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On the origin and diversification of Venezuelan freshwater fishes: the genus *Gephyrocharax* (Ostariophysi: Characidae) a case study

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We conducted a phylogeographic analysis of the genus *Gephyrocharax* in Venezuela to evaluate geomorphologic evidence for the formation of the country's main watersheds and to establish a biogeographical hypothesis of possible diversification mechanisms of the Neotropical freshwater fish fauna. We assayed eight enzyme systems and general proteins to estimate genetic variability (*H, P*), intraspecific structuring in several *Gephyrocharax valencia* and *G. venezuelae* populations (F_{IS}, F_{IT}, and F_{ST}), and a phylogenetic approach for the three species of *Gephyrocharax* in Venezuela, using *Corynopoma riisei* as the external group. Fourteen presumptive *loci* indicate that populations of the three species of *Gephyrocharax* analyzed show a clear genetic inter-specific differentiation, determined by four *loci* with fixed alleles (*GPI-B**, *IDH**, *ME-1**, and *ME-2**). The resulting cladogram shows two major clades: a monophyletic group consisting of *Gephyrocharax* n. sp. and *G. venezuelae* (restricted to the northwest of the country) and a group formed exclusively by *G. valencia* (distributed along the largest geographic range). Speciation of the Venezuelan lineages of the genus *Gephyrocharax* could be explained by the origin and course movements of the present Orinoco River together with geomorphologic processes that have occurred in northern Venezuela since the Miocene.

Foi feita uma análise filogeográfica do gênero *Gephyrocharax* na Venezuela a fim de avaliar as evidências geomorfológicas que levaram à formação dos principais sistemas hidrográficos do país, além de estabelecer uma hipótese biogeográfica com os possíveis mecanismos de diversificação da fauna de peixes de água doce Neotropical. Foram analisados oito sistemas enzimáticos e proteínas gerais para conhecer a variabilidade genética (*H*, *P*), estruturação intraespecífica em populações de *Gephyrocharax valencia* e *G. venezuelae* (F_{IS}, F_{IT} e F_{ST}), e uma abordagem filogenética com base na análise isozimática para as três espécies de *Gephyrocharax* na Venezuela, com *Corynopoma riisei* como grupo externo. Quatorze *loci* presumíveis indicam que as populações das três espécies de *Gephyrocharax* analisados revelam uma diferenciação inter-específica genética, determinada por quatro *loci* com alelos fixos (*GPI-B*, IDH*, ME-1* e ME-2**). O cladograma resultante apresenta dois clados principais: um grupo monofilético composto por *Gephyrocharax* n. sp. e *G. venezuelae* (restrita ao noroeste do país) e um grupo formado exclusivamente por *G. valencia* (distribuídos ao longo da maior área geográfica). A especiação das linhagens de *Gephyrocharax* na Venezuela poderia ser explicada pela origem e movimentos do curso atual da bacia do rio Orinoco, associado a processos geomorfológicos que ocorrem no norte da Venezuela desde o Mioceno.

Key words: Isoenzymes, Speciation, Venezuela, Vicariance.

Introduction

The high diversity of tropical freshwater fishes has been mainly attributed to allopatric speciation with subsequent mixing given the appropriate physical conditions (Lowe-McConnell, 1969). However, Lundberg *et al.* (1998) suggested that vicariant events produced by the movement and division of drainages in large rivers represented a significant source of diversification of neotropical freshwater fishes. Moreover, Lovejoy *et al.* (1998, 2006) suggested that marine transgressions during the Early Miocene (15-23 million years) profoundly affected the structure and diversification of neotropical fish communities and indicated that some species of South American freshwater fishes had originated from marine groups by means of massive movements of seawater into the upper Amazon region.

A Parsimony Analysis of Endemicity by Hubert & Renno (2006) proposed that the establishment of the freshwater fish fauna of South America was the result of the interaction of various processes such as marine incursions, the elevation of mountain systems and historical connections that allowed dispersal between drainages. They emphasized the presence

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of seven dispersion routes scattered throughout the Amazon, Orinoco, and Paraná rivers. In their review of the phylogenetic patterns of fishes of the rivers of northwestern South America, Albert *et al.* (2006) concluded that these patterns are highly consistent with geological information regarding the isolation time of these drainages. They also suggested that the species composition of these river basins was modern by the time the late Middle Miocene tectonics events occurred.

In recent studies of species differentiation it has become common to use a phylogenetic approach to integrate information on genetic and morphological differentiation and geographical distribution patterns, so as to establish a more robust hypothesis of their relationships (Avise *et al.*, 1987; McKay & Miller, 1991; Dimmick & Lawson, 1991; Carmona *et al.*, 2000; Pouyaud *et al.*, 2000; Morris *et al.*, 2001; Bonilla *et al.*, 2002; Wang *et al.*, 2004; Strecker *et al.*, 2004; Salzburger *et al.*, 2005). For example, Carmona *et al.* (2000) studied *Chondrostoma lemmingii*, a cyprinid endemic to the Iberian Peninsula, using genetic criteria (allozymes and cytochrome c) and determined that the phenetic and phylogenetic relationships found supported the hypothesis that the process of differentiation between their populations was due to several vicariant events including endorheism and hydrological isolation of the drainages studied. On the other hand, there are examples where geographical isolation does not lead to detectable genetic differentiation: Revaldaves *et al.* (1997) studied the isoenzyme variability of *Prochilodus lineatus* (Characidae) from three isolated localities of the Paraná River, Brazil, and found a relatively high average heterozygosity (H = 13%) but a low level of differentiation (F_{ST} = 0.018) between the populations studied.

