Polymorphism of mitochondrial genes in populations of *Leporinus friderici* (Bloch, 1794): intraspecific structure and zoogeography of the Neotropical fish

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Abstract

On the basis of faunistic and floristic inventories, various authors have developed the forest refuge theory to explain species diversity in humid tropical regions. Renno *et al.* (1990) used electrophoretic markers to study the genetic structure of *Leporinus friderici* and suggested the existence of an aquatic refuge on the Guiana shield. In the present study, mitochondrial markers (RFLP) confirmed and complemented the previous electrophoretic study. Four multimorphs were evidenced, allowing the populations to be separated into two sets on either side of the eastern edge of the Guiana refuge, i.e. the Kourou river region in French Guiana.

Introduction

The current state of the tropical forest ecosystems is not sufficient to explain the extensive biological diversification in these regions. Indeed, according to the forest refuge theory, proposed by Haffer (1969, 1978, 1982) and developed by Brown (1975), Brown and Ab'Sabbar (1979), Weitzman and Weitzman (1982), Livingstone (1982), and Endler (1982), the organization of humid tropical ecosystems is the result of paleoecological disturbances. The theory proposes that during climatic variations in the Quaternary, glacial periods at extreme latitudes were reflected in tropical regions by a climate that was colder and drier than at present. The forest was broken up into patches separated by savanna, which constituted refuges for many populations. In contrast, the interglacial periods resulted in higher temperatures and humidity in the tropical regions, allowing extension of the forest and the species inhabiting it, and favoring the isolation of savanna populations. Hence, the tropical forest underwent successive cycles of extension and reduction corresponding to rainfall variations. The formation of forest refuges, allowing allopatric speciation, is thus considered to be one of the main causes of species diversity in humid tropical ecosystems.

Until recently, the study of refuges was mainly based on inventories of species belonging to very different groups, i.e. birds (Haffer, 1969), plants (Prance, 1973; De Granville, 1982), reptiles (Vanzolini & Williams, 1970; Vanzolini, 1973), butterflies (Brown, 1975), batrachians (Lescure, 1975; Descamp *et al.*, 1978), and fishes (Weitzman & Weitzman, 1982). There is generally a close geographic correspondence between refuges, and differences of form and surface according to the group can be attributed to differences in the ecological preferences of the species studied. Few studies have been devoted to the effect of Neotropical refuges on the genetic structure of the species. Renno *et al.* (1989) carried out a multispecies genetic study in French Guiana, to verify the reproductive isolation of species in the genus *Leporinus*, which are morphologically very similar. The biological species status of *L. friderici* was thereby confirmed before undertaking an intraspecific genetic study. The genetic structure of this species could have been formed by an aquatic refuge, coinciding with a forest refuge described by other authors, on the Guiana shield west of the Kourou river region (Renno *et al.*, 1990). However, only a small number of electro-

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phoretic markers were used to determine the intra-specific structure.

In recent years, the development of molecular methods has provided new information concerning the genetic structure of species. For instance, mito-chonrial DNA, which shows high polymorphism and a high rate of clonal evolution in certain groups (Brown *et al.*, 1979), has furnished markers that are very useful in phylogeographic studies involving the reconstruction of recent biogeographic history leading to the current genetic structure of species (Boursot & Bonhomme, 1986). Avise and Vrijenhoek (1987) considered that as a general rule, if two populations show allozymic divergences, it is highly probable that they have fixed differences at the many sites of their mitochondrial DNA.

