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Delimiting species by reproductive isolation: the genetic structure of epigean and hypogean *Trichomycterus* spp. (Teleostei, Siluriformes) in the restricted area of Torotoro (Upper Amazon, Bolivia)

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Abstract Genetic variability of *Trichomycterus* from the region of Torotoro (Bolivia, Upper Amazon), distributed in the same watershed where the habitat is structured by waterfalls, canyons and a cave, was studied by allozyme (twelve putative loci) and RFLP-mtDNA (DLoop and cytochrome *b*) analyses. Alloenzymatic variation studied by Correspondence Analysis and Maximum Likelihood Analysis revealed a four-group structure, which was largely congruent with the distribution of the 14 mtDNA haplotypes. Two of these four clusters (*I* and *II*) were differentiated by two diagnostic loci (IDH and G3PDH), two semi-diagnostic loci (PGM and 6PGDH) and consequently a very high F_{st} value (estimator $\theta = 0.77$). Therefore, clusters *I* and *II* are reproductively isolated. The distribution limit of these two (sibling) species does not correspond to those of the morphological species of *Trichomycterus* identified in this region: the epigean *T. cf. barbouri* and the hypogean *T. chaberti*. However, hypogean fish exhibited two mtDNA haplotypes, a private one and another shared with the epigean *Trichomycterus* from upstream reaches.

Keywords *Trichomycterus* · Allozymes · mtDNA · Amazon · Cavernicolous · Speciation · Maximum Likelihood Analysis

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Introduction

Teleosts are the most speciose group of cavernicolous vertebrates, with no less than 86 species that are troglomorphic (i.e., displaying specialisation for underground environments; Romero and Paulson 2001) and another 117 species reported as troglophiles (i.e., normally epigean but frequently visiting and exploiting hypogean resources; Poly and Boucher 1996; Poly 2001). There are at least 86 species of troglomorphic fishes, 23 being endemic to the North American continent and 16 to South America (Weber 2000; Romero and Paulson 2001). The apparent difference in the number of species according to latitude is mostly due to differences in survey effort. Nowadays most discoveries are taking place in tropical areas, so it is expected that hypogean diversity will be consistent with that of biodiversity in general, i.e., much higher in tropical than in temperate latitudes (see Romero 2001 for the historical context; Romero and Green 2005 for a latitudinal analysis).

Catfishes represent 26% of the 22 troglomorphic species of clariids, ictalurids, loricariids, pimelodids, silurids and trichomycterids (Baras and Laleye 2003; Trajano 2003), a proportion that is similar to their contribution to epigean species. However, knowledge of subterranean aquatic habitats is limited, and it is likely that the number and proportion of hypogean species of catfishes are greater, especially in tropical regions and among families or genera that comprise mainly or exclusively small-sized fish that may pass unnoticed, for example, the trichomycterids.

The Trichomycteridae, also known as pencil catfishes, are all endemic to South America. They comprise 171 species (Reis, Kullander and Ferraris 2003), including some parasite species as the parasitic *candirus* (or vampire catfish), and three hypogean species (Romero and Paulson 2001) that all belong to the genus *Trichomycterus*: *T. itacarambiensis* (Trajano and de Pinna 1996) in the Minas Gerais (Brazil), *T. conradi* (Eigenmann 1912) in Venezuela and *T. chaberti* (Durand 1968) in Bolivia. *T. chaberti* is endemic to a single cave of the Umajalanta stream in the Torotoro area. As with most other hypogean fish species, its description was based on morphological criteria (Durand 1968), including the reduction of the ocular apparatus and accentuated depigmentation, both traits being common to all hypogean species (Eigenmann 1909; Breder 1942; Poulson 1969; Yoshiyuki and Jeffery 2000). In the Torotoro area, an epigean *Trichomycterus* has been identified as *Trichomycterus cf. barbouri* (Eigenmann 1911), a species which has a broad distribution in the Bolivian Andes and their piedmont (Eigenman 1918; Arratia 1983; Arratia and Menu-Marque 1984; Burgess 1989; Fernández 1999). Pouilly and Miranda (2003) provided evidence that the life-histories of hypogean *T. chaberti* and epigean *T. cf. barbouri* differed substantially.

Mallet (1995) reviewed the different concepts used to define a species and discussed the limitation of their utilization according to the reproductive system (sexual or asexual) and the geographical situation (sympatric or allopatric) of the taxa. Mayden (1997) recognized the lineage-based evolutionary species concepts as a general theoretical definition of species, a point of view emphasized by Sites and Marshall (2003, 2004) who presented tree-based and non-tree based methods to identify species, the latter including the genetic distances. However, the phylogenetic species concept does not provide efficient methods in a restricted area of the geographical distribution of sympatric species, such as the Torotoro area for *Trichomycterus*. We therefore considered the use of the ''biological species concept'' BSC that is more suitable in this study for conceptual (sexual species, sympatric situation) and methodological reasons. All in all, the concept of biological species (Mayr 1942), far away from being obsolete, remains the most relevant in a sympatric situation in the case of sexual species, in a restricted area of the geographical distribution of the species (here, a small watershed). Distinct species are then identified from their inability to exchange genes or produce fertile progeny in a sympatric context, with no need to identify ecological, morphological or other kinds of differences between them.