There are many freshwater fishes suitable for studying the influence of vicariant fractioning on isolated populations, in particular, species of the genus *Gephyrocharax* (Characidae, Stevardiinae; Weismann *et al.*, 2005) that present a predominantly allopatric geographical distribution in South and Central America (Fig. 1A). In Venezuela three species have been described: *Gephyrocharax valencia* was originally described from Lake Valencia in north-central Venezuela, but is widely distributed throughout the Orinoco River basin, as well as in the Tocuyo, Aroa, Yaracuy, Urama, San Esteban, and Neverí rivers, all draining into the Caribbean Basin. *G. venezuelae* is restricted to Lake Maracaibo (Schultz, 1944) and the western Caribe Basin: Cueva del Indio, Cuare (Falcón) (López *et al.*, 1996), Urama River (Bonilla & López, 2001), Aroa, Yaracuy and Tocuyo rivers (Rodríguez *et al.*, 2006). The Urama River forms the eastern boundary of *G. venezuelae* in the entire Caribe Basin. The taxonomic identity of a third venezuelan species of *Gephyrocharax*, identified by several authors as *G. melanocheir* deserves a separate consideration as its characteristics do not coincide with any of the valid species described for Venezuela (Eigenmann, 1912; Schultz, 1944; Dahl, 1971). Differences in color pattern suggest it could be a new species, and consequently will be referred to in this manuscript as *Gephyrocharax* n. sp. It will be described in a separate paper in preparation. *Gephyrocharax* n. sp. has been reported only in some drainages in Zulia (Taphorn & Lilyestrom, 1984) and Falcón States (Sierra de San Luis; J. Moscó, unpublished data) belonging to Venezuela's western Caribe basin. Despite the coincidental distribution of *G. venezuelae* and *Gephyrocharax* n. sp. in the Lake Maracaibo and Caribe basins, so far these two species have not been found sympatrically. *G. valencia* and *G. venezuelae*, on the other hand, are sympatric in drainages of the Caribe Basin between the Tocuyo and Urama rivers.

The main objective of this study was to conduct an isoenzymatic analysis of the species of the genus *Gephyrocharax* in Venezuela to compare the information obtained with geomorphological evidence for the formation of the major watersheds in the country. The resulting biogeographic hypothesis could be used as a model to explain the potential mechanisms involved in the diversification of the country's freshwater fish fauna.

Material and Methods

Specimens studied are representative of allopatric populations of the three species of the genus *Gephyrocharax*; additionally, two sympatric populations of *G. valencia* and *G. venezuelae* from the Aroa and Taría rivers are included (populations 3 and 4; Fig. 1b). The population of *Corynopoma riisei* (population 6, Fig. 1b) used as the external group is allopatric with respect to those of *Gephyrocharax*.

Material examined. *Gephyrocharax valencia*, MBUCV-V-35679, 15, 18.3-24.3 mm SL, río Aroa, Falcón, km 26 vía Palma Sola-Boca de Aroa, 10º38'02"N 68º28'01"W.; río Las Peñas de Taría, Yaracuy, afluente del río Yaracuy, 8 km pasando el pueblo Taría, 10º22.6'N 68º32.9'W, MBUCV-V-35682, 17, 17.8-22.4 mm SL; río Guapo, Miranda, antes de llegar a la planta de tratamiento vía represa El Guamito, MBUCV-V-35683, 20, 20.9-33.5 mm SL; río Pao, Anzoátegui, MBUCV-V- 35684, 11, 22.7-34.2 mm SL. *Gephyrocharax venezuelae:* río La Pedregosa, Zulia, afluente del río Machango, cruce de la carretera desde el pueblo de Sipayare, 10º09.321'N 70º53.841'W, MBUCV-V- 35685, 7, 25.0-37.4 mm SL; río Aroa, Falcón, km 26, vía Palma Sola-Boca de Aroa, 10º38'2" N 68º28'1"W, MBUCV-V- 35686, 7, 27.2-30.5 mm SL; río Las Peñas de Taría, Yaracuy, afluente del río Yaracuy, 8 km pasando el pueblo Taría, 10º22.6' N 68º32.9' W, MBUCV-V-35687, 39, 22.8-29.9 mm SL; río Alpargatón, Carabobo, cantera, MBUCV-V-35688, 31, 30.9-22.9 mm SL. *Gephyrocharax* sp.n.?: río Meachiche, Falcón, balneario Meachiche, MBUCV-V-35689, 19, 31.5-38.1 mm SL. *Corynopoma riisei:* río La Cumaca, Carabobo, afluente del río San Diego, entrando por la Urbanización Villas de San Diego, sector La Cumaca, San Diego, MBUCV-V-35690, 9, 29.2-39.00 mm SL.

