

Intraspecific genetic differentiation of *Leporinus friderici* (Anostomidae, Pisces) in French Guiana and Brazil: a genetic approach to the refuge theory

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A genetic study was carried out on a neotropical fish species to illustrate the refuge theory, whose main principles are summarized. The geographical structure of the anostomid species *Leporinus friderici* was analysed in French Guiana and Brazil by horizontal starch gel electrophoresis. Fifteen enzymatic systems corresponding to 21 loci were studied, revealing the following particularities: (1) specimens of fish from six independent coastal rivers of French Guiana form two groups geographically situated on either side of the Kourou River; (2) two alleles can serve as geographic markers, *Ldh2*(130) showing an affinity between the populations in eastern French Guiana and Brazil, and *Mel*(300) being specific to the west.

These observations are probably related to the refuges that were formed in the Quaternary in South America, especially on the Guiana Shield. We consider that the populations of French Guiana are separated into two groups at the boundary between the two migratory flows, one arising in the west from the refuge of the Guiana Shield and the other in the east from a nearby undetermined Amazonian refuge.

Key words: biochemical genetics; refuge theory; anostomid fish; neotropical ecosystem; South America; French Guiana.

I. INTRODUCTION

The neotropical region shows an extraordinary abundance of plant and animal species, including fish. Until the end of the sixties, it was generally assumed that neotropical species diversity arose from the presumed ecological stability of the forests (Darlington, 1957; Schwabe, 1968; Simpson, 1969). More recently, Haffer (1982) pointed out several complementary theories based on the instability and heterogeneity of the Amazonian environment, as follows.

According to island theory, hyperspeciation arose from multiple isolations due to changes in uplifted land and ocean inlets during large orogenic episodes, variations in sea level, and Tertiary climatic disruptions. This theory stipulates that no speciation took place in the Quaternary, during which these environments were stable in this respect (e.g. Emsley, 1965, Croizat, 1976).

According to the river theory, the speciations were caused by the formation of large rivers during the Quaternary. Because of their width, they acted as barriers between the populations on opposite banks. This theory has been referred to by many authors but has never been clearly developed. Without actually refuting the theory, Haffer (1982) considered it unable to explain current Amazonian fauna.

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Thirdly, refuge theory was first mentioned by Gentili (1949) and was then developed by Ab'Saber (1977), Simpson & Haffer (1978) and Brown & Ab'Saber (1979). A synthesis was proposed in 1982 (Haffer, 1982; Weitzman *et al.*, 1982; Livingstone, 1982; Endler, 1982). This theory proposes that climatic fluctuations in the Quaternary, involving alternating glacial and interglacial epochs in temperate latitudes, also could have affected the neotropical regions. It states that during cold dry periods, the forest cover was replaced by open vegetation (savannas). Only several remnant forests constituted refuge areas for species living in these ecosystems. These species were thereby fragmented into allopatric populations that diverged to the point of speciation. In warm wet periods, the refuge areas recovered ground lost to open vegetation, until they coalesced and reformed the homogeneous forest cover existing today. During this wet phase, the areas of dry vegetation constituted refuges for certain species. The repetition of these phenomena was the cause of current species diversity.

One of these forest refuges is the Guiana Shield. The works of Haffer (1969) on birds, Prance (1973) on plants, and Vanzolini & Williams (1970) and Vanzolini (1973) on reptiles show that there was a forest refuge there in the Upper Pleistocene. On the other hand, Weitzman & Weitzman (1982) studied two characoid fish genera that are strictly Amazonian and forest-related, *Carnegiella* and *Nannostomus*, and established that the formation of species in these genera appears to be independent of known refuges. Only their intraspecific differentiation could have been related to forest refuges. Considering these findings, it would appear that in certain fishes more or less related to the forest ecosystem, intraspecific differentiation may be the level of organization retaining traces of the influence of Quaternary refuges.