It is widely admitted that hypogean fish originated from epigean relatives (Bichuette and Trajano 2003), and that the ancestors of troglomorphic species possessed one or several pre-adaptations to a hypogean life (Poulson 2001; Trajano 2001). However, Romero and Green (2005) argued that the ''evolution of hypogean fauna can be explained by well-known mechanisms within the current context of evolutionary biology''. In the absence of genetic studies, it is uncertain whether the hypogean fish currently described as *T. chaberti* in the Umajalanta cave, constitute a biological species that is distinct from *T. cf. barbouri*, or if they are the result of phenotypic plasticity. The goal of this research is to evaluate the gene flow among hypogean and various epigean samples of *Trichomycterus* in Torotoro park, a small part of its geographical distribution. In this small area the physical structure of the aquatic environment including caves, canyons and waterfalls could play an important role in genetic variability of the local trichomycterids. This study aims to test (i) whether morphological descriptions of epigean and hypogean *Trichomycterus* in the Torotoro National Park are supported by genetic differences and (ii) whether epigean *Trichomycterus* in this very limited Andean region are genetically structured, neither of which have been tested before.

Materials and methods

Study area

The study area is located at 18°05′ S, 65°45′ W, at an altitude ranging between 1,950 m and 3,000 m in the drainage basin of the Upper Bolivian Amazon in the Torotoro National Park (Department of Potosi, Bolivia). The Torotoro Park comprises a small (15 by 3 km) massif of Cretaceous limestone characterised by karstic phenomena, including caves and canyons. The local river network comprises four almost parallel rocky streams, which are located in canyons and flow

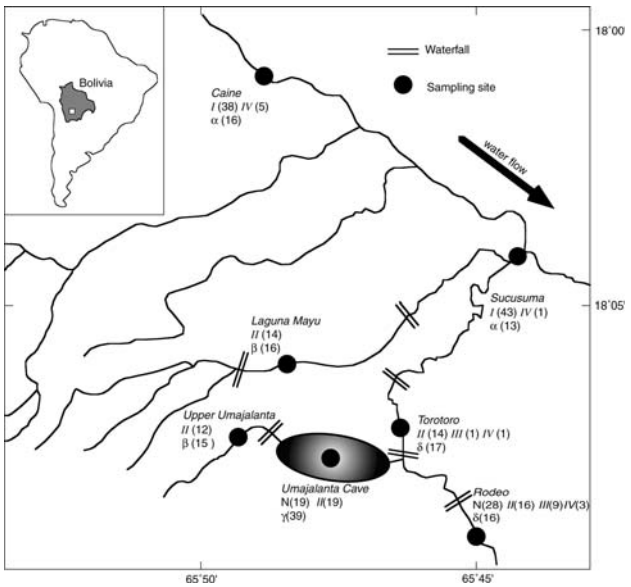

Fig. 1 Geographical distribution of epigean and hypogean *Trichomycterus* in the sampling area. The haplotypic variability of mtDNA is structured into four groups α, β, γ and δ and the isoenzymatic variability (nuclear DNA) into four clusters *I, II, III* and *IV*. The number of fish analysed in each cluster and each group is indicated between parentheses

into the Caine River, a tributary of the Rio Grande River (Fig. 1). The upper part of the southernmost stream, the Torotoro stream, is formed by two tributaries, namely the Rodeo and Umajalanta streams. The latter has a 3-km long subterranean stretch and flows into the Torotoro canyon through a 20 m high waterfall. The Umajalanta cave descends as far as 140 m into Upper Cretaceous formations (Chumacero 1991).

Sampling

Epigean *Trichomycterus* were sampled in six stations, located in six different streams (Fig. 1). The rivers Caine and Sucusuma are located in the valley, at altitudes of 1,980 and 2,020 m, respectively. The Laguna Mayu and Torotoro streams are located in canyons, at altitudes of 2,200 and 2,300 m, respectively. The Rodeo and Upper Umajalanta streams are located in small headwater valleys, at altitudes of 2,740 and 2,820 m, respectively. The subterranean part of the Umajalanta stream (altitude 2,500 m) was sampled as far as 0.5 km inside the cave.

All samples were collected in November 1998 and July 1999. Fish were collected with backpack electrofishing gear (Smith-Root Inc., http://www.smith-root.com), the electrode deployed near the bottom delivering a current of between 300 and 600 V. Electrofishing is a satisfactory method for this kind of habitat made up of rocky streams, as well as for studies of fragile or small populations.

In all study sites, fish of variable sizes (20–110 mm standard length) were captured in successive passages over sites of a few tens of metres in length. Sampling was continued until a maximum of 30 fish per site was captured, since the sizes of the populations were unknown. For logistical reasons, only a piece of epaxial muscle dissected on the site was preserved in 96° ethanol for mtDNA analysis during the first sampling (November 1998), whereas during the second sampling campaign (July 1999), whole fish were frozen in liquid nitrogen for allozyme assays, then stored in the laboratory for subsequent mtDNA analyses. Reference specimens were preserved at the Bolivian Collection of Fauna of the National Museum of Natural History of La Paz (Bolivia).

Polymorphism analysis of mitochondrial DNA

The total DNA was extracted using the phenol/chloroform technique of Sambrook, Fritsch and Maniatis (1989), and preserved at –20°C until analysis. An approximately 2-kb fragment of mtDNA, including the control region (D-Loop) and the gene coding for cytochrome *b*, was amplified by polymerase chain reaction (PCR) using the primers HN20 and VGLU that were identified by Bernatchez and Danzman (1993) and Briolay et al. (1998), respectively. The amplification conditions of each extract of DNA were: 5 µl of DNA extracted, 2.5 units of Taq polymerase with 1X buffer, 0.2 mM of dNTP, 1.5 mM of magnesium chloride, 0.5 µM of each primer, in a final volume of 50 µl. After a pre-PCR at 91°C for 1 min, 35 cycles were run from denaturation at 95°C for 1 min and annealing at 50°C for 1.5 min to elongation at 72°C for 2.5 min. The PCR was finished by a final elongation at 72°C for 10 min.