For the genetic analysis skeletal muscle was homogenized with buffer Tris/0.001 M 0.1 M EDTA pH 7.0 and analyzed by 10% starch gel electrophoresis in a horizontal system. Gels were prepared following a modification of the microwave cooking method described in Murphy *et al.* (1990): gels were cooked for 12 minutes with manual agitation every 30 seconds until they thickened, then were allowed to boil and degasified with a vacuum pump. We assayed 8 specific enzymes and

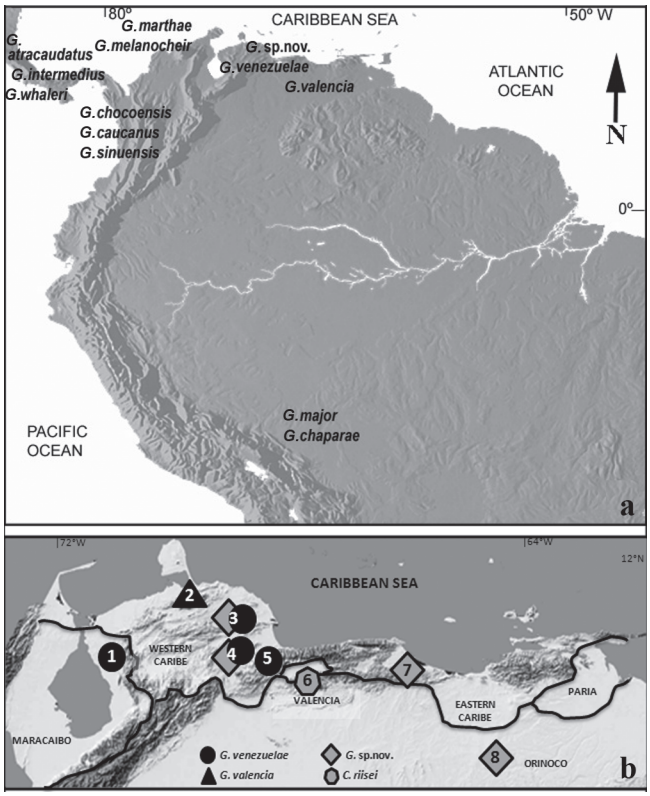

Fig. 1. a. Geographical distribution of species of *Gephyrocharax*. **b.** Sampling sites for *Gephyrocharax* and *Corynopoma riisei* in Venezuela. 1. La Pedregosa River, Machango River basin, Zulia State. 2. Meachiche River, Falcón State. 3. Aroa River, km 26, Palma Sola, Falcón State. 4. Las Peñas de Taría River, Yaracuy River basin, Yaracuy State. 5. Alpargatón River, Urama River basin, Carabobo State. 6. La Cumaca River, San Diego River basin, Carabobo State. 7. La Maleja creek, Guapo River basin, Miranda State. 8. Pao River, Orinoco River basin, Anzoátegui State. In sites 3 and 4 *G. venezuelae* and *G. valencia* occur sympatrically. Limits between major river basins (Lake Maracaibo, Caribe, Paria, and Orinoco) follow Mago-Leccia (1970) and are indicated with a solid line.

general proteins with five different electrophoretic buffers; details of the enzyme systems more fully revealed and electrophoretic conditions are listed in Table 1. Nomenclature of presumptive *loci*, as well as their corresponding alleles, follow Shaklee *et al.* (1990) for electrophoretic studies in fish. Data analysis was performed using BIOSYS-1 (Swofford & Selander, 1981) to determine allelic frequencies, percentage of polymorphic *loci* (*P*), average heterozygosity (*H*), and deviations from the Hardy-Weinberg equilibrium for each polymorphic *locus* in each population analyzed, using Levene's correction for small sample size. Addititionally, we calculated the statistics F_{IS}, F_{IT}, and F_{ST} to determine intraspecific structuring in *G. valencia* and *G. venezuelae*. The statistic F was also calculated for a pool of the three species so as to determine the levels of interspecific differentiation. No calculations for population structure were made for *Gephyrocharax* n. sp. as we were only able to sample one single population (population 2; Fig.1b). Using a hierarchical geographical array analysis the genetic distance coefficient (Cavalli-Sforza & Edwards, 1967 arc distance, *D*) and level of population structure (F) statistics (Wright, 1978) were calculated for *G. valencia* and *G. venezuelae* to determine the differences between: 1. Western (populations 1, 3, 4 and 5; Fig. 1b) and Eastern (populations 7 and 8; Fig. 1b) REGIONS compared to the TOTAL (F_{RT}); 2. Populations within each Region (F_{PR}); 3. Basins compared to the Total (F_{BT}); and 4. All Populations in relation to the Total (F_{PT}). In the latter case the value of F_{PT} represents an estimate of population structure equivalent to the statistic F_{ST}. The product between the effective population size (N_e) and the rate of gene flow (m) was used as an estimator of the extent of genetic structuring between the populations studied (N_em), using the formula $F_{ST} = 1/1 + 4N_e m$ (Wright, 1931). Values of $N_e m > 1$ indicate lack of migrant interchanges that could prevent differentiation due to genetic drift (Slatkin 1985); *i.e.*, due to a high gene flow there is no genetic structure in the population.

The most parsimonious tree was calculated with the Wagner Distance method using the Cavalli-Sforza & Edwards (1967) arc distance index. This index has been reported as the most appropriate to derive phylogenetic trees from allelic

Table 1. Enzyme systems studied, enzyme commission code number, buffer and running conditions, reference, and *loci* scored for *Gephyrocharax* and *Corynopoma* species.