Genetic markers such as isoenzymes can reveal this influence within a species. We chose the species *Leporinus friderici* for the present study. The genus *Leporinus* is widely distributed in South America, comprising about 70 species (Garavello, 1979). Renno *et al.* (in press) have demonstrated reproductive isolation between close species of French Guiana belonging to this genus (*L. friderici*, *L. granii*, *L. aff. steyermarki*, and *L. lebailli*). *L. friderici* is found in the rivers of the Guiana Shield and in the basins of the Amazon, the Parana (Nomura, 1984), and the Orinoco (Mago-Lecchia, 1971).

Samples from six areas in French Guiana and two in Brazil were studied by electrophoresis of enzyme proteins to define the genetic structure of the species. The objective of this study centred in French Guiana was to investigate the influence the Guiana refuge may have had on the recent genetic differentiation of the species.

II. MATERIALS AND METHODS

SAMPLING

Between January 1987 and June 1988, specimens were taken from six main river basins of French Guiana (Oyapock, Approuague, Comté, Sinnamary, Iracoubo, Mana), Lake Januaca (Brazil) connected to the Solimoes River in the Amazon basin, and the Mongi-Guaçu River (Brazil) in the Parana basin (Fig. 1). Adult fish were caught with trammels (30–60 mm mesh), and young fish were obtained by poisoning small streams with rotenone. The protocol was adapted from Boujard & Rojas-Beltran (in press).

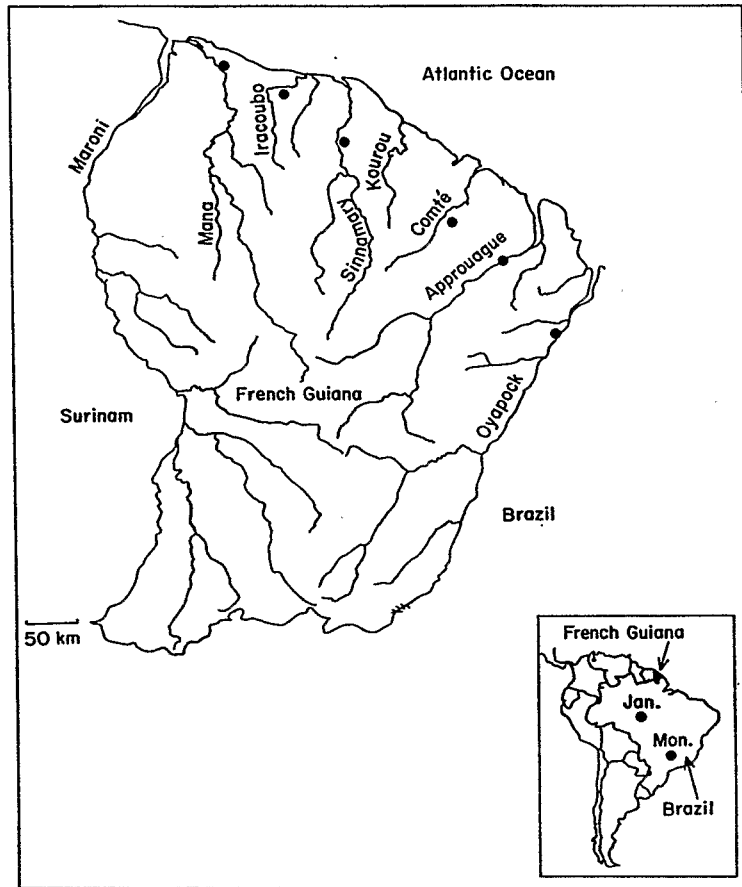


FIG. 1. Sampling points for *Leporinus friderici* in French Guiana and in Brazil. Jan.: Lake Januaca; Mon.: Mongi-Guacu River.

DETERMINATION

Fishes were identified according to the keys established by Géry (1977, unpubl. data) and Le Bail *et al.* (1984). The morphomeric characters, the number of scales from top to bottom, along the lateral line, and around the caudal peduncle, as well as the colouring (distribution of black and red patterns), were the most practical criteria for determination.

ISOENZYME ANALYSES

A sample (1–2 g) of muscle and liver from each fish was removed and placed in liquid nitrogen for transfer to the laboratory where it was stored at -40°C . The protocol for enzyme electrophoresis on horizontal starch gel was adapted from Guyomard & Krieg (1982) and Pasteur *et al.* (1987). The enzyme systems studied are shown in Table I; the nomenclature is that described by Renno *et al.* (in press).