For these essays mtDNA of 132 fish were analysed by three restriction enzymes: Alu I; Rsa I and Hinf I. For this, 10 µl of the amplified product of PCR was digested by 2.5 units of endonuclease enzyme in 1X buffer. The digestion ran overnight at 37°C. The digested DNA was revealed on a 3% agarose gel stained by ethidium bromide.

Polymorphism analysis of nuclear DNA (allozymes)

The enzymatic electrophoresis on starch gels and the staining of gels were performed as described by Pasteur et al. (1987). The enzyme extracts were randomly placed on the gel so as not to influence the reading by the geographical origin of the samples.

When an individual fish could not be assigned a particular genotype, the genotype was ranked into the category of missing data so as to minimize the risk of error. The use of several controls on each gel facilitated the reading, while serving as stable references between gels. A total of 176 fish was analysed for 10 enzyme systems in the epaxial muscle: aspartate aminotransferase (AAT), adenylate kinase (AK), creatine kinase (CK), glucose 3-phosphate dehydrogenase (G3PDH), glucose phosphate isomerase (GPI), isocitrate dehydrogenase (IDH), malic enzyme (ME), mannose phosphate isomerase (MPI), 6-phosphogluconic dehydrogenase (6PGDH) and phosphoglucomutase (PGM).

Data analysis

The variability of mtDNA in the sampling was analysed through haplotypes that were defined by different combinations of restriction patterns (DNA fragment sets obtained after digestion by a restriction enzyme) according to Table 1. The differences in the haplotypic composition of samples taken two-by-two were measured by the θ estimator of F_{st} (Weir and Cockerham 1984). The significance of the observed value of θ was compared to the theoretical θ values obtained under the assumption there was no structure (null hypothesis). For this purpose, pair-wise comparisons between samples were tested over 1,000 permutations of the individual haplotypes. Finally, the percentage of theoretical θ values greater than the observed θ values gave the probability P that the observed value of F_{st} was due to chance.

For each sample at each locus, the enzyme data were used to calculate the rate of observed (H_{obs}) and expected unbiased (H_{nb}) heterozygosity (Nei 1978). A departure from mono- and multilocus (global equilibrium) panmixia was estimated by taxa using Weir and Cockerham's (1984) f estimator of F_{is} (Wright 1951). The significance of F_{is} was tested by permuting alleles in each taxon, for each locus individually and then for all of them to simulate panmixia.

Using the θ estimator of F_{st} (Weir and Cockerham 1984), the differentiation between samples belonging *a priori* to the same species was assessed by pair-wise comparisons. The differentiation between the different samples compared two-by-two (all the combinations using two samples) was tested using pair-wise permutations of the individual multilocus genotypes (see above).

The rates of observed (H_{obs}) and expected unbiased (H_{nb}) heterozygosity, the θ estimators of F_{st} and the f estimators of F_{is} were calculated using Genetix 3.0 software (Belkhir et al. 1998).

Within a sample, a departure from global equilibrium can indicate the existence of different equilibrated subpopulations in a same geographical

Table 1 Distribution of the haplotypes among the sampling sites classified into four (α, β, δ, and γ) groups

	Haplotypes	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Rsa I	a (600/400/300/300/250/250/130/80)	x	x												
	b (600/530/400/300/250/130/80			x	x	x									
	c (850/400/380/250/160/150/130/80)						x	x	x						
	d (1000/400/300/200/130/80)									x	x	x	x		
	e (1000/530/400/200/80)													x	x
Hinf l	a (1400/530/480)						x	x							
	b (1400/580/250)	x	x	x	x	x			x	x	x	x			
	c (1400/400/350)													x	x
	d (1000/580/400/350)												x		
Alu I	a (1200/650/130/100/80)						x		x						
	b (1040/650/200/130/100/80)							x		x					
	c (1040/480/200/130/100/80)	x		x							x		x	x	
	d (1040/600/200/130/100/80)		x		x							x			x
	e (1040/350/280/200/130/100/80)					x									
Caine, α		0	2	0	0	1	0	0	0	1	7	1	1	1	2
Sucusuma, α		0	0	0	0	3	0	0	0	1	4	0	1	0	4
Laguna Mayu, β		0	0	0	0	0	16	0	0	0	0	0	0	0	0
Upper Umajalanta, β		0	0	0	0	0	15	0	0	0	0	0	0	0	0
Umaja. Cave, γ		0	0	0	0	0	27	12	0	0	0	0	0	0	0
Rodeo, δ		4	0	1	3	0	6	0	2	0	0	0	0	0	0
Torotoro, δ		1	5		2	0	5	0	4	0	0	0	0	0	0

The 14 haplotypes are indicated by a capital letter from A to N. Each haplotype is determined by a combination of three restriction enzyme patterns marked by a cross among 14 possible patterns indicated by a lower-case letter. For each restriction enzyme pattern the estimated weights of the observed mtDNA fragments are given in parentheses. In each site and group the number of haplotype is indicated in the bottom portion of the table

sample. To detect such hidden subpopulation structure and assign individuals to their original taxon, two types of analyses were used independently with the same data: the Correspondence Analysis (CA) and the Maximum Likelihood analysis (ML), using Genetix 3.0 software (Belkhir et al. 1998) and PartitionML software (Belkhir and Bonhomme 2002; available at http://www. http://www.univ-montp2.fr/%7Egenetix/labo.htm#programmes), respectively.