Enzyme / Abreviature / Structure	E.C.	Buffer system	*Loci*	Reference
Creatine kinase / CK / dimer	2.7.3.2	Tris/Borate/EDTA pH: 8.0; 90 mA, 3 hrs.	*CK**	Shaw & Prasad (1970) System III.
Isocitrate dehydrogenase / IDH / dimer	1.1.1.42	Phosphate/citrate pH: 7.0; 90 mA, 4 hrs.	*IDH**	Buth & Murphy (1990)
Glucose dehydrogenase / GCDH / dimer	1.1.1.47	Tris/Citrate pH: 7.0; 200 v, 2 hrs.	*GCDH**	Shaw & Prasad (1970) System I.
Glucose-6-phosphate isomerase / GPI / dimer	5.3.1.9	Phosphate/citrate pH: 7.0; 90 mA, 4 hrs.	*GPI-1* GPI-2**	Buth & Murphy (1990)
Malate dehydrogenase / MDH / dimer	1.11.37	Phosphate/citrate pH: 7.0; 90 mA, 4 hrs.	*MDH-1* MDH-2* MDH-3* MDH-4**	Buth & Murphy (1990)
Malic enzyme / ME / tetramer	1.1.1.40	Phosphate/citrate pH: 7.0; 90 mA, 4 hrs.	*ME-1* ME-2**	Buth & Murphy (1990)
Lactate dehydrogenase / LDH / tetramer	1.1.1.27	Tris/Citrate pH: 7.0; 200 v, 2 hrs.	*LDH-1**	Shaw & Prasad (1970) System I.
Phosphoglucomutase / PGM / monomer	5.4.2.2	Phosphate/citrate pH: 7.0; 90 mA, 4 hrs.	*GCDH-1**	Buth & Murphy (1990)
General Proteins / GP	non-specific	Tris/Citrate pH: 7.0; 200 v, 2 hrs.	*GP**	Shaw & Prasad (1970) System I.

frequency data (Roger, 1986). *Corynopoma riisei*, a species of the tribe Corynopomini, which includes *Gephyrocharax*, *Corynopoma*, and *Pterobrycon* (Weitzman & Menezes, 1998), was used as an external group. The results of the hierarchical genetic analysis were reviewed in conjunction with the geomorphological information available for northwestern South America (Lundberg *et al.*, 1998; Albert *et al.*, 2006) and Venezuela (Rod, 1981; Mendez, 1985; Díaz de Gamero, 1996) in order to offer further biogeographical evidence for the probable vicariant origin of the Orinoco, Lake Maracaibo, and Caribe basins.

Results

Eight enzyme systems and the general proteins (GP) showed well-defined bands that allowed us to establish iso and aloenzymatic patterns for 14 presumptive *loci*. *Loci GPI-A** and *PGM** were polymorphic (*100** allele with a frequency of less than 0.990) in at least one of the populations of each species studied, while *loci CK**, *GCDH**, *GP-1**, *GPI-B**, *IDH**, *LDH-A**, *MDH-1**, *MDH-2**, *MDH-3**, *MDH-4**, *ME-1**, and *ME-2** were monomorphic. However four of them (*GPI-B**, *IDH**, *ME-1**, and *ME-2**) had different mobilities between species and/or between populations of the same species (Table 2). Because of their fixed allelic differences these may represent diagnostic *loci* between species of *Gephyrocharax*, and between *Gephyrocharax* and *Corynopoma*. For example, *GPI-B** differentiates *Corynopoma riisei* from the three species of *Gephyrocharax* studied; *IDH** differentiates *G. valencia* and *Gephyrocharax* n. sp. from *G. venezuelae* and *C. riisei*; *ME-1** and *ME-2** differentiate *G. valencia* and *C. riisei* from *G. venezuelae* and *Gephyrocharax* n. sp.

Genetic variability, expressed as the proportion of polymorphic *loci* (*P*) and average frequency of heterozygotic *loci* for individuals for each population analyzed are shown in Table 2. *P* values for the *Gephyrocharax* populations studied varied between 7.1 and 14.3% with two polymorphic *loci*: *GPI-A** and *PGM**. Polymorphism level for the *Corynopoma riisei* population was 0.00%. *H* values were low and very similar for all populations studied. In populations of *G. venezuelae* and *Gephyrocharax* n. sp. *H* values were higher than those of populations of *G. valencia* and *C. riisei*, albeit with a very low level of differentiation. The χ^2 test showed with a 99% confidence level that the *locus GPI-A** in six out of nine populations of *Gephyrocharax* studied were not in accordance with the Hardy-Weinberg law. All populations, except La Pedregosa, revealed a clear heterozygosity deficiency for the *GPI-A* locus* with negative values of the parameter D equal to minus one (-1.000) or very close to this maximum value. Results for the fixation index (F) show a high degree of inbreeding for these populations with maximum values (1.000) of this parameter in all populations, except for the population of *G. venezuelae* from La Pedregosa River, with a value of the statistic F equal to -0.077. The *PGM* locus* deviated significantly from the Hardy-Weinberg equilibrium in two populations of *G. valencia*: Aroa y Taría (Table 2).

Interspecific comparisons (Cavalli-Sforza & Edwards, 1967 arc distance index) resulted in a closer genetic proximity between *G. venezuelae* and *Gephyrocharax* n. sp. (D = 0.371 [0.359- 0.377]), whereas the most dissimilar species were *G. valencia* and *G. venezuelae* (D = 0.537[0.517-0 .556]). Distance values for *Corynopoma riisei* and the *Gephyrocharax* species were 0.470, 0.484, and 0.593 for *G. venezuelae*, *G. valencia*, and *Gephyrocharax* n. sp., respectively (Table 3). The level of genetic differentiation among the three species of *Gephyrocharax* in Venezuela is within the average range for full species (Nei, 1975). A high average value (F_{IS} = 0.703; F_{IT} = 0.952; F_{ST} = 0.839) of the Wright F statistic indicates a clear genetic differentiation among the species of

Table 2. Allelic frequencies, heterozygosity per *locus* (*h*; unbiased estimation according to Nei, 1978) for the polymorphic *locus* and the diagnostic *loci* in the *Gephyrocharax* and *Corynopoma* populations studied. (N) number of individuals studied, (*P*) percentage of polymorphism, (*H*) average heterozygosity (standard errors in parentheses), (Obs) heterozygotes observed, (Esp) heterozygotes expected, (χ^2) chi squared, (D) heterozygote deficiency coefficient, (F) fixation index, (F_{ST}) population differentiation coefficient. (1) Negative values indicate heterozygote deficiency. (2) Direct-count. A *locus* is considered polymorphic if the frequency of the most common allele (**100*) is lower than 0.990. (3)significative with a 99% confidence.

