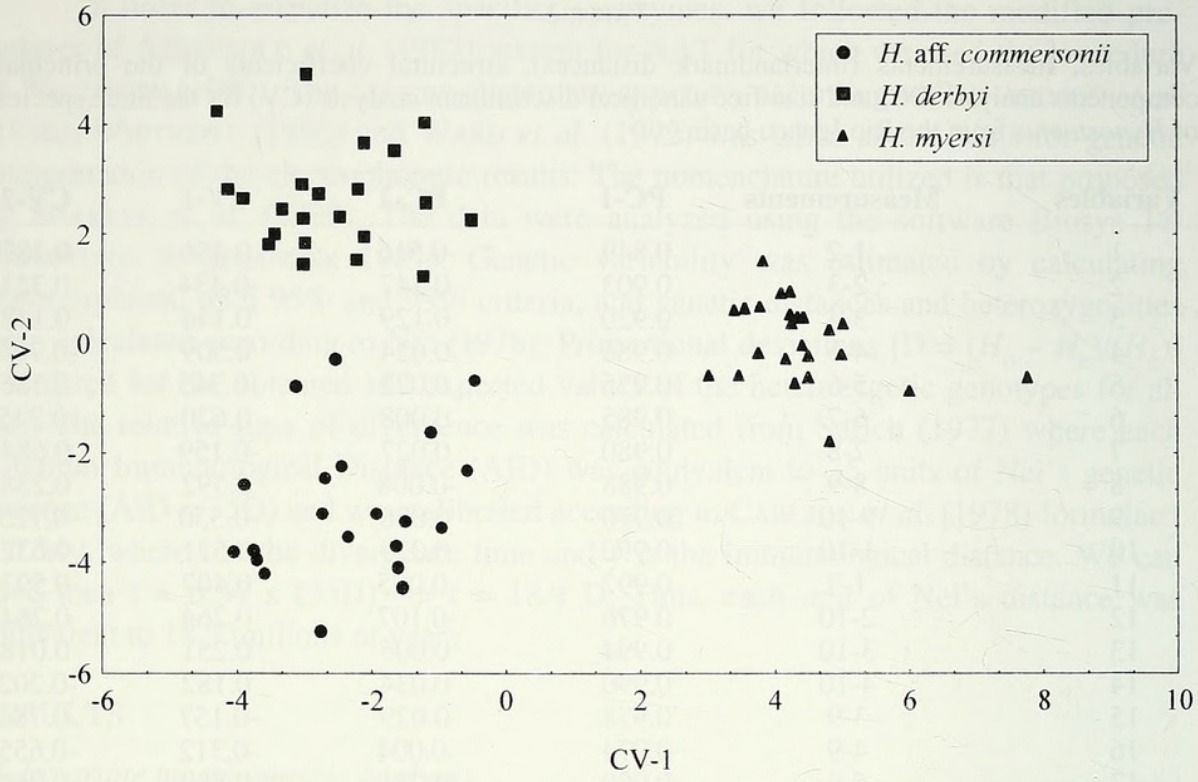

FIG. 3

Graphic representation of the scores of the first (CV-1) and second (CV-2) canonical variables from the three *Hypostomus* species analyzed.

TABLE 3

Allele frequencies for the 26 loci of *Hypostomus* of the Rio Iguaçu basin. N indicates number of individuals analyzed.