The CA is not a statistical test *sensu stricto*, as it gives no probability but it provides structural information and identifies the responsible variables. It was carried out to define consistent genetic groups, each of them being constituted of genetically similar individuals, as defined by their allelic combinations or by private alleles. To run this analysis for each locus and for each individual, the alleles were coded 0 when absent, 1 when heterozygous, and 2 when homozygous. As a corollary, a homozygous allele has twice as much weight as a heterozygous one.

The ML calculation is similar to that in the genetic mixture analysis model used by Smouse, Waples and Tworek (1990) on predefined stocks and wild populations of Pacific salmon. Alternatively, and without any *a priori* knowledge about the source populations, the software PartitionML implements a ''Simulated Annealing'' method (Kirkpatrick, Gelatt and Vecchi 1983). This method runs several numbers of underlying source populations contributing to a sample (while assigning individuals to each of them). The most probable partition is structured in various clusters, each of which provides the minimum departure from panmixia.

In this study, the modified genetic clusters revealed by the CA and the ML were then compared and treated again by the θ estimator of F_{st} and by the f estimator of F_{is}. Null hypotheses were rejected at $P \leq 0.05$.

No phylogenetic analyses are proposed because of the small area studied compared with the large geographical distribution and the number of *Trichomycterus* species in the Andes.

Results

Mitochondrial DNA

Fourteen haplotypes (denoted A to N) were revealed. They had a geographical distribution in four (α, β, γ and δ) groups (Fig. 1): (α) Caine and Sucusuma Rivers; (β) Upper Umajalanta (upstream of the cave) and Laguna Mayu streams; (γ) Umajalanta cave; and (δ) Rodeo and Torotoro streams. The pair-wise distributions using the F_{st} estimator were always significant at $P \leq 0.001$.

The upstream β-group was characterized by the sole F-haplotype. In the γ-group, the F-haplotype (69%) was accompanied by the G-haplotype (31%). The α-group was characterized by the E, I, J, K, L, M and N private haplotypes. The δ group, formed by the samples from the Rodeo and Torotoro rivers, was characterized by the A, C, D and H private haplotypes, and shared the B-haplotype with the α-group, and the F-haplotype with the β- and γ-groups (Table 1).

Allozymic variability

The 10 enzyme systems revealed 12 putative loci, of which 11 were polymorphic (Table 2). A strong differentiation between all the sampling sites was observed, with the F_{st} estimator θ values ranging from 0.21 ($P \leq 0.001$) to 0.79 ($P \leq 0.001$), except between three pairwise comparisons: Rodeo and Torotoro rivers ($\theta = 0.09$, $P \leq 0.05$), Upper Umajalanta and Umajalanta Cave ($\theta = 0.04$, $P > 0.05$) and Sucusuma and Caine rivers ($\theta = 0.00$, $P > 0.05$). In the polymorphic samples, all of them had significant F_{is} multilocus values, ranging between 0.33 and 0.76 except for the Laguna Mayu stream. Samples from the Upper Umajalanta stream and Umajalanta cave showed almost null F_{is} values, because they were almost monomorphic (Table 2).

Partial congruence between allozymic and mitochondrial structure

The first three axes of the CA (Fig. 2a, b) accounted for 19%, 18% and 9% of total variance, respectively. The statistically robust PartitionML test ($P < 0.001$, d.f. = 30) and the CA differentiated the same four genetic clusters, with only two individuals out of 176 (1.14%) assigned differently by the two methods.

Cluster (*I*) was composed of 81 fish from the downstream reaches, distributed in the Caine (47%) and Sucusuma Rivers (53%). It had the same geographical distribution as the α-haplotype group. In the CA, cluster *I* was positioned on the positive side of axis *I* because of a fixed allele (IDH (046)) with a frequency (freq.) = 1, that was absent from the opposite cluster *II* and private alleles with a high frequency (6PGDH (225), freq. = 0.96; PGM (153), freq. = 0.92) that were absent from cluster *II* (Table 2).

Cluster (*II*) was composed of 75 fish from the upstream reaches, distributed in the Laguna Mayu (19%), Upper Umajalanta (16%), Rodeo (21%) and Torotoro streams (19%), and in the Umajalanta cave

Table 2 Allelic frequencies for each allozymic locus for each of the geographical samples and genetic clusters