		G. venezuelae				*G.valencia*				*G.* n. sp.	*C. riisei*
	allele	Pedregosa	Aroa	Taría	Alpargatón	Aroa	Taría	Guapo	Pao	Meachiche	Cumaca
Locus	(N)	7	7	18	19	19	19	7	15	20	7
*GPI-A**	**100*	0.929	0.786	0.806	0.737	0.158	0.474	0.786	0.533	0.300	1.000
	**98*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	**102*	0.071	0.214	0.194	0.263	0.842	0.526	0.214	0.467	0.000	0.000
	**-100*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.700	0.000
h		0.133	0.337	0.313	0.388	0.266	0.499	0.337	0.498	0.420	0.000
Obs		1	1	1	0	0	2	1	2	0	-
Exp		1.000	2.538	5.800	7.568	7.568	9.730	2.538	7.724	8.615	-
χ^2		0.00	3.636	[3]14.069	[3]20.74	[3]8.42	[3]12.66	3.64	[3]8.83	[3]21.50	-
$D^{(1)}$		0.000	-0.606	-0.787	-1.000	-0.615	-0.794	-0.606	-0.741	-1.000	-
F		-0.077	0.576	0.823	1.000	1.000	0.789	0.576	0.732	1.000	-
F_{ST}	0.033										
	(N)	7	7	20	19	20	20	7	15	20	7
*GPI-B**	**100*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
	**98*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
h		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F_{ST}	1.000										
	(N)	7	7	20	18	20	20	7	15	20	7
*PGM**	**100*	0.000	0.000	0.000	0.056	0.125	0.125	0.643	1.000	0.825	0.000
	**98*	0.000	0.000	0.000	0.000	0.875	0.875	0.357	0.000	0.000	0.000
	**102*	1.000	1.000	1.000	0.944	0.000	0.000	0.000	0.000	0.175	1.000
h		0.000	0.000	0.000	0.105	0.219	0.219	0.459	0.000	0.289	0.000
Obs		-	-	-	2	1	1	1	-	3	-
Exp		-	-	-	1.943	4.487	4.487	3.462	-	5.923	-
χ^2		-	-	-	0.030	[3]14.766	[3]14.766	4.267	-	5.567	-
$D^{(1)}$		-	-	-	0.029	-0.777	-0.777	-0.711	-	-0.494	-
F		-	-	-	-0.059	0.771	0.771	0.689	-	0.481	-
F_{ST}	0.042										
	(N)	7	3	16	19	16	16	3	11	20	7
*IDH**	**100*	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	0.000
	**102*	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	1.000
h		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F_{ST}	1.000										
	(N)	7	7	20	19	20	20	7	15	20	7
*ME-1**	**100*	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000
	**98*	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	0.000	1.000
h		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F_{ST}	1.000										
	(N)	7	7	20	19	20	20	7	15	20	7
*ME-2**	**100*	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	0.000	1.000
	**98*	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000
h		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F_{ST}	1.000										
						Mean population values					
$P^{(3)}$		7.1	7.1	7.1	14.3	14.3	14.3	14.3	7.1	14.3	0.00
$H^{(2)}$		0.010 (0.010)	0.010 (0.010)	0.004 (0.004)	0.008 (0.008)	0.011 (0.008)	0.011 (0.008)	0.020 (0.014)	0.010 (0.010)	0.011 (0.011)	0.000 (0.000)
F_{ST}	0.839										

Gephyrocharax. These values are determined mainly by the maximum contribution (F_{ST} = 1.000) of the *GPI-2**, *IDH**, *ME-1**, and *ME-2* loci*, which could be regarded as diagnostic *loci* for the differentiation of the three species.

A hierarchical analysis of genetic distances using the Cavalli-Sforza & Edwards (1967) arc distance index between the three species of *Gephyrocharax* studied showed differentiation across the three major river basins sampled.

Table 3. Cavalli-Sforza & Edwards (1967) arc distance index for the *Gephyrocharax* and *Corynopoma* populations studied.

		Population									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	*G.venezuelae* Pedregosa	-	0.036	0.032	0.061	0.556	0.542	536	0.541	0.373	0.465
2	*G. venezuelae* Aroa		-	**0.004**	0.042	0.547	0.537	0.535	0.537	0.377	0.470
3	*G. venezuelae* Taría			-	0.043	0.548	0.538	0.535	0.537	0.376	0.469
4	*G. venezuelae* Alpargatón				-	0.538	0.530	0.519	0.517	0.359	0.474
5	*G. valencia* Aroa					-	0.060	0.151	0.217	0.490	0.503
6	*G. valencia* Taría						-	0.112	0.206	0.478	0.483
7	*G. valencia* Guapo							-	0.118	0.438	0.470
8	*G. valencia* Pao								-	0.433	0.480
9	*G.* sp.nov. Meachiche									-	**0.593**
10	*C. riisei* Cumaca										-