In the fishes, intraspecific differentiation, as re-vealed by electrophoretic variants, is more pronounced in strictly freshwater species than in anadromous species, and is more pronounced in anadromous species than in marine species (Gyllensten, 1985). This was also observed by Avise (1989), who used mito-chondrial markers. The genetic structure of a species is thus highly dependent on its possiblities for dis-persion, which affect the intensity and duration of genetic isolation of populations. *L. friderici* populati-ons isolated in different watersheds could have been in contact during the paleoclimatic variations of the Quaternary, and their divergence may thus be recent. Consequently, we considered that a study of the restriction fragment length polymorphism (RFLP) of their mitochondrial DNA would be of great value in confirming and complementing the description of the genetic structure of this species.
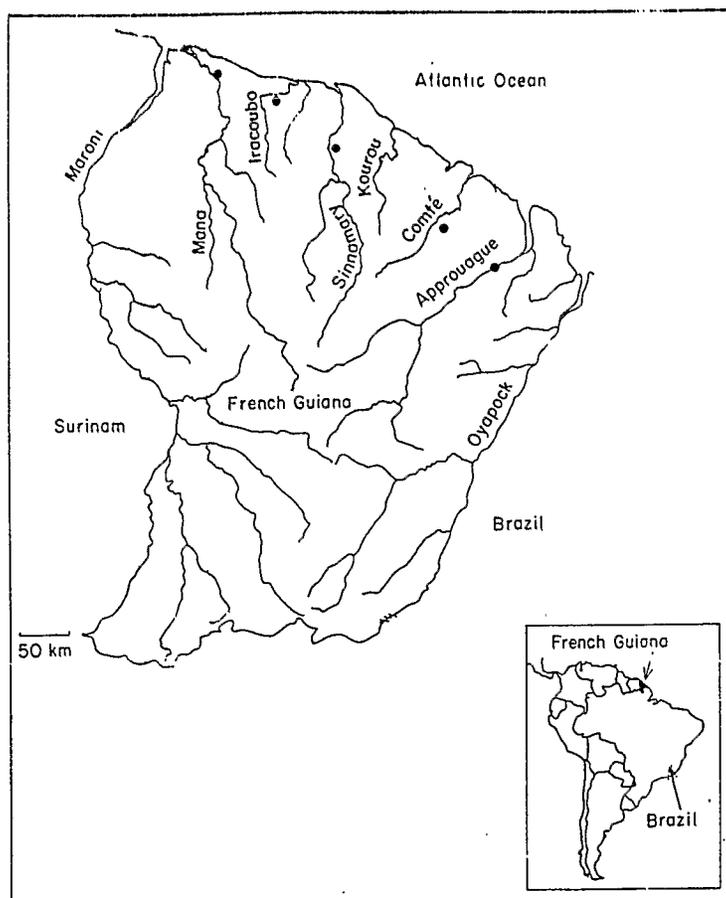
Fig. 1. Distribution of specimens in French Guiana.

Materials and methods

Specimens of *L. friderici* were captured in 5 rivers of Guiana located on either side of the Kourou river, i.e. the Mana, Iracoubo, and Sinnamary to the west, and the Comté and Approuague to the east (Fig. 1). About 5 g of liver or ova was removed from each fish immediately following death. Samples were stored for several months in liquid nitrogen. During air transport, they were kept in dry ice.

A 'rapid method' for extraction and revelation with ethidium bromide has been developed in the mouse (Boursot *et al.*, 1987). It was adapted to the study of marine fish by Blanquer (1990) after comparing different extraction methods. We used this final adaptation for analyzing RFLP of the mtDNA in our samples.

Results

Seven restriction enzymes recognizing sequences of 6 base pairs were tested, i.e. HindIII, HincII, EcoRI, XbaI, SmaI, and AccI, which all showed different patterns of restriction, and EcoRV, which does not shows any difference between the patterns, but was only tested on a very small number of individuals.

In the five populations, 12 restriction patterns were revealed for the 6 enzymes (Fig. 2). The mean size of the mitochondrial genome was 16.000 base pairs. A letter was attributed to each restriction pattern of each enzyme, and a 6-letter code described the multimorph of each individual. Individuals with the same multi-enzyme pattern were considered to belong to the same clonal lineage of mitochondrial DNA (the same ancestral female). A total of 30 fishes were analysed, 19 of them by the 6 endonucleases showing different patterns, and 11 by fewer than 6 endonucleases (Table 1). The analytical technique used was equally valid for liver and ova. Four lineages were thereby revealed, based on the restriction patterns of the 19 individuals analyzed for all endonucleases (Table 2).