	Caine	Sucusuma	Laguna. Mayu	Upper Umajalanta	Umaja. Cave	Rodeo	Torotoro	Cluster *I*	Cluster *II*	Cluster *III*	Cluster *IV*
AAT1											
N	*41*	*44*	*14*	*12*	*19*	*28*	*16*	*79*	*75*	*10*	*10*
044	0.11	0.10	0.00	0.00	0.00	0.02	0.00	0.10	0.00	0.00	0.15
065	0.78	0.85	0.00	0.00	0.00	0.02	0.00	0.85	0.00	0.00	0.25
082	0.01	0.03	0.00	0.00	0.00	0.25	0.06	0.03	0.00	0.80	0.00
100	0.04	0.01	1.00	1.00	1.00	0.64	0.88	0.01	1.00	0.20	0.10
105	0.06	0.00	0.00	0.00	0.00	0.07	0.06	0.01	0.00	0.00	0.50
H_{obs}	0.24	0.30	0.00	0.00	0.00	0.03	0.00	0.29	0.00	0.00	0.10
H_{nb}	0.38	0.26	0.00	0.00	0.00	0.53	0.23	0.26	0.00	0.33	0.68
F_{is}	0.36**	–0.12	–	–	–	0.93***	1.00***	–0.12	–	1.00*	0.86***
AAT2											
N	*43*	*43*	*14*	*12*	*19*	*27*	*16*	*80*	*74*	*10*	*10*
090	0.00	0.00	0.00	0.00	0.00	0.11	0.06	0.00	0.01	0.30	0.00
100	0.95	0.98	1.00	1.00	1.00	0.70	0.31	0.99	0.78	0.70	0.80
125	0.05	0.02	0.00	0.00	0.00	0.19	0.63	0.01	0.20	0.00	0.20
H_{obs}	0.00	0.00	0.00	0.00	0.00	0.22	0.38	0.00	0.16	0.00	0.00
H_{nb}	0.09	0.05	0.00	0.00	0.00	0.47	0.52	0.02	0.35	0.44	0.34
F_{is}	1.00***	1.00**	–	–	–	0.52**	0.29	1.00**	0.53***	1.00***	1.00*
AK											
N	*43*	*44*	*14*	*12*	*19*	*28*	*16*	*81*	*75*	*10*	*10*
100	1.00	1.00	1.00	1.00	1.00	0.96	1.00	1.00	1.00	0.90	1.00
105	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.10	0.00
H_{obs}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H_{nb}	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.19	0.00
F_{is}	–	–	–	–	–	1.00*	–	–	–	1.00	–
CK											
N	*43*	*44*	*13*	*12*	*18*	*28*	*16*	*81*	*73*	*10*	*10*
089	0.01	0.00	0.00	0.08	0.00	0.00	0.00	0.01	0.01	0.00	0.00
100	0.87	0.93	1.00	0.92	1.00	0.93	0.94	0.97	0.99	1.00	0.10
105	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
132	0.12	0.02	0.00	0.00	0.00	0.07	0.06	0.00	0.00	0.00	0.90
H_{obs}	0.02	0.00	0.00	0.17	0.00	0.00	0.00	0.01	0.03	0.00	0.00
H_{nb}	0.23	0.23	0.00	0.16	0.00	0.13	0.12	0.06	0.03	0.00	0.18
F_{is}	0.90***	1.00***	–	–0.05	–	1.00***	1.00***	0.79***	–0.01	–	1.00
G3PDH											
N	*26*	*37*	*13*	*11*	*17*	*26*	*13*	*62*	*68*	*8*	*5*
010	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.38	0.00
050	0.96	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00
100	0.04	0.00	1.00	1.00	1.00	0.81	1.00	0.00	1.00	0.38	1.00
200	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.25	0.00
H_{obs}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H_{nb}	0.08	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.70	0.00
F_{is}	1.00**	–	–	–	–	1.00***	–	–	–	1.00***	–
GPI 1											
N	*40*	*36*	*9*	*9*	*16*	*19*	*14*	*70*	*58*	*6*	*9*
003	0.49	0.51	0.22	0.00	0.03	0.26	0.04	0.54	0.07	0.67	0.06
006	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
100	0.50	0.47	0.78	1.00	0.97	0.74	0.96	0.45	0.93	0.33	0.94
H_{obs}	0.38	0.36	0.22	0.00	0.06	0.21	0.07	0.39	0.10	0.33	0.11
H_{nb}	0.52	0.52	0.37	0.00	0.06	0.40	0.07	0.51	0.12	0.48	0.11
F_{is}	0.28	0.31	0.41	–	0.00	0.48	0.00	0.25*	0.20	0.33	0.00
GPI 2											
N	*38*	*34*	*10*	*9*	*16*	*16*	*13*	*67*	*54*	*7*	*8*
010	0.03	0.01	0.00	0.00	0.00	0.53	0.31	0.02	0.17	0.50	0.00
100	0.54	0.49	1.00	1.00	1.00	0.34	0.62	0.49	0.83	0.50	0.56
200	0.43	0.50	0.00	0.00	0.00	0.13	0.08	0.49	0.00	0.00	0.44
H_{obs}	0.23	0.29	0.00	0.00	0.00	0.44	0.00	0.26	0.07	0.42	0.12
H_{nb}	0.52	0.52	0.00	0.00	0.00	0.60	0.54	0.52	0.28	0.53	0.52
F_{is}	0.55***	0.44*	–	–	–	0.28	1.00***	0.49***	0.73***	0.22	0.77