Most different were populations from the Orinoco and Maracaibo basins (D = 0.541 [0.541-0.541]); populations from the Caribe and Orinoco basins are genetically closer to each other (D = 0.366 [0.118-0.537]), as are those of the Caribe and Maracaibo basins (D = 0.305 [0.032-0.556]). Results from the hierarchical intraspecific analysis showed that *G. valencia* populations were genetically more differentiated (D = 0.144 [0.060-0.217]) than *G. venezuelae* populations (D = 0.036 [0.004-0.061]). Hierarchical genetic analyses of *G. valencia* and *G. venezuelae* revealed differences in the level and extent of the structuring of the populations studied (Table 4). *G. valencia* showed evidence of interpopulation differentiation and low genetic flow between populations of the western and eastern regions as well as between those of the Caribe and Orinoco basins, and between all populations with respect to the total; nevertheless populations within each region were relatively homogeneous. For *G. venezuelae* the structure level was very low for all hierarchical levels compared and with gene flow values corresponding to those of connected populations (Wright, 1943).

A phylogenetic approach based on isoenzyme analysis for the three species of *Gephyrocharax* in Venezuela yielded the most parsimonious cladogram using Cavalli-Sforza & Edwards (1967) arc distance index (Fig. 2). The tree was rooted using *Corynopoma riisei* as the external group. The resulting cladogram shows a well-defined group with two major clades: a monophyletic group (a) consisting of *Gephyrocharax* n. sp. and *G. venezuelae* and a group (b) formed exclusively by *G. valencia*. The latter is distributed along the largest geographic range while the most distant species from the external group, *Gephyrocharax* n. sp. and *G. venezuelae*, are geographically restricted to the northwest of the country. In the cladogram obtained all nodes are fully resolved, except for the populations of *G. venezuelae* from the Taría and Aroa rivers which cannot be distinguished from the *loci* revealed in this study.

Table 4. Variance components and hierarchical F statistics for all *loci* of *G. venezuelae* and *G. valencia*. Negative values are considered non-defined and are the result of higher intra-population (as opposed to inter-population) variability (Lessios *et al.*, 1998).

Comparison level	Species					
	G. venezuelae			*G. valencia*		
	Variance	F_{XY}	N_em	Variance	F_{XY}	N_em
F_{RT}	0.00903	-0.020	-	0.20596	0.206	0.96
F_{PR}	-0.00213	0.052	4.56	0.15317	0.193	1.05
F_{BT}	0.00146	0.033	7.33	0.27460	0.275	0.66
F_{PT}	0.00689	0.033	7.33	0.35913	0.360	0.44

Discussion

In this study we selected populations of the three species of *Gephyrocharax* distributed in different watersheds under conditions of sympatry and allopatry. Analyses of differentiation and genetic distances were performed using a hierarchical approach. This is important when dealing with data from different populations belonging to different species in different geographic areas, because it allows for the evaluation of intra and inter-specific variability in a geographical context (Trexler, 1988; Johnson, 2001).

The *Gephyrocharax* species studied showed a clear interspecific genetic differentiation determined by the presence of four different *loci* with fixed alleles (*GPI-B**, *IDH**, *ME-1**, and *ME-2**) together with conditions of very high inbreeding. Regarding the geographical relationships between basins, the hierarchical analysis showed greater genetic affinity between the eastern Caribe and Orinoco basins, and between the western Caribe and Maracaibo basins, probably as a consequence of a longer isolation period between the Lake Maracaibo and Orinoco basins than that between the Caribe and Orinoco basins, mainly in the eastern region of the country. Evaluation of gene flow (N_em) indicated that populations of *G. venezuelae* retain connections and that the small differences observed could represent a case of independent evolution under strong selective pressures. In the case of *G. valencia*, there is clear evidence for isolation between the eastern and western populations, whose N_em values denote a low level of gene flow between disconnected populations, possibly due to the differentiation of neutral alleles. Significantly, western populations of *G. valencia* sampled are found in the Caribe basin, whereas eastern populations of the same species are distributed both in

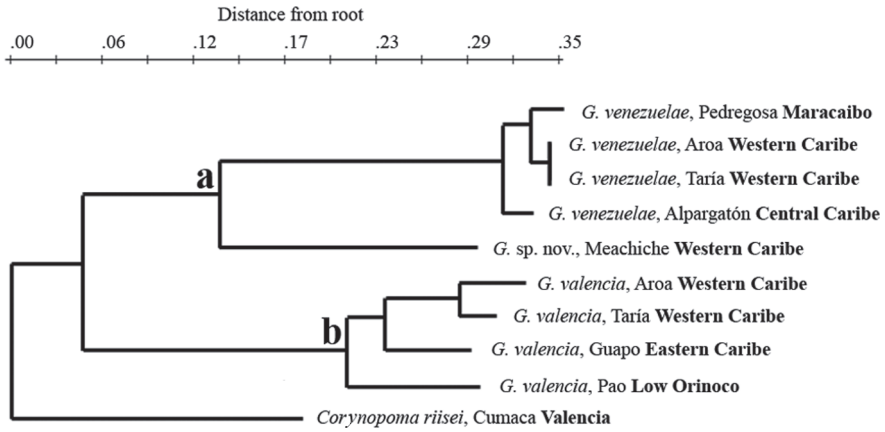

Fig. 2. Phylogenetic approach for the three species of *Gephyrocharax* in Venezuela. *Corynopoma riisei* is included as the external group (Names in bold denote major river basins referred to in the text).