The populations of the Approuague and Comté rivers located to the east of the Kourou river region, were characterized by the majority multimorph ACEGIK (ME). A minority multimorph ADEGIK (mE) was found in the Approuague. The populations of the Mana, Sinnamary, and Iracoubo rivers located to the west of the Kourou basin, were characterized by the majority multimorph BDFHJL (MW). The Mana also had the majority multimorph of the Approuague and Comté populations (ME) as well as another minority multimorph ADEGJK (mW). The different multimorphs included 35 restriction fragments, with a variable percentage of shared fragments (Nei & Li, 1979): MW and ME (37%), MW and mW (63%), MW and mE (45%), ME and mW (71%), ME and mE (93%), and mW and mE (79%).
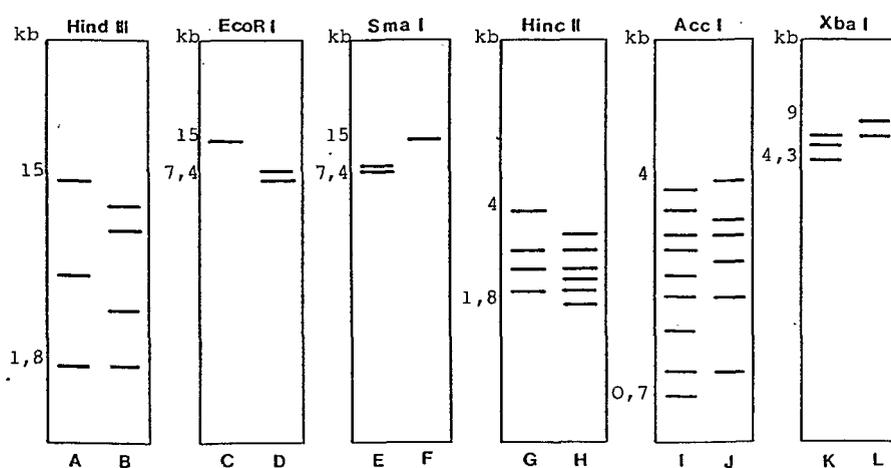
Fig. 2. Twelve restriction patterns corresponding to 6 enzymes showing polymorphism. Each pattern is denoted by a letter (mean size of the mitochondrial genome, 16 kb).

Table 1. Distribution of the 12 restriction patterns in the 5 populations studied. A total of 30 specimens were analysed, 19 of them by the 6 endonucleases and 11 by fewer than 6 endonucleases. A sign (–) indicates a missing result.

		Hind III (A,B)		Eco RI (C,D)		Sma I (E,F)		Hinc II (G,H)		Acc I (I,J)		Xba I (K,L)		
Populations		A	B	C	D	E	F	G	H	I	J	K	L	Multimorphe
Mana	1		+		+		+		+		+		+	BDFHJL
	2	+		+		+		+		+		+		ACEGIK
	3	+			+	+		+			+	+		ADEGJK
	4				+									–D——
Sinnamary	5		+		+		+		+		+		+	BDFHJL
	6		+		+		+		+		+		+	BDFHJL
	7		+		+		+		+		+		+	BDFHJL
	8		+		+		+							BDF——
	9		+		+									BD——
	10		+		+									BD——
Iracoubo	11		+		+		+		+		+		+	BDFHJL
	12		+		+		+		+		+		+	BDFHJL
	13		+		+		+		+		+		+	BDFHJL
	14		+		+		+		+		+		+	BDFHJL
	15		+		+									BD——
	16		+		+									BD——
	17		+											B———
	18		+		+									BD——
Comté	19	+		+		+		+		+		+		ACEGIK
	20	+		+		+		+		+		+		ACEGIK
	21	+		+		+		+		+		+		ACEGIK
	22	+		+		+		+		+		+		ACEGIK
	23	+		+		+		+		+		+		ACEGIK
	24	+		+		+		+		+		+		ACEGIK
	25	+		+										AC——
	26	+								+				A——I—
	27	+										+		A——K
Approuague	28	+		+		+		+		+		+		ACEGIK
	29	+		+		+		+		+		+		ACEGIK
	30	+			+	+		+		+		+		ADEGIK

Based on a table of presences or absences of each fragment in each multimorph, a system of parsimony according to the algorithm of Wagner, used in the Phylip program developed by Felsenstein (1989), located the different multimorphs so that they in-

Table 2. Number of individuals per population and per multimorph, designated by 6 letters.

	BDFHJL	ACEGIK	ADEGJK	ADEGIK
Mana	1	1	1	–
Sinnamary	3	–	–	–
Iracoubo	4	–	–	–
Comté	–	6	–	–·–
Approuague	–	2	–	1

volved the minimum of possible mutational events. This method is approximate since the presence or absence of fragments does not represent independent characters. The four multimorphs fall on a line whose root can be assumed to be equidistant from the ends (if the mutation rate is constant in populations of the same species) (Fig. 3). The two majority multimorphs ME and MW are situated at the two ends. The two minority multimorphs mE and mW are at intermediate positions. The relative position of the multimorphs corresponds to the percentage of fragments they share. According to the method proposed by Slatkin and Maddison (1989), there are at least 3 migration events consistent with our observations.