Locus	**Allele**	***H.* aff. *commersonii***	***H. derbyi***	***H. myersi***
*AAT-1**	A	1,000	1,000	1,000
*AAT-2**	A	1,000	1,000	—
	B	—	—	1,000
*AAT-3**	A	1,000	1,000	—
	B	—	—	1,000
*ACP-1**	A	1,000	1,000	—
	B	—	—	1,000
*ADH-1**	A	0,100	1,000	—
	B	0,900	—	1,000
*GDH-1**	A	—	—	0,033
	B	—	—	0,967
	C	—	0,036	—
	D	1,000	0,964	—
*GPI-1**	A	—	—	1,000
	B	1,000	1,000	—
*GPI-2**	A	1,000	—	1,000
	B	—	0,929	—
	C	—	0,071	—

*G3PDH-1**	A	1,000	1,000	1,000
*G3PDH-2**	A	1,000	1,000	1,000
*G6PDH-1**	A	—	—	1,000
	B	1,000	1,000	—
*G6PDH-2**	A	—	—	1,000
	B	1,000	1,000	—
*IDHP-1**	A	—	—	1,000
	B	1,000	0,964	—
	C	—	0,036	—
*IDHP-2**	A	—	—	1,000
	B	1,000	1,000	—
*LDH-1**	A	0,100	1,000	—
	B	0,900	—	1,000
*LDH-2**	A	1,000	1,000	1,000
*MDH-1**	A	0,800	1,000	0,933
	B	0,200	—	0,067
*MDH-2**	A	0,900	1,000	1,000
	B	0,100	—	—
*MDH-3**	A	1,000	1,000	—
	B	—	—	1,000
*ME-1**	A	1,000	1,000	—
	B	—	—	1,000
*ME-2**	A	1,000	1,000	1,000
*PGM-1**	A	0,900	—	—
	B	0,100	1,000	0,867
	C	—	—	0,133
*PER-1**	A	1,000	1,000	1,000
*PER-2**	A	1,000	1,000	1,000
*SOD-1**	A	1,000	1,000	1,000
*SOD-2**	A	1,000	1,000	1,000
N		5	14	15

TABLE 4

Statistical analyses of the genetic variation examined for the three species of *Hypostomus*. L, number of loci studied; N, number of individuals examined; AN, mean number of alleles per locus; P0.99 and P0.95, percentages of polymorphic loci using the relaxed and stringent criteria respectively; H_O and H_e, mean observed and expected heterozygosity per locus (non-tendentious estimate of Nei, 1978); D, proportional deviation. Numbers in parentheses refer to respective standard errors.

Variable	*H.* aff. *commersonii*	*H. derbyi*	*H. myersi*
L	26	26	26
N	5	14	15
AN	1.20 (0.10)	1.10 (0.10)	1.10 (0.10)
P0.99	19.23	11.54	11.54
P0.95	19.23	3.85	7.69
H_o	0.031 (0.014)	0.011 (0.007)	0.008 (0.006)
H_e	0.44 (0.019)	0.011 (0.006)	0.017 (0.010)
D	-0.295	0.000	-0.529

TABLE 5

Nei's coefficients of genetic similarity (upper diagonal) and genetic distance (lower diagonal) (NEI 1978) between the species studied. Values in parentheses refer to relative time of divergence of the species, in millions of years (Carlson *et al.* 1978).

Species	*H.* aff. *commersonii*	*H. derbyi*	*H. myersi*
H. aff. *commersonii*	—	0.867	0.538
H. derbyi	0.142 (2,684)	—	0.460
H. myersi	0.621 (11,737)	0.776 (14,666)	—

and similar magnitude, and is therefore interpreted as a variable of general size (NEFF & MARCUS 1980). The remaining principal components possess positive and negative coefficients, and are usually interpreted as containing information relative to form (REIS *et al.* 1987). Because the independence of principal components is a consequence of the mathematical deduction of the method, the relationship of the principal components to size and shape may be arbitrary and therefore without biological meaning (MORRISON 1976; NEFF & MARCUS 1980). For the species studied, the position along the second principal component of the smaller individuals was much different from that of larger individuals, thus indicating a strong tendency toward allometric increments.

According to GOULD (1971, 1975), allometry is an almost universal phenomenon, in ontogenetic development as much as in interspecific scaling of bodily differences among species. BOOKSTEIN *et al.* (1985) commented that discrimination among species must be inherently robust against tendentious samples, in which differences in size frequency are an inevitable aspect.