Venezuela's Caribe and Orinoco basins, which are more than 500 km apart. By contrast, the value of N_em for *G. venezuelae* indicates a trend towards panmixia within each region, possibly due to the more recent connections maintained by the rivers analyzed within each region and their geographical proximity.

In Venezuela, a succession of geologic events coupled with different water divisions, largely related to the Orinoco River basin, has determined the formation of the country's watersheds. Fig. 3 schematically represents a proposal for the possible origin and diversification of species of the genus *Gephyrocharax* in Venezuela, taking into consideration the formation of the major river basins and a phylogenetic analysis based on isoenzyme characters. Within clade (a), *Gephyrocharax* n. sp. + *G. venezuelae* (Fig. 2), populations of *G. venezuelae* analyzed are distinct: the population of Alpargatón (Carabobo State) is the sister group of populations from Taría-Aroa-Pedregosa (Yaracuy-Falcón-Zulia States). The species *G. venezuelae* probably originated via vicariant allopatric speciation (Bush, 1975) as a consequence of the splitting of the watershed of the current Magdalena River (Colombia) and the Proto-Orinoco River (11.8-10 Ma) (Lundberg *et al.*, 1998; Albert *et al.*, 2006). Remnant populations are found in the present-day Lake Maracaibo region as well as in Falcón and Yaracuy States. Subsequent isolation of the Maracaibo and Falcón basins (approximately between 2 to 1 My.; Yoris & Ostos, 1997) causes the distribution of *G. venezuelae* to become disjunct as the mountains that form the Falcón-Lara-Yaracuy cordillera rise to their current state; this is evidenced by the presence of *G. venezuelae* in the Lake Maracaibo basin and in the drainages of the depression that was formed between the mountains of Falcón-Lara-Yaracuy and Cordillera de la Costa (Fig. 3).

The continental relief of the Lara-Falcón-Yaracuy region is characterized by the presence of hills oriented in a general east-west direction, except for the Siruma Sierra, which has a north-south orientation and is closer to Zulia State. It should be noted that the Aroa-Taría group is the most inclusive, its localities being more closely together in the geographical distribution range of *G. venezuelae* examined in this work. In this respect, the populations of *G. venezuelae* studied come from rivers that are currently in the region covering the ancient Proto-Orinoco delta, which extended from the Maracaibo Depression to the western limit of the Cordillera de la Costa (Zulia, Falcón, and Carabobo States). Upon the definitive rise of the Lara-Falcón-Yaracuy Sierra some waterways in lowland drainages became elevated to mountainous areas with a consequent change in their environmental conditions. This change may have fostered or molded changes in the genetic structure of the populations of fishes in these mountain rivers, as opposed to the fish populations of those rivers that were not affected by major environmental changes. Among those mountain rivers are the Taría and Aroa, whereas the Alpargatón and Pedregosa rivers are in lowland areas. The current geomorphological configuration of the Falcón State region probably caused the emergence via vicariance of a more recent lineage, *Gephyrocharax* n. sp., distributed in the small rivers originating in the mountains to the north and draining directly into the Caribbean Sea. The presence of *Gephyrocharax* n. sp. is associated with more recent geological formations (Pliocene; PDVSA, 2008) as opposed to those where *G. venezuelae* occur (Middle to Late Miocene; PDVSA, 2008).

Clade b (*G. valencia*) also shows structuring for the populations studied: the groups of Venezuela's western Caribe basin (Taría-Aroa) are related among themselves and form the sister group of the clade Guapo + Pao (Venezuela's eastern Caribe-Orinoco basins) with the Guapo population from the eastern Caribe basin as its sister group and the population of Pao (Orinoco) as the basal group of *G. valencia*.

Subsequent changes to the Orinoco River course, in which the Maracaibo and Falcón basins became isolated, would