Discussion

RFLP study of the mtDNA of *L. friderici* revealed a marked biogeographic structure. The interruption of gene flow between two sets of Guianese populations, i.e. an eastern group (Comté and Approuague) and a western group (Sinnamary, Iracoubo, Mana), was much clearer than that previously defined by protein electrophoresis. Two multimorphs were in the majority and each characterized a set of populations. Two others were in the minority and were found only once, one in the Approuague population and the other in the Mana population. Too few individuals and populations were involved to allow conclusions on the significance of the minority multimorphs. However, the Mana population is remarkable for its high polymorphism. The three individuals analyzed each belonged to different mitochondrial lineages, and one of the multimorphs was found nowhere else.

Other authors have found analogous divisions between populations of continental fishes. Berming-

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BDFHJL●─────────────── ADEGJK ●─────── ADEGIK ────●ACEGIK
                              ‖
Mana                    Mana  root      Approuague  Approuague
Sinnamary                               Comté
Iracoubo                                           Mana
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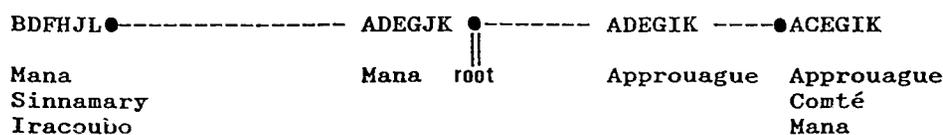
Fig. 3. Positions of the four multimorphs according to the algorithm of Wagner. The length of segments is proportional to the number of events. Under each multimorph the populations in which it was found are indicated. The solid circles indicate where migration events were counted according to the method proposed by Slatkin and Maddison (1989).

ham and Avise (1986) have studied the mtDNA of four species in the southeastern United States. They found three main geographic borders characterized by genetic discontinuities. The authors pointed out the correspondence between the intraspecific genetic structures observed in the four species, and between the genetic structures of the species and the zoogeographic distribution of the fishes studied by Şwift *et al.* (1985) in this region. They described a scenario related to the topographic disturbances of watersheds during marine regressions and transgressions in the Quaternary and proposed a time for the divergence between the populations.

In the case of *Leporinus*, the RFLP study of mtDNA confirmed the discontinuity of gene flow in the central region of French Guiana, but the data are not sufficient to determine whether there was an ancient genetic division of the species or a more recent separation following a bottleneck in either of the geographic sets of populations. Further study of mitochrondial polymorphism combined with electrophoretic analysis should make it possible to determine possible founder effects in the regions recently colonized by *L. friderici* and to evaluate current or fossil genetic flows.

According to the minimum number of migratory events (Slatkin & Maddison, 1989), the multimorph ME in the Mana population could indicate an eastern immigration, while MW and mW would have an other origin. It follows that the populations with the most abundant mitochondrial lineages, such as that of the Mana, could be populations that remained in refuges during Quaternary paleoclimatic disturbances. Their genetic pool has been enriched several times during periods favorable to species dispersion, from surrounding regions with diverse faunistic characteristics. The present study supports the hypothesis of a Guianese aquatic refuge, whose eastern edge is the Kourou river region.

References

Avise, J. C., 1989. Gene trees and organismal histories: a phylogenetic approach to population biology. Evolution 43 (6): 1192-1208.

Avise, J. C. & Vrijenhoek, R. C., 1987. Mode of inheritance and variation of mitochondrial DNA in hybridogenetic fishes of the genus Poeciliopsis. Mol. Biol. Evol. 4 (5): 514-525.

Bermingham, E. & Avise, J. C., 1986. Molecular zoogeography of freshwater fishes in the southern United States. Genetics 113: 939-965.

Blanquer, A., 1990. Phylogégraphie intraspécifique d'un poisson marin, le flet Platichthys flesus L. (Heterosomata). Polymorphisme des marquers nucléaires et mitochondriaux. Thèse de doctorat, USTL. 157 p.

Boursot, P. & Bonhomme, F., 1986. Génétique et évolution du génome mitochondrial des Métazoaire. Génét. Sél. Evol. 18: 73-98.

Boursot, P., Yonekawa, H. & Bonhomme, F., 1987. Heteroplasmy in mice with deletion of a large coding region of mitochondrial DNA. Mol. Biol. Evol. 4 (1): 46-55.