In order to resolve this problem, several authors have refined the techniques of multivariate morphometry so that the separation of the groups does not suffer the effect of size of the organisms, but any separation results from differences in shape between the groups studied (HUMPHRIES *et al.* 1981; SOMERS 1986; REIS *et al.* 1990b).

Size-free discriminant canonical analysis (REIS *et al.* 1990b) was shown to be efficient when applied to the three species analyzed, clearly demonstrating the separation of the groups obtained primarily by PCA. In Fig. 3 the individual scores in the reduced space of the CV-1 and CV-2 resulted in total separation of the species, demonstrating that when the size effect is eliminated, *H.* aff. *commersonii*, *H. derbyi* and *H. myersi* can be completely distinguished by their shape. The most morphologically divergent *H. myersi*, was discriminated by the others principally by some measures related to length of the body, that are smallers (6-7) length of the caudal peduncule, (8-9) toraxic length and (1-10) length of the head. And *H.* aff. *commersonii* was discriminated by the others principally by having a grater fin dorsal length (4-5).

Genetic variability

From the values of heterozygosities presented in WARD *et al.* (1992) we calculated a mean hetorozygosity using the same 14 enzyme systems assayed in this work.

We found a value of 0.051, which is higher than the values exhibited by the three *Hypostomus* species. However, those authors obtained this value from several previous works on both marine and freshwater fishes. GYLLENSTEN (1985), comparing marine, anadromous and freshwater fishes, found a lower mean heterozygosity for freshwater and anadromous fishes ($H = 0.041$) than for marine fishes ($H = 0.063$). Compared to the mean for freshwater fishes, the heterozygosity values estimated for the three species of *Hypostomus* are still low. In spite of the small number of specimens examined, a good sum of loci, as the 26 scored here, make possible a reliable estimate of the population genetic variability, useful in interspecific comparisons and phylogenetic inferences (SARICH 1977; NEI 1978; GORMAN & RENZI 1979). Several models have been developed to explain the variation in heterozygosity between different groups of organisms (SELANDER 1976; NEVO 1978; SMITH & FUJIO 1982; NEI & GRAUR 1984 and DA SILVA *et al.* 1992). Among these, DA SILVA *et al.* (1992) declared that most variation in protein heterozygosity between different taxa is attributable to the species-protein interaction, where neither the function nor the structure of the enzyme would help to explain such interaction. Because of this, it becomes difficult to compare mean heterozygosities for different species in distinct habitats. According to those authors, it would be necessary to compare species with regard to a single protein, or to compare proteins among individuals of one species.

A possible explanation for the low heterozygosity encountered may be the sedentary mode of life of these species, which contributes to high endogamy. Corroborating this hypothesis are the low values for D (Table 4). According to Nei (1977), high values of heterozygosity and polymorphism are expected in species that occupy a broad niche, in heterogeneous environments, and which have a large population size, lessening the possibility for interbreeding. Considering that these species appear to have evolved in a relatively stable environment, with low gene flow between populations and an apparent high rate of endogamy, natural selection apparently favored low genetic variability as the best strategy to exploit the environment. However, in order to test this hypothesis, further studies on each species would be necessary, analyzing a higher number of individuals in different localities throughout their geographic ranges.

Genetic distance and time of divergence

Nei's coefficients of genetic distance and identity (NEI 1978) indicate that the two most morphologically similar species, *H.* aff. *commersonii* and *H. derbyi*, are also the less genetically divergent (Table 5), while *H. myersi* is shown to be more distant from both of them. Although the value of genetic identity (I) of *H.* aff. *commersonii* and *H. derbyi* (0.867) is at the upper limit of the interval for congeneric species of THORPE (1982), where I varies from about 0.35 to 0.85, the locus *GPI-2* was shown to be fixed for different alleles in the two species, showing that they are really genetically different sympatric species. The identity values of *H. myersi* with these two species agree with Thorpe's values for congeneric species.