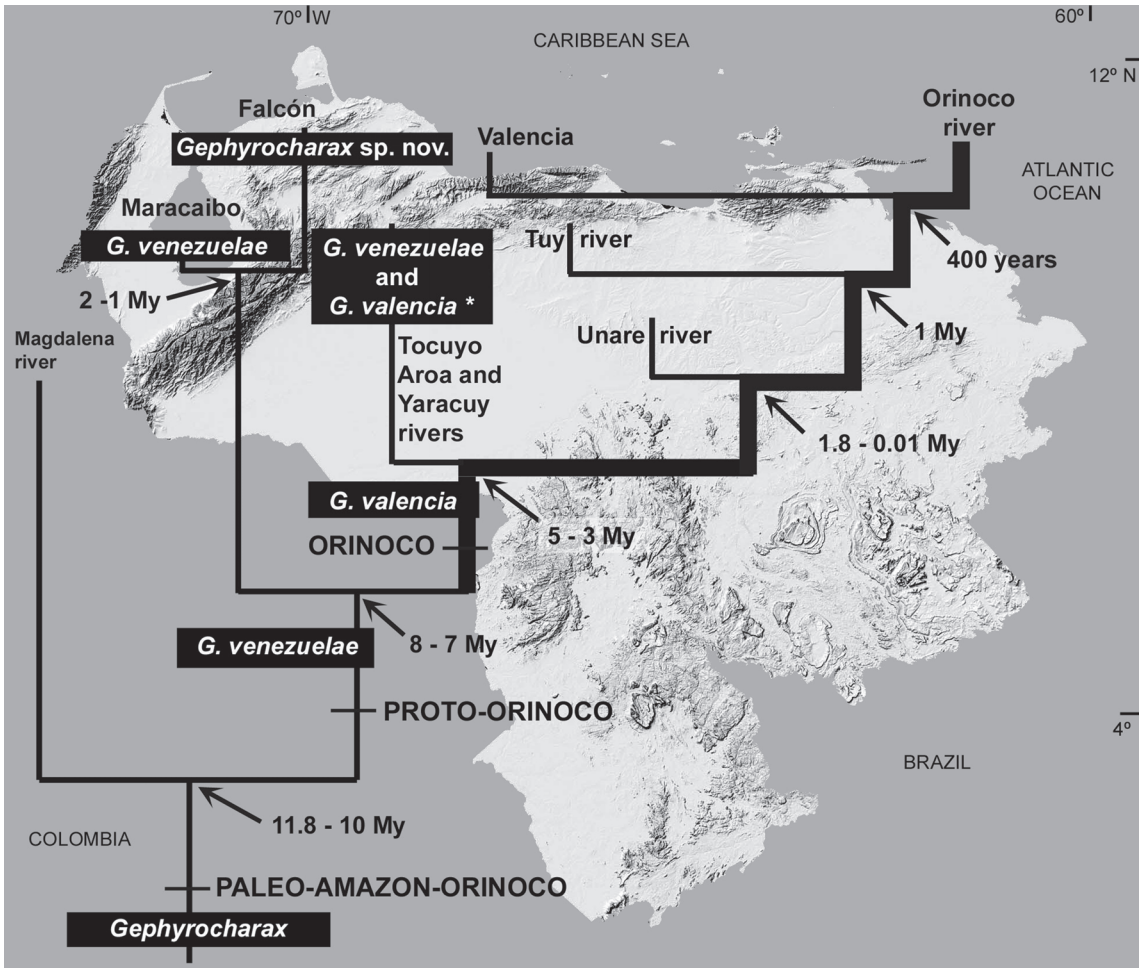

Fig. 3. Scheme for the possible origin of the species of *Gephyrocharax* in Venezuela. Hypothesized changes in the course of the Orinoco River in Venezuela and how they may have influenced the formation of major watersheds within the country follow Rod (1981), Díaz de Gamero (1996), and Lundberg *et al.* (1998). Major river basins in Venezuela are depicted in Fig. 1. (*) indicates simpatry.

result in the Orinoco River draining into the current east-central Llanos region, with connections to Venezuela's eastern Caribe (current Unare River basin, mainly) and Lake Valencia basins (Yoris & Ostos, 1997; Hung, 2005). The species *G. valencia* probably originated through allopatric speciation after this last major course change and then dispersed into rivers located between the Andes and Cordillera de la Costa, the only localities where *G. venezuelae* and *G. valencia* occur in sympatry.

The geographical distribution and isoenzymatic relationships of *Gephyrocharax valencia* are consistent with the geomorphological facts described above. The current distribution of this species includes Venezuela's Caribe basin (western region in the Depression occupied by the Aroa, Tocuyo, Yaracuy, and Urama rivers, and eastern region with the Tuy, Guapo, Unare, and Neverí river basins), the Valencia Lake basin and Orinoco River basin. The current Neverí and Manzanares rivers in northeastern Venezuela could have been drainages (or portions thereof) already existing in the eastern inner Cordillera flowing south-north at least since the Pliocene, period which accounts for the late orogenesis in most of the country (Hung, 2005). It is probable that channels of the Orinoco in their eastward movement captured the courses of various drainages of the eastern inner Cordillera (north and south flanks), which could explain the presence of *Gephyrocharax* in the Neverí and Manzanares rivers. The above mentioned events could also explain the current geographic distribution of *Corynopoma riisei*, a monospecific genus used as external group for the genetic analysis in this study. *C. riisei* is also present in Venezuela's Orinoco, Caribe (Tuy and Manzanares rivers), and Lake Valencia (La Cumaca and San Diego rivers) basins as well as in Colombia (Meta River and Colombia's Orinoco basin) and the island of Trinidad.

The genus *Gephyrocharax* should be of at least Middle Miocene age consistent with the drainage of the late Paleo-

Orinoco-Amazon in northwestern South America. The combined analysis of morphological and genetic information for this group along with biogeographic evidence, points toward a process of speciation with morphological stasis (Larson, 1989; Lundberg *et al.*, 1986). In Venezuela there seem to be three genetically distinct lineages that maintain very little differences in body shape.

Based on the results for the three species of *Gephyrocharax* in Venezuela, we propose that the dominant process in the diversification and distribution of the current Venezuelan continental ichthyofauna appears to be associated with changes in the course of the Orinoco River, whose basin represents 70.5% of the national territory (Mago-Leccia, 1970). We suggest that the freshwater fishes of Lake Maracaibo and the Venezuelan western Caribe region had a vicariant origin from those of the Paleo-Orinoco-Amazon. Older and more complex processes such as the formation of paleoarchs and marine incursions and regressions (Hubert & Renno, 2006) could explain the conformation of ichthyic regions in Maracaibo-Falcón and the northern slopes of the Cordillera de la Costa. The regions and ichthyofauna most closely related should correspond to Venezuela's eastern Caribe and Orinoco basins, since they have been most recently connected.

Due to the wide range of palaeogeographic processes associated with the origin and distribution of the continental fishes of South America in general (Hubert & Renno, 2006) and those of Venezuela in particular, it is important to carry out studies that incorporate morphological, genetic, and geomorphological aspects in order to answer questions about the processes that have led to the different taxa and to understand the possible factors that have shaped the distribution patterns of current freshwater fishes.

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