Brown, K. S. Jr., 1975. Geographical patterns of evolution in neotropical Lepidoptera. Systematic and derivation of known and new Heliconii (Nymphalidae: Nymphalinae). J. Ent. 44: 201-242.

Brown, K. S. & Ab'Saber, A. N., 1979. Ice-age forest refuges and evolution in the neotropics: correlation of paleoclimatological, geomorphological and pedological data with modern biological endemism. Paleoclimas (Sao Paulo) 5: 1-30.

Brown, W. M., George, M. & Wilson, A. C., 1979. Rapid evolution of animal mitochondrial DNA. Proc. Atl. Acad. Sci. USA. 76 (4): 1967-1971.

De Granville, J. J., 1982. Rain forest and xeric flora refuges in

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French Guiana. In: Biological diversification in tropics. (G. T. Prance, ed), pp. 159-181. New York: Columbia University Press.

Descamps, M., Gasc, J. P., Lescure, J. & Sastre, C., 1978. Etude des écosystèmes guyanais. II Données biogéographiques sur la partie orientale des Guyanes. C. R. Soc. Biogeogr. 467: 55-82.

Endler, J. A., 1982. Pleistocene forest refuges: fact or fancy? in: Biological Diversification in the Tropics (G. T. Prance, ed), pp. 641-657. New York: Columbia University Press.

Felsenstein, J., 1989. PHYLIP, Phylogenic Inference Package, version 3.2, Department of genetics, SK 50. University of Washington, Seattle, Washington 98195, USA.

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

Haffer, J., 1969. Speciation in Amazonian forest birds. Science. 165: 131-137.

Haffer, J., 1978. Distribution of Amazon forest birds. Bonn. Zoo. Beitr. 29: 38-78.

Haffer, J., 1982. General aspects of the refuge theory. In: Biological Diversification in the Tropics (G. T. Prance, ed), pp. 6-26. New-York: Columbia University Press.

Lescure, J., 1975. Biogéographie et écologie des amphibiens de Guyane française. C. R. Soc. Biogeogr. 440: 68-82.

Livingstone, D. A., 1982. Quaternary geography of Africa and the refuge theory. In: Biological Diversification in the Tropics (G. T. France, ed), pp. 523-536. New-York: Columbia University Press.

Nei, M. & Li, W. H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA. 76 (10): 5269-5273.

Prance, G. T., 1973. Phytogeographic support for the theory of Pleistocene forest refuges in the Amazon basin, based on evidence from distribution patterns in Caryoraceae, Chrysobalanaceae, Dichapetalaceae and Lecythidaceae. Acta Amazônica 3: 5-28.

Renno, J.-F., Guyomard, R., Boujard, T. & Bastide, C., 1989. Genetic differenciation among four species of Leporinus (Anostomidae, Pisces) in French Guiana, Aquat. Liv. Resour. 2: 127-134.

Renno, J.-F., Berrebi, P., Boujard, T. & Guyomard, R., 1990. Intraspecific genetic differenciation of Leporinus friderici (Anostomidae, Pisces) in French Guyana and Brazil: A genetic approach to the refuge theory. J. Fish Biol. 36: 85-95.

Slatkin, M. & Maddison, P., 1989. A cladistic measure of gene flow inferred from the phylogenies of alleles. Genetics, 123: 603-613.

Swift, C. C., Gilbert, C. R., Bortone, S. A., Burgess, G. H. & Yerger, R. W., 1985. Zoogeography of the freshwater fishes of southeastern United States: Savannah River to lake Ponchartrain. In: Zoogeography of North American Freshwater Fishes (C. H. Hocutt and E. O. Wiley, John Wiley & Son, ed.) pp. 213-265. New York.

Vanzolini, P. E. & Williams, E. E., 1970. South American Anoles: the geographic differenciation and evolution of Anolis chrysolepis species groups (Sauria, Iguanidae). Arq. Zool. S. Paulo. 19: 1-298.

Vanzolini, P. E., 1973. Paleoclimates, relief and species multiplication in equatorial forest. In: Tropical Forest Ecosystems in Africa and South America (B. J. Meggers, E. Agensu & R. Duckworth, ed), pp. 255-258. Washington, D.C. Smithsonian Inst. Press.

Weitzman S. H. & Weitzman, M., 1982. Biogeography and evolutionary diversification in neotropical freshwater fishes, with comments on the refuges theory. In: Biological Diversification in the Tropics (G. T. Prance, ed.), pp. 403-422. New York: Colombia University Press.