III. RESULTS

In all samples, the 21 loci producing 53 allelomorphs were counted and allelic frequencies were calculated (Table II). Using the χ^2 method, the Hardy–Weinberg structure was tested at all loci in the samples from French Guiana and the Parana.

TABLE I. Enzymatic systems and corresponding migration buffers. A, morpholine-citrate buffer (MC2) (after Clayton & Tretiak, 1972); B, tris-(hydroxymethyl)-aminoethane 0.1 M adjusted to pH 7.4 with NaH₂PO₄ (TPO₄); C, discontinued buffer tris-citrate-borate (RID) (after Ridway *et al.*, 1970). The molecular structure (monomeric, dimeric or tetrameric) is deduced from the phenotype of the heterozygote

Enzymatic systems	Loci	Buffer	'Zygote'	Bands	Molecule	Comments
Aspartate aminotransferase	<i>Aat1(m)</i>	A	homo.	1	?	
	<i>Aat2(l)</i>	A	homo.	1	?	
α -Glycerophosphate dehydrogenase	<i>aGpdh(m)</i>	B	homo.	1	?	4 echo bands
Adenylate kinase	<i>Ak(m)</i>	A	homo.	1	?	
Creatine phosphate Esterase	<i>Cpk(m)</i>	C	homo.	1	?	
	<i>Est(m)</i>	C	homo.	1	monomeric	
			hetero.	1		
Fumarase	<i>Fum(m)</i>	A	homo.	2	tetrameric	Twice, 5 merged bands
			hetero.	10		
Isocitrate dehydrogenase	<i>Idh1(m)</i>	B	homo.	1	dimeric	
			hetero.	3		
	<i>Idh2(l)</i>	A	homo.	1	dimeric	
			hetero.	3		
Lactate dehydrogenase	<i>Ldh1(m)</i>	A	homo.	1	?	
	<i>Ldh2(l)</i>	A	homo.	1	tetrameric	
			hetero.	5		
Malate dehydrogenase	<i>Mdh1(l)</i>	A	homo.	2	?	merged bands
	<i>Mdh2(m)</i>	A	homo.	1	?	
Malic enzyme	<i>Me1(l)</i>	A	homo.	1	tetrameric	merged bands
			hetero.	5		
	<i>Me2(m)</i>	A	homo.	1	tetrameric	merged bands
			hetero.	5		merged bands
6-Phosphogluconate dehydrogenase	<i>6Pgdh(l)</i>	A	homo.	1	dimeric	
			hetero.	3		
Phosphoglucose isomerase	<i>Pgi1(m)</i>	C	homo.	1	dimeric	
			hetero.	3		
	<i>Pgi2(l)</i>	C	homo.	1	dimeric	
			hetero.	3		
Phosphoglucomutase	<i>Pgm(m)</i>	C	homo.	1	monomeric	
			hetero.	1		
Phosphomannose isomerase	<i>Pmi(m)</i>	B	homo.	1	?	
Superoxide dismutase	<i>Sod(l)</i>	C	homo.	1	dimeric	
			hetero.	3		

The test showed no deviation from panmixia, indicating that each was taken from a single population. The test could not be used with the Januaca samples because of their small size. Thus, for convenience, the term 'population' can be used to designate the samples.

Nei genetic distances (Nei, 1977) were calculated (Table III) and a UPGMA dendrogram (Fig. 2) was constructed. Three levels of differentiation appeared: (1) the populations of the Sinnamary, Iracoubo, and Mana Rivers were separated from those of the Oyapock, Approuague, and Comté Rivers at a distance of 0.029; (2) the populations of French Guiana were separated from those of Brazil at a distance of 0.222; (3) the two Brazilian populations were separated at a distance of 0.239.