Table 2 continued

	Caine	Sucusuma	Laguna. Mayu	Upper Umajalanta	Umaja. Cave	Rodeo	Torotoro	Cluster *I*	Cluster *II*	Cluster *III*	Cluster *IV*
IDH											
N	*43*	*44*	*14*	*12*	*19*	*28*	*16*	*81*	*75*	*10*	*10*
046	0.88	0.98	0.00	0.00	0.00	0.32	0.06	1.00	0.00	1.00	0.00
100	0.12	0.02	1.00	1.00	1.00	0.68	0.94	0.00	1.00	0.00	1.00
H_{obs}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H_{nb}	0.21	0.04	0.00	0.00	0.00	0.44	0.12	0.00	0.00	0.00	0.00
F_{is}	1.00***	1.00**	–	–	–	1.00***	1.00*	–	–	–	–
ME											
N	*42*	*44*	*14*	*12*	*19*	*27*	*16*	*81*	*75*	*10*	*8*
060	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.15	0.13
100	1.00	1.00	1.00	1.00	1.00	0.91	1.00	1.00	1.00	0.85	0.88
H_{obs}	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.27	0.23
H_{nb}	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.25	0.22
F_{is}	–	–	–	–	–	0.36	–	–	–	–0.13	1.00
MPI											
N	*43*	*43*	*14*	*12*	*18*	*28*	*15*	*80*	*73*	*10*	*10*
084	0.14	0.13	0.00	0.00	0.00	0.29	0.07	0.13	0.00	0.90	0.10
100	0.86	0.87	1.00	1.00	1.00	0.71	0.93	0.87	1.00	0.10	0.90
H_{obs}	0.14	0.16	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.00
H_{nb}	0.24	0.22	0.00	0.00	0.00	0.41	0.13	0.23	0.00	0.19	0.19
F_{is}	0.43*	0.28	–	–	–	1.00***	1.00***	0.29	–	1.00	1.00
6PGDH											
N	*16*	*32*	*6*	*11*	*15*	*21*	*8*	*48*	*51*	*7*	*3*
050	0.06	0.00	0.75	0.00	0.00	0.07	0.19	0.02	0.15	0.00	0.00
100	0.00	0.00	0.25	1.00	1.00	0.60	0.81	0.00	0.85	0.00	1.00
150	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
225	0.91	0.98	0.00	0.00	0.00	0.14	0.00	0.96	0.00	0.43	0.00
230	0.00	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.57	0.00
255	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
H_{obs}	0.06	0.03	0.50	0.00	0.00	0.14	0.12	0.04	0.13	0.00	0.00
H_{nb}	0.18	0.03	0.41	0.00	0.00	0.59	0.33	0.08	0.25	0.52	0.00
Fis	0.66	0.00	–0.25	–	–	0.76***	0.63	0.49*	0.46**	1.00**	–
PGM											
N	*38*	*36*	*13*	*12*	*16*	*25*	*14*	*70*	*68*	*8*	*8*
062	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
080	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
100	0.08	0.00	0.81	1.00	1.00	0.60	0.86	0.00	0.96	0.00	0.38
112	0.00	0.00	0.00	0.00	0.00	0.08	0.07	0.00	0.00	0.00	0.38
135	0.08	0.07	0.04	0.00	0.00	0.00	0.00	0.08	0.01	0.00	0.00
153	0.84	0.93	0.00	0.00	0.00	0.32	0.07	0.92	0.00	1.00	0.25
H_{obs}	0.16	0.14	0.23	0.00	0.00	0.00	0.00	0.16	0.04	0.00	0.00
H_{nb}	0.28	0.13	0.34	0.00	0.00	0.54	0.26	0.15	0.07	0.00	0.70
F_{is}	0.44*	–0.06	0.34	–	–	1.00***	1.00**	60.08	0.39	–	1.00***
Multilocus H_{obs}	0.10	0.11	0.08	0.01	0.01	0.10	0.05	0.11	0.05	0.09	0.03
Multilocus H_{nb}	0.23	0.16	0.09	0.01	0.01	0.39	0.19	0.15	0.09	0.31	0.25
Multilocus F_{is}	0.55***	0.33***	0.15	–0.04	0.00	0.75***	0.76***	0.29***	0.51***	0.72***	0.89***

An allele is indicated by a three-digit number, *N* indicates the number of fish analysed, H_{nb} is the unbiased average expected heterozygosity, H_{obs} is the average observed heterozygosity, F_{is} is the estimator *f* of the departure from panmixia, with *, ** and *** representing a discrepancy from panmixia at $P \leq 0.05$, $P \leq 0.001$ and $P \leq 0.001$, respectively

(25%). Its geographical distribution overlapped totally with those of the β- and γ-groups of haplotypes and partially with the δ-group. In the CA, cluster *II* was opposed to cluster *I* on the first axis (negative coordinates) because they had different fixed alleles (G3PDH (050), IDH (046) in cluster *I*) and (G3PDH (100), IDH (100) in cluster *II*). The frequent allele (AAT1 (100), freq. = 1) in cluster *II* was rare in the cluster *I* (freq. = 0.01). Private alleles with a high frequency in the cluster *II* (6PGDH (100),

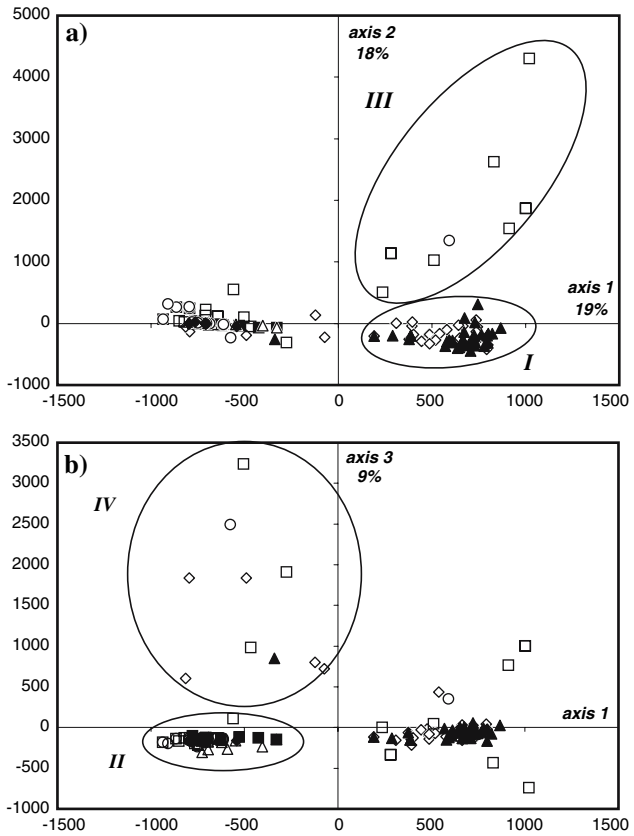

Fig. 2 Factorial correspondence analysis (CA), showing the projection of the 176 sampled fish according to their allelic combinations for each of the 12 loci on the factorial plane, of the axes 1 and 2 (**a**) and on the factorial plane of the axes 1 and 3 (**b**). In each factorial plane the positioning of each fish is indicated by a symbol in relation with its geographical origin: (◆) Upper Umajalanta, (■) Umajalanta cave system, (◇) Caine, (△) Laguna Mayu, (□) Rodeo, (▲) Sucusuma and (◯) Torotoro streams. Each ellipse delimits a cluster named *I*, *II*, *III* or *IV*

freq. = 0.85; PGM (100), freq. = 0.96) were absent from cluster *I* (Table 2).