From genetic and morphological patterns, some hypotheses can be suggested regarding the origin of the species studied. We can return about the separation of the Rio Iguaçu basin from the rest of the Rio Paraná basin, from which, according to

HASEMAN (1911) and SAMPAIO (1988), the ichthyofauna of the Iguaÿu basin appears to have originated. Geomorphological data are still incomplete, but SEVERI & CORDEIRO (1994) estimated the formation of Iguaçu Falls at about 22 million years ago, and SAMPAIO (1988) affirmed that the isolation of the ichthyofauna of the Iguaçu basin from the rest of the Paraná basin happened in the Oligocene (about 38 million years ago).

Considering the values for genetic distance as a relative measure of time of divergence of the taxa involved (Table 5), the species *H.* aff. *commersonii* and *H. derbyi* have initiated speciation approximately 2.7 million years ago, in the Rio Iguaçu basin, long after the formation of Iguaçu Falls.

Despite many authors as HILLIS (1987) do not agree with SARICH (1977) and CARLSON *et al.* (1978)'s calculations of relative time of divergence based on proteins, we found this approach suitable to elaborate an evolutionary hypothesis, since the estimated time for the separation of the two basins was dated long before the estimated time of divergence between the species of *Hypostomus*. Thus, an ancestral "*derbyi-commersonii*" population in the Rio Paraná drainage was isolated by a vicariance event (the Iguaçu Falls uplift), and later, within the Iguaçu basin, this population underwent speciation, originating *H. derbyi* and *H.* aff. *commersonii*. The hypothesis of the formation of these two species within the Rio Iguaçu basin is strengthened by the statement of SAMPAIO (1988) that the formation of the ichthyofauna of the Rio Iguaçu basin, after its isolation, was not subject to influences from neighboring basins.

There is morphological evidence that, within the genus *Hypostomus*, a "*commersonii*" lineage was broadly distributed through South American hydrographic basins (excluding the Amazonian basin) before these became separated and the present hydrographic configuration was formed. Valenciennes, in CUVIER & VALENCIENNES (1840) described *H. commersonii* based on specimens from Rio de la Plata basin, Montevideo, Uruguay and from Rio São Francisco basin. Later, the type-locality was restricted to the Rio de la Plata (WEBER 1986). GOSLINE (1947) included in the "*commersonii*" group, the species *H. derbyi*, endemic to the Rio Iguaçu (HASEMAN 1911); *H. affinis*, restricted to the Paraíba do Sul basin (MAZZONI *et al.* 1994); and *H. punctatus*, occurring in coastal rivers of the State of Rio de Janeiro (MAZZONI *et al.* 1994). WEBER (1985) compared *H. dlouhyi*, apparently endemic to the Rio Acayary, Paraná basin, and *H. cordovae*, cited by RINGUELET *et al.* (1967) for Argentina and Paraguay to the "*commersonii*" group. Also, EIGEMANN & KENNEDY (1903) *apud* WEBER (1986) noticed in the original description the close relationship of *H. commersonii* to *H. boulengeri*, *H. plecostomus* and *H. scabriceps*.

According to data in literature, the species of a "*commersonii*" group are relatively morphologically conservative even inhabiting different hydrographic basins. However, in spite of its close phenotypic similarity to *H. commersonii* (sensu Valenciennes), *H.* aff. *commersonii* from the Iguaçu basin, has the dorsal fin always reaching the adiposa fin when laying it down (even in the smaller specimens) and this is not seen in *H. commersonii*. Thus, *H.* aff. *commersonii* may have followed an evolutionary path independent from the Rio Paraná populations, to the point of meriting closer investigation of its taxonomic status. Ideally, a revision of the "commersonii" group should be done and its genetics data compared.