Concerning the geographic distribution of the alleles, the populations of the Sinnamary, Iracoubo, and Mana Rivers (western French Guiana) had allele *Me1*(300) with high frequencies (0.22–0.26) but not allele *Ldh2*(130), whereas the populations of the Oyapock, Approuague, and Comté Rivers (to the east) had allele *Ldh2*(130) with high frequencies (0.29–0.49), but not allele *Me1*(300). The populations of Brazilian rivers had allele *Ldh*(130) with high frequencies (0.46–1.00) but not allele *Me1*(300).

These allelic peculiarities link the populations in the rivers of eastern French Guiana and Brazil, and separate the populations in the eastern rivers from those in the western rivers. It should be noted that a number of alleles were limited to Brazil (at loci *aGpdh*, *Idh1*, *Ldh1*, *Me1*, *Me2*, *6Pgdh*, and *Sod*).

The allelic difference between eastern and western French Guiana appears to be correlated with the level of heterozygosity (Table II) which doubles between the eastern rivers ($0.04 > H > 0.07$) and western rivers ($0.10 > H > 0.11$). The break in allelic frequencies and heterozygosity is located in both cases between the Comté and Sinnamary Rivers, at the Kourou River, where *L. friderici* appears to be absent.

IV. DISCUSSION AND CONCLUSION

According to refuge theory (Haffer, 1969, 1974, 1982; Simpson & Haffer, 1978; Vanzolini & Williams, 1970; Vuilleumier, 1971), tropical ecosystems were periodically broken up into refuge areas during paleoclimatic cycles. In periods of forest regression, different populations of the same species became isolated, separated by impassable zones. Allopatric differentiation then developed, which sometimes reached a level sufficient for reproductive isolation (speciation) depending on (i) the biological peculiarities of the species (especially its evolutionary rate) and (ii) the length of separation. When these refuges spread and again merged (the current state of the Amazonian forest) these isolated populations followed the movement.

The disappearance of refuge areas was clearly not instantaneous. Contacts between refuges were partial and localized at first, imposing migration directions for the species belonging to these ecosystems. The consequences of these phenomena were investigated in the present study.

By considering the intraspecific genetic variations of *L. friderici*, we deliberately chose a situation in which there is no speciation. Our findings are as follows.

(i) The genetic distances indicate a closer resemblance between the populations of eastern and western French Guiana than between those of western French

TABLE II. The allelic frequencies observed in the eight populations of *Leporinus friderici* from the Mongi-Guaçu (Mon.), Oyapock (Oya.), Approuague (App.), Comté (Com.), Sinnamary (Sin.), Iracoubo (Ira.), Mana (Man.) Rivers and Lake Januaca (Jan.). The number of observed alleles is given in parentheses