Two loci were therefore diagnostic between cluster *I* and *II* (G3PDH, IDH), two were semi-diagnostic with private alleles having a frequency greater than 0.80 (6PGDH, PGM) and one was close to being diagnostic (AAT1).

Cluster (*III*) was composed of 10 fish from the upstream reaches, distributed in the Rodeo (90%) and Torotoro streams (10%). It had a geographical distribution inside that of the δ-group. In the CA, cluster *III* was located on positive coordinates of both the first and second axes, because of private alleles with a high frequency (AAT1 (82), freq. = 0.80; AAT2 (90), freq. = 0.30; AK (105), freq. = 0.10; G3PDH1 (200), freq. = 0.25; G3PDH1 (10), freq. = 0.38; 6PGDH (230), freq. = 0.57) that were absent from cluster *IV* and because of a higher allelic frequency of MPI1 (84) in cluster *III* than in cluster *IV* of 0.90 and 0.10, respectively (Table 2).

Cluster (*IV*) included 10 fish from the Caine (50%), Sucusuma (10%), Rodeo (30%) and Torotoro (10%) rivers, but none from the Umajalanta and Laguna Mayu streams. It had a geographical distribution overlapping those of the δ- and α-groups. In the CA, cluster *IV* was positioned on the negative side of the second axis and on the positive side of the third axis, because of private alleles with a high frequency (AAT1 (105), freq. = 0.50; CK (132), freq. = 0.90; PGM (112), freq. = 0.38) that were absent from cluster *III* (Table 2).

Inside the clusters *I* and *II* defined by the CA and PartitionML, only the F_{is} in the Rodeo stream (belonging to cluster *II*) was not significantly different from zero, providing no basis for rejecting the hypothesis of panmixia. The sampling component of the clusters *III* and *IV* was not analysed by F_{is} because the sample sizes were too small. Inside all the clusters the F_{st} differentiation between geographical samples was maintained, except between the Sucusuma and Caine rivers in cluster *I*.

Pair-wise comparisons of F_{st} estimators θ of all four clusters ranged from 0.56 ($P \leq 0.001$) to 0.78 ($P \leq 0.001$), indicating significant differences between all clusters. Furthermore, all clusters differed significantly from panmictic equilibrium (Table 2).

Clusters *III* and *IV* which occurred in sympatry in the Torotoro and Rodeo streams had a complex genome, which was mainly composed of homozygote genotypes for each locus with alleles observed in clusters *I* and *II* or with private alleles. This produced a mosaic of homozygote and mainly cluster-specific loci that largely accounted for the high F_{is} values.

Discussion

The CA and PartitionML analyses independently showed strong genetic structure in the *Trichomycterus* of Torotoro National Park, with various levels of genetic differentiation ranging from population to species. These contrasting results indicated that the taxonomic organization of local populations should be revised, at least in part.

Alloenzymatic analyses revealed no difference between the *Trichomycterus* from the Umajalanta cave and those from the upper Umajalanta stream, and indicated that both groups of fish were almost monomorphic. This low degree of polymorphism contrasts with the marked morphological differences between the epigean and hypogean *Trichomycterus*, and among

hypogean *Trichomycterus* (Pouilly and Miranda 2003). Morphological differences between epigean and hypogean fish could be the consequence of a phenotypic plasticity, as observed in certain forms of *Astyanax* (Herwig 1976) or of a much higher speed of morphological differentiation than genetic differentiation (Culver 1969; Berrebi and Valiushok 1998). Cases of low variability in the nuclear DNA of hypogean species or populations have been reported in many taxonomically distant families, either from RAPD (Balitoridae: Borowsky and Vidthayanon 2001) or isoenzymatic analyses (Characidae: Avise and Selander 1972; Amblyopsidae: Swofford, Branson and Sievert 1980; Trichomycteridae: Perez and Moodie 1993). Furthermore, Perez and Moodie (1993) found no genetic difference between the hypogean and epigean populations of *Trichomycterus* spp. in the Guacharo area (Venezuela), as was the case in the present study on Bolivian *Trichomycterus*. Using RAPDs and microsatellites, Panaram and Borowsky (2005) observed considerable genetic diversity in both hypogean and epigean *Astyanax mexicanus* populations and suggest that there was an introgression of alleles from surface populations to hypogean populations. This last observation could explain partially the apparent nuclear DNA homogenization between the *Trichomycterus* from the upper Umajalanta stream and those from the Umajalanta cave. However, the absence of polymorphism cannot be used to test whether the hypogean fish are reproductively isolated. *T. chaberti* would either be a recently formed species, in the beginning of differentiation, or must be considered as a synonym of *T. cf. barbouri*, the epigean species from the upper Umajalanta.