Although *H. myersi* have been considered endemic to the Iguaçu basin (GOSLINE 1947) and the calculations for the estimated time of divergence also suggest that *H. myersi* originated within the Iguaçu basin after its isolation. The high number of diagnostic loci separating *H. myersi* from the other two species may lead to an underestimate of genetic distance and time of divergence, since these diagnostic loci may contain more than one change event (mutations), which are not detected by the isoenzyme electrophoresis technique. Besides, the high degree of morphological differentiation observed to the other two species and the cited close relationship to *H. paulinus* from the Rio Paraná basin by GOSLINE (1947) leads to doubts as to whether *H. myersi* originated from a "*derbyi-commersonii*" lineage after isolation of the Rio Iguaçu basin.

Extending electrophoretic studies to other species of the genus *Hypostomus* in the Rio Paraná basin may lead to improved clarification of their phylogenetic relationships. In addition, these data can be compared to data from other groups of fishes, in an attempt to establish a broad and reliable biogeographic hypothesis for the area under study.

ACKNOWLEDGEMENTS

We are grateful to Drs. Horácio F. Júlio Jr, Leda M. K. Sodré de Lima and Claude Weber for valuable suggestions and Carla S. Pavanelli for reviewing the manuscript. We also wish to thank Janet W. Reid for revising the English text.. Logistic support: Nupélia at the State Unviversity of Maringá.

REFERENCES

AEBERSOLD, P.B., G.A. WINANS, D.J. TELL, G.B. MILNER & F.M UTTER. 1987. Manual for starch gel electrophoresis: a method for the detection of genetic variation. *NOAA Technical Report NMFS*, 61: 1-19.

AVISE, J.C. 1974. Systematic value of electrophoretic data. *Systematic Zoology* 23: 465-481.

BOOKSTEIN, F.L., B. CHERNOFF, R.L. ELDER, J.M. HUMPHRIES, G.R. SMITH & R.E. STRAUSS. 1985. Morphometrics in evolutionary biology: the geometry of size and shape change with examples from fishes. *Academy of Natural Sciences of Philadelphia*, Spec. Publ., p.15.

BOYER, S.H., D.C. FAINER & M.A. NAUGHTON. 1963. Myoglobin: inherited structural variation in man. *Science* 140: 1228-1231.

CARLSON, S.S., A.C. WILSON & R.D. MAXSON. 1978. Do Albumin clock Run on Time? *Science* 200: 1182-1185.

CUVIER, G. & A. VALENCIENNES. 1840. Histoire naturelle des poissons. Paris: Ch. Pitois, 540 pp.

DA SILVA, J., G. BELL & A. BURT. 1992. Sources of variance in protein heterozigosity: the importance of the species-protein interaction. *Heredity* 68: 241-252.

GORMAN, G.C. & J. RENZI JR. 1979. Genetic distance and heterozygosity estimates in electrophoretic studies: effects of sample size. *Copeia* 2: 242-249.

GOSLINE, W.A. 1947. Contributions to the classification of the loricariid catfishes. *Arquivos do Museu Nacional do Rio de Janeiro* 41: 79-134.

GOULD, S.J. 1971. Geometric similarity in allometric growth: A contribution to the problem of scaling in the evolution of size. *American Naturalist* 105: 113-136.

GOULD, S.J. 1975. On the scaling of tooth size in mammals. *American Zoologist* 15: 351-362.

GYLLENSTEN, U. 1985. The genetic structure of fish: differences in the intraspecific distribution of biochemical genetic variation between marine, anadromous, and freshwater species. *Journal of Fish Biology* 26: 691-699.

HASEMAN, J.D. 1911. Some new species of fishes from the rio Iguassú. *Annals of Carnegie Museum* 7(3-4): 374-387.

HEDGECOCK, D., E.S. HUTCHINSON, G. LI, F.L. SLY & K. NELSON. 1989. Genetic and morphometric variation in the pacific sardine, *Sardinops sagax caerulea*: comparisons and contrasts with historical data and with variability in the northern anchovy, *Engraulis mordax*. *Fishery Bulletin* 87(3): 653-671.