Loci Allele	Mon.	Jan.	Oya.	App.	Com.	Sin.	Ira.	Man.
<i>Aat1</i> 100	1 (26)	1 (16)	1 (40)	1 (44)	1 (104)	1 (98)	1 (80)	1 (120)
<i>Aat2</i> 100	1 (16)	1 (6)	1 (34)	1 (28)	1 (104)	1 (20)	1 (20)	1 (162)
<i>Gpdh</i> 090	0	0.33	0	0	0	0	0	0
100	1 (40)	0.67 (6)	1 (42)	1 (70)	1 (104)	1 (92)	1 (62)	1 (202)
<i>Ak</i> 100	1 (30)	1 (14)	1 (42)	1 (38)	1	1 (104)	1 (80)	1 (172)
<i>Ck</i> 100	1 (48)	1 (14)	1 (42)	1 (68)	1 (104)	1 (104)	1 (80)	1 (194)
<i>Est</i> 095	0	0	0	0.04	0	0.01	0.11	0.03
100	1 (4)	1 (8)	1 (42)	0.96 (46)	1 (78)	0.99 (94)	0.90 (76)	0.97 (166)
<i>Fum</i> 100	1 (48)	1 (6)	1 (42)	1 (62)	1 (104)	1 (84)	1 (70)	1 (214)
<i>Idh1</i> 100	0	1	1	1	1	0.95	1	1
150	1	0	0	0	0	0.01	0	0
200	0 (36)	0 (14)	0 (42)	0 (70)	0 (104)	0.04 (92)	0 (80)	0 (214)
<i>Idhf</i> 025	0	0	0	0	0	0	0.08	0.02
050	0	0	0	0	0	0	0	0.02
100	1	1	1	1	1	1	0.89	0.94
150	0 (42)	0 (14)	0 (16)	0 (48)	0 (48)	0 (88)	0.04 (26)	0.02 (92)
<i>Ldh1</i> 000	0.02	0	0.05	0	0	0	0	0
100	0	0.06	0.95	1	1	1	1	1
125	0.98 (48)	0.94 (16)	0 (38)	0 (70)	0 (104)	0 (98)	0 (62)	0 (110)
<i>Ldh2</i> 100	0.55	0	0.71	0.52	0.60	1	1	1
130	0.46 (44)	1 (16)	0.29 (38)	0.49 (68)	0.40 (104)	0 (98)	0 (80)	0 (222)
<i>Mdh1</i> 100	1 (44)	1 (46)	1 (42)	1 (70)	1 (104)	1 (96)	1 (80)	1 (224)
<i>Mdh2</i> 100	1 (44)	1 (16)	1 (42)	1 (70)	1 (104)	1 (96)	1 (80)	1 (206)
<i>Mel</i> 000	0	0.33	0	0	0	0	0	0
100	1	0.50	1	1	1	0.74	0.78	0.77
110	0	0.17	0	0	0	0	0	0
300	0 (14)	0 (12)	0 (42)	0 (70)	0 (104)	0.26 (46)	0.22 (50)	0.23 (214)
<i>Me2</i> 100	1	0	1	1	1	1	1	1
130	0	0.14	0	0	0	0	0	0
140	0	0.43	0	0	0	0	0	0
400	0 (16)	0.43 (14)	0 (32)	0 (70)	0 (94)	0 (86)	0 (80)	0 (220)

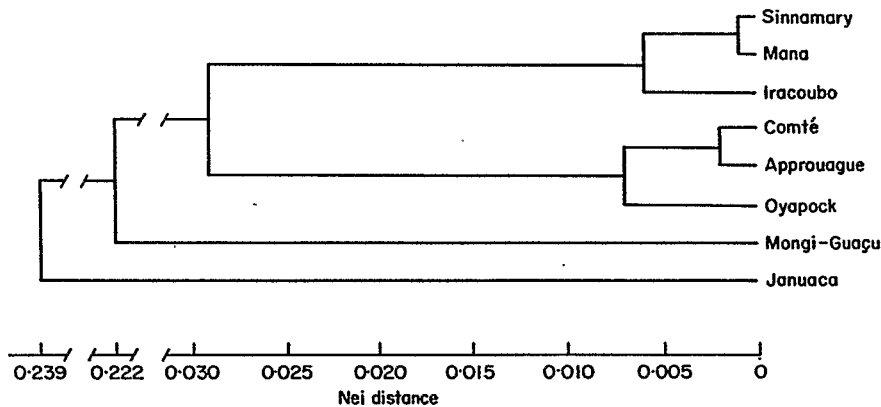


FIG. 2. Dendrogram of linkages, by Nei distances, among the *Leporinus friderici* populations from eight river systems in French Guiana and Brazil (UPGMA).

Guiana and Brazil. This appears to comply with the obvious geographical situation, i.e. the populations of French Guiana are separated by only several tens of kilometres, whereas the Brazilian populations are at least 1500 km away.

(ii) The separation of the Guianese populations defined by the dendrogram of genetic distance, the level of heterozygosity, and the distribution of the two marker alleles always occurs as on either side of the Kourou region.

(iii) The presence of alleles *Ldh2*(130) and *Me1*(300) in certain populations is far from random and we consider that they constitute markers of the paleogeographic history of the species linking eastern French Guianese and Brazilian populations.

