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Short Communication

Molecular phylogeny of the genus *Pseudoplatystoma* (Bleeker, 1862): Biogeographic and evolutionary implications

J.P. Torrico [a,d,*], N. Hubert [a,1], E. Desmarais [d], F. Duponchelle [b,1], J. Nuñez Rodriguez [a,1], J. Montoya-Burgos [e], C. Garcia Davila [c], F.M. Carvajal-Vallejos [b,g], A.A. Grajales [f], F. Bonhomme [d], J.-F. Renno [a,1]

[a] IRD UR 175/IBMB, Universidad Mayor San Andrés, Facultad de Ciencias Puras y Naturales, Campus Universitario Cota Cota, La Paz, Murillo, Bolivia
[b] IRD UR 175/ULRA, Universidad Mayor San Simón, Cochabamba, Bolivia
[c] Instituto de Investigaciones de la Amazonía Peruana (IIAP), Laboratorio de Biología Molecular y Biotecnología (LBMB), Av. Abelardo Quiñónes km. 2.5, Iquitos, Peru
[d] Département Biologie Intégrative, Institut des Sciences de l'Evolution, UMR 5554 Université de Montpellier 2 cc 63 Pl. E Bataillon F34095 Montpellier Cedex 5, France
[e] Département de Zoologie et Biologie Animale, Université de Genève, 30 quai Ernest Ansermet, 1211 Genève 4, Switzerland
[f] Departamento de Sistemas de Produccion Agropecuaria, Programa de Medicina Veterinaria y Zootecnia, Facultad de Ciencias Agropecuarias, Universidad de Caldas. Calle 65 No. 26-10 Manizales, Colombia
[g] Asociación FAUNAGUA, final Av. Max Fernández, Zona Arocagua, Sacaba, Cochabamba, Bolivia

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1. Introduction

In the last years molecular genetics approaches have allowed to assess cryptic patterns of diversity within and among remnant populations of threatened and endangered species. Along with inferred levels of current or historic gene flow, and demographic history, molecular data could help planning and executing conservation policies (Vrijenhoek, 1998). This is particularly true with groups of migratory freshwater fishes of high economic value such as some species of the family Pimelodidae, one of the most speciose groups of Neotropical Siluriformes (50–60 genera, 300 species; Reis et al., 2004). Largely distributed throughout South and Central America, this group of piscivorous and carnivorous species contains some of the largest and most important species for commercial and subsistence fisheries. The genus *Pseudoplatystoma* (Bleeker, 1862) is, in addition, a resource of growing importance for aquaculture (Nuñez et al., 2008). *Pseudoplatystoma* species are known to undertake complex lateral migrations between rivers, lakes and river floodplains as well as longitudinal movements (300–700 km) along river channels (Barthem and Goulding, 1997; Loubens and Panfili, 2000; Coronel et al., 2004). It is worth noting that some *Pseudoplatystoma* populations are already considered threatened due to overexploitation, hydroelectric projects, mining, deforestation and contamination (Carolsfeld et al., 2003).

The large distribution ranges of the *Pseudoplatystoma* species, encompassing the main drainages of South America, has led to postulate that some level of cryptic diversity may exist beyond an apparent morphological homogeneity (Buitrago-Suarez and Burr, 2007). Large geomorphologic and physiographic processes have transformed South American river drainages through the entire Miocene and Pliocene providing opportunities for vicariance but also for secondary contact through headwater captures, as evidenced for several groups (Montoya-Burgos, 2003; Albert et al., 2006; Lovejoy and Araujo, 2000; Hubert and Renno, 2006; Hubert et al., 2007; Willis et al., 2007). This idea has fostered a comprehensive reassessment of morphological and anatomical characters within the genus *Pseudoplatystoma* (Buitrago-Suarez and Burr, 2007). It has been proposed that the inability to recognize cryptic or sibling species using traditional morphological characters may hinder the understanding of ecological and evolutionary processes with negative consequences such as the underestimation of species richness, the overestimation of potential for long-distance dispersal, the failure to recognize biological invasions and the misinterpretation of ecological and paleoecological data (Rocha-Olivares et al., 2001). Until very recently, three species were recognized within the genus *Pseudoplatystoma* (Fig. 1a–d): *P. fasciatum* (Linnaeus 1766), widely distributed in the Paraná, Amazon, Orinoco, Magdalena basins and Guyana shield's rivers; *P. tigrinum* (Valenciennes 1840) in Orinoco and Amazon basins and *P. corruscans* (Spix and Agassiz 1829) restricted to the Atlantic basins, Paraná and São Francisco. Buitrago-Suarez and Burr (2007) have then raised the number of recognized species to eight on the basis of morphological analyses: *P. punctifer* and *P. tigrinum*

* Corresponding author. Address: IRD UR 175/IBMB, Universidad Mayor San Andrés, Facultad de Ciencias Puras y Naturales, Campus Universitario Cota Cota, La Paz, Murillo, Bolivia.
E-mail address: jptb_bioevol@yahoo.com (J.P. Torrico).
[1] Present address: IRD, UR 175, BP 5095, 361 rue J.F. Breton, 34196 Montpellier Cedex 05, France.

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