HILLIS, D.M. 1987. Molecular versus morphological approaches to systematics. *Annual Review of Ecology and Systematics* 18: 23-42.

HUMPHRIES, J.M., F.L. BOOKSTEIN, B. CHERNOFF, G.R. SMITH, R.L. ELDER & S.G. POSS. 1981. Multivariate discrimination by shape in relation to size. *Systematic Zoology* 30: 291-308.

HUNTER, R.L. & C.L. MARKERT. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science* 125: 1294-1295.

JULIO JR, H.F., C.C. BONECKER & A.A. AGOSTINHO. 1997. Reservatório de Segredo e sua inserção na bacia do rio Iguaçu. *In*: Reservatório de Segredo: bases ecológicas para o manejo, (Agostinho, A.A. & L.C. Gomes, eds). *EDUEM, Maringá-PR*, pp.1-17.

LOPEZ-LEMUS, L.G. 1991. Systematic studies of the *Opistonema* species complex (Pisces: Clupeidae): statistical multivariant analysis of external morphology, genetic relationship and speciation. *In*: Trends in Ichthyology, (Schröder, J.H., J. Bauer & M. Schartl, eds). *GSF-Forschungszentrum, Neuherberg*, 213-236 pp.

MAZZONI, R., U. CARAMASCHI & C. WEBER. 1994. Taxonomical revision of the species of *Hypostomus* Lacépède, 1803 (Siluriformes, Loricariidae) from the Lower rio Paraíba do Sul, State of Rio de Janeiro, Brazil. *Revue suisse de Zoologie* 10(1): 3-18.

MORRISON, D.F. 1976. Multivariate statistical methods. *McGraw-Hill*, New York, 415 pp.

NEFF, N.A. & L.F. MARCUS. 1980. A survey for multivariate methods for systematics. *Privately published, New York*, 1980.

NEI, M. 1977. F-statistics and analysis of gene diversity in subdivided populations. *Annals of Human Genetics* 41: 225-233.

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

NEI, M. & D. GRAUR. 1984. Extent of protein polymorphism and the neutral mutation theory. *Evolutionary Biology* 17: 73-118.

NEVO, E. 1978. Genetic variation in natural populations: patterns and theory. *Theoretical of Populational Biology* 13: 121-177.

O'MAOILEIDIGH, N., S. CAWDERY, J.J. BRACKEN & A. FERGUSON. 1988. A. Morphometric, meristic character and electrophoretic analyses of two Irish populations of twaite shad, *Alosa fallax* (Lacépède). *Journal of Fish Biology* 32: 355-366.

PASTEUR, N., G. PASTEUR, F. BONHOMME, J. CATALAN & J. BRITTON-DAVIDIAN.1988. Practical isozyme genetics. *Ellis Horwood Limited, Chichester*, 215pp.

REIS, R.E.; C. WEBER & L.R. MALABARBA. 1990a. Review of the genus *Hypostomus* Lacépède, 1803 from Southern Brazil, with descriptions of three new species (Pisces, Siluriformes, Loricariidae). *Revue suisse de Zoologie* 97: 729-766.

REIS, S.F., R.A. CUNHA, J.C. GARAVELLO & A.S. ABE. 1987. Discriminação pela forma em relação ao tamanho: um exemplo com peixes do gênero *Leporinus*. *Ciência e Cultura* 39: 757-761.

REIS, S.F., L.M. PESSÔA & R.E. STRAUSS. 1990b. Application of size-free canonical discriminant analysis to studies of geographic differentiation. *Revista Brasileira de Genética* 13(3): 509-520.

RICHARDSON, B.J., P.R. BAVERSTOCK & M. ADAMS. 1986. Allozyme electrophoresis: a handbook for animal systematics and population studies. *Academic Press, Sydney*, 410 pp.

RINGUELET, R.A., R.H. ARAMBURU & A.A. ARAMBURU. 1967. Los peces argentinos de agua dulce. *Comision de Investigacion Cientifica, Buenos Aires*, 602 pp.