De Panepucci *et al.* (1984, 1987) have shown variations of the allelic frequencies at a locus of lactate dehydrogenase (probably the locus designated here as *Ldh2*) in *L. friderici* over a period of 4 years. According to these authors, the variations have an adaptive character with respect to the thermal conditions of the environment. However, this hypothesis was formulated in the context of an experimental protocol (*in vitro* at temperatures exceeding 45° C) that was quite different from past or present natural conditions. Moreover, the presence of allele *Ldh2*(130) in Brazil and eastern French Guiana and its absence in western French Guiana cannot be explained by ecological evidence, or (even less so) by heat conditions.

The bipartition of the two groups of Guianese populations reflects a recent separation of two allopatric populations. The low rate of heterozygosity in the eastern populations indicates that they are colonizers, whereas the western populations appear to be more indigenous and therefore genetically closer to the inhabitants of the Guiana Shield refuge, whose geometric centre is to the west of French Guiana.

The relatively large genetic distance between the populations of Guiana and Brazil suggests a much older separation. Nevertheless, since the Amazonian populations show the same high frequencies of allele *Ldh2*(130) as the populations of eastern Guiana, it is probable that the latter originate from a North-Amazonian refuge contiguous with the watersheds of the Amazon and south-eastern Guiana. Lescure (1975) and Descamps *et al.* (1976) have observed a faunistic similarity between the eastern Guiana Shield and Brazil.

Thus, populations originating from a former nearby Amazonian refuge could have colonized the Guiana Shield via its eastern part, perhaps by the marsh belt along the coastline. In contrast, the populations of western French Guiana could be the remnants of a former Quaternary refuge to the west of the Kourou region.

Géry *et al.* (in press) have proposed separating *L. friderici* into two subspecies: *L. f. friderici*, found everywhere on the Guiana Shield except for the Oyapock river basin, and *L. f. acutidens*, found in the Oyapock, Amazon, and Parana river basins. The distribution of these subspecies partly coincide with the distribution of the electrophoretic markers.

In Brazil, reproduction is seasonal and displacements of mature adults between the river and the flooded forest have been observed (Boujard *et al.*, 1988). Their omnivorous diet consists mainly of plants and fallen fruit (Goulding, 1980; Santos, 1982). In the dry season, when the plants along the banks are no longer accessible, the rate of stomach filling in *L. friderici* drops to almost zero, which indicates that the species eats practically nothing while waiting for the next floodwaters to make the forest accessible (Boujard *et al.*, in press). If there is a strict dependence between fish and forest, a fragmentation of the forest could lead to a fragmentation of the species.

But even if the fish-forest relationship is not strict, the formation of refuges has a strong influence on the distribution of aquatic species. The formation of a forest refuge is related to topographic features (altitude, exposure, distance from the sea) which directly affect the density and extent of the drainage system. In interglacial periods (wet), the drainage system is dense, with connections between the river basins. At present, for instance, anastomoses link the main watersheds of South America: between the Orinoco in Venezuela and the Rio Negro in the Amazon basin (Mago-Leccia, 1971); between the Rio Branco in the Amazon basin and the Essequibo River in Guiana (McConnel, 1964); between the Rio Guapore in the Amazon basin and the Rio Paraguay; and between the Rio Tocantins in the Amazon basin and the Rio Parana. Smaller, less noticeable connections probably form temporarily between smaller river basins when rainfall is heavy. This situation probably favours the dispersion of aquatic species. In contrast, during glacial periods (dry) that favour the speciation of forest species, the drainage system shrinks and the connections between the river basins disappear. The aquatic populations are then in isolated situations that favour speciation. Thus, the same causes (paleoclimatic variations) can have the same effects on all forest and aquatic species, and a forest refuge can also coincide with an aquatic refuge.

The correlation between particular topographic features and the formation of refuges results in a geographic stability of refuges, which can appear and disappear during climatic variations, until a major orographic event disturbs the topography. This explains why these refuges have affected the Amazonian populations at different levels, i.e. at the species level, for refuges stable over long periods of time, and at the intraspecific level, for short-lived ones. The refuge which promoted the actual intraspecific genetic divergence of *L. friderici* was probably in operation during the last ice age. It was located at the same place as older ones which may have functioned off and on as early as the formation of the Guiana Shield. The most durable of these refuges induced, at different times, the speciation events which are the source of the present extraordinary specific diversity.

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