While no genetic difference could be detected from the analysis of allozyme variation, the mtDNA analysis indicated that 31% of the *Trichomycterus* sampled in the cave had a private G-haplotype, and that the other 69% had the F-haplotype which is typical of the *Trichomycterus* in the Upper Umajalanta and Laguna Mayu streams. This last observation suggests that exchanges between the populations of these two headwater streams occurred recently or are ongoing, possibly through one or several unrevealed subterranean streams. Population mixing between hypogean and epigean populations has already been observed. This situation could led to hybridization, as observed in the Characidae *Astyanax fasciatus* between a blind depigmented population at the cave locality and eyed pigmented fish from a nearby surface population (Romero 1983), or to the full replacement of the hypogean population by an epigean of the same species in little as 50 years, as observed in the Pimelodidae *Rhamdia quelen* (Romero et al. 2002). The G-haplotype in the cave might indicate the beginning of differentiation in the subterranean environment. The D-loop, in particular its control region, varies much faster than the nuclear genome (Shedlock et al. 1992; Simon and Francesco 1994; Lee et al. 1995), thereby being a good marker of recent phenomena. Mitochondrial DNA, in contrast to nuclear DNA, is maternally transmitted. The occurrence, in the cave population, of a private mtDNA haplotype and the absence of nuclear DNA variation specifying allozymes might therefore simply imply that gene flow is sex-dependent with females being more sedentary than males. The occurrence of the G- and F-haplotypes in the cave, in contrast to the systematic occurrence of the F-haplotype and the absence of the G-haplotype in the upstream reaches, might indicate that the gene flow between hypogean and epigean fish is strongly asymmetric because the waterfalls impede or prevent upstream dispersion.

Speciation

This study revealed the existence of four clusters, whose distributions are mainly delimited by waterfalls: cluster *I* occurs in the lower reaches, clusters *II* and *III* in the upper reaches and cluster *IV* is found in all streams, except in the Umajalanta and Laguna Mayu streams. Waterfalls could limit upstream but not downstream dispersion by eggs, larvae, juveniles or adults that could survive the downstream passage. In spite of the possible contact between fish from clusters *I* and *II* living in the same watershed with possible rare migratory exchanges between upstream and downstream (about 1.14%), these clusters are separated by two diagnostic loci with fixed alleles and by two semi-diagnostic loci with private alleles having high frequencies (>0.80) in one cluster and absent in the other. Therefore, clusters *I* and *II* are reproductively isolated in the same watershed. According to Mayr's (1942) definition of biological species, as applied to sympatric sexual species, clusters *I* and *II* therefore constitute two different species, whose limits do not correspond to those of the morphological species *Trichomycterus cf. barbouri* and *T. chaberti*. In the absence of detailed morphological descriptions, it is uncertain whether these two species refer to one or two new species of *Trichomycterus*. These data emphasize the importance of using genetic tools to quantify the degree of reproductive isolation for solving issues pertaining to biosystematics and associated disciplines, in particular when sibling species are concerned. Other recent discoveries of sibling species, based on molecular analyses, can be found in several fish families, as

for example in the South American catfishes of the family Loricariidae (Zawadzki, Reis and Renesto 2000; Fish-Muller, Mazzoni and Weber 2001; Zawadzki et al. 2004).

This study revealed a departure from mono- and multilocus (global equilibrium) panmixia from the significant deficit of heterozygotes in all four clusters of *Trichomycterus*, and in almost all sampling sites. This indicates that gene exchange does not occur randomly within clusters or within sites, as if there were subpopulations within each of them (Wahlund effect) or selection against heterozygotes (Hartl and Clark 1989; Gouyon and Henry 1998). However, a more detailed analysis of clusters *III* and *IV* indicates that most individual genotypes are organised as a mosaic of homospecific and mainly homozygote loci, which can be accounted for neither by the subpopulation hypothesis nor by the counterselection of heterozygotes. Another hypothesis, which would match all cases described here, could be a special breeding system combining facultative self-fertilizing hermaphrodism and outcrossing-fertilization. Laughlin et al. (1995) reported the existence of self-fertilizing hermaphrodism in the opportunistic strategist *Rivulus marmoratus* (Rivulidae), which lives in very small populations in small temporary marshes. Situations where the probability of encountering sexual partners is low or null as, for example, if no more than a single fish survives in a marsh, might have favoured the selection of self-fertilising hermaphrodism. This selection does not preclude the possibility of outcrossing fertilisation, which is eventually necessary to regenerate genetic variability. A self-fertilising hermaphrodism system is totally compatible with limited dispersal behaviour explaining the mtDNA structure observed. At present, there is not a single study on how these trichomycterids breed and on whether self-fertilising hermaphrodism might occur. Nevertheless, it is tempting to suggest that this breeding system would be a major advantage for colonising new environments, including the hypogean ones, where the probability of meeting sexually mature partners is probably lower than in any other environment. It is worth noting that this hypothesis does not contradict the mechanisms depicted above for the colonisation of the cave and for the maintenance of private mtDNA haplotypes inside the cave.

Perspectives

This study raised many questions and functional hypotheses, which will require further field and experimental studies. In particular, sampling effort should be extended geographically so as to determine the genetic variability and phylogeography of clusters (or sibling species) using sequences of mtDNA. A higher variable molecular marker such as microsatellites would be more informative for further study and more likely to reveal Umajalanta cave/stream *Trichomycterus* relationships. Experimental studies of captive *Trichomycterus* of epigean and hypogean origin in controlled environments would also be valuable to further characterise their ecophysiology and behaviour, and to evaluate the respective contributions of the environment and genotype on life-history and morphological traits to test for the effects of phenotypic plasticity. Rearing fish in captivity could also determine whether the self-fertilising hypothesis and hermaphrodism that was formulated on the basis of the genetic mosaics found in the wild is relevant. The answers to these questions will not be provided soon, because of the difficulties in sampling remote nature reserves, as well as the difficulties in the captive breeding of hypogean fishes due to long generation lengths. Captive stocks exist for only six of the 26 ''species'' of hypogean catfishes, and only those of two species of *Rhamdia* breed regularly in captivity (Proudlove 2001).

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