ROBY, D., J.D. LAMBERT & J.M. SÉVIGNY. 1991. Morphometric and electrophoretic approaches to discrimination of capelin (*Mallotus villosus*) populations in the Estuary and Gulf of St. Lawrence. *Canadian Journal of Fishery and Aquatic Science* 48: 2040-2050.

SAMPAIO, F.A.A. 1988. Estudos taxonômicos preliminares dos Characiformes (Teleostei, Ostariophysi) da bacia do rio Iguaçu, com comentários sobre endemismo dessa fauna. *UFSCAR, São Carlos*, 175 pp. Thesis (Master degree).

SARICH, V.M. 1977. Rates, sample sizes, and the neutrality hypothesis for electrophoresis in evolutionary studies. *Nature* 265: 24-28.

SELANDER, R.K. 1976. Genetic variation in natural populations. *In:* Molecular Evolution. (AYALA, F.J., eds). *Sinauer, Sunderland, Massachusetts*, pp. 21-45.

SEVERI, W. & A.A.M. CORDEIRO. 1994. Catálogo de peixes da bacia do rio Iguaçu. *IAP/GTZ, Curitiba*, 128 pp.

SHAKLEE, J.B., F.W. ALLENDORF, D.C. MORIZOT & G.S WHITT. 1990. Gene Nomenclature for Protein-Coding Loci in Fish. *Transactions of American Fishery Society* 119: 2-15.

SHAKLEE, J.B. & C.S. TAMARU. 1981. Biochemical and morphological evolution of Hawaiian bonefishes (*Albula*). *Systematic Zoology* 30(2): 125-146.

SHAW, C.R. & R. PRASAD. 1970. Starch gel electrophoresis of enzymes - A compilation of recipes. *Biochemical Genetics* 4: 297-320.

SMITH, P.J. & Y. FUJIO. 1982. Genetic variation in marine teleosts: high variability in habitat specialists and low variability in habitat generalists. *Marine Biology* 69: 7-20.

SOMERS, K.M. 1986. Multivariate allometry and removal of size with principal components analysis. *Systematic Zoology* 35: 359-368.

STRAUSS, R.E. 1985. Evolutionary allometry and variation in body form in the South American catfish *Corydoras* (Calichthyidae). *Systematic Zoology* 34: 381-396.

STRAUSS, R.E. & F.L. BOOKSTEIN. 1982. The truss: body form reconstructions in morphometrics. *Systematic Zoology* 31: 113-135.

SWOFFORD, D.L. & R.B. SELANDER. 1981. BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *Journal of Heredity* 72: 281-283.

THORPE, J.P. 1982. The molecular clock hypothesis: biochemical evolution, genetic differentiation and systematics. *Annual Review of Ecology and Systematics* 13: 139-168.

THORPE, J.P. & A.M. SOLÉ-CAVA. 1994. The use of allozyme electrophoresis in invertebrate systematics. *Zoological Scripta* 23: 3-18.

WARD, R.D., D.O.F. SKIBINSKI & M. WOODWARD. 1992. Protein heterozygosity, protein structure, and taxonomic differentiation. *Evolutionary Biology* 26: 73-159.

WEBER, C. 1985. *Hypostomus dlouhyi*, nouvelle espèce de poisson-chat cuirassé du Paraguay (Pisces, Siluriformes, Loricariidae). *Revue suisse de Zoologie* 92: 955-968.

WEBER, C. 1986. Revision de *Hypostomus boulengeri* (Eigenmann & Kennedy) et deux espèces nouvelles de poissons-chats du Paraguay (Pisces, Siluriformes, Loricariidae). *Revue suisse de Zoologie* 93: 979-1007.

WHITMORE, D.H. 1990. Electrophoretic and isoelectric focusing techniques in fisheries management. *CRC Press, Boca Raton, Florida*, 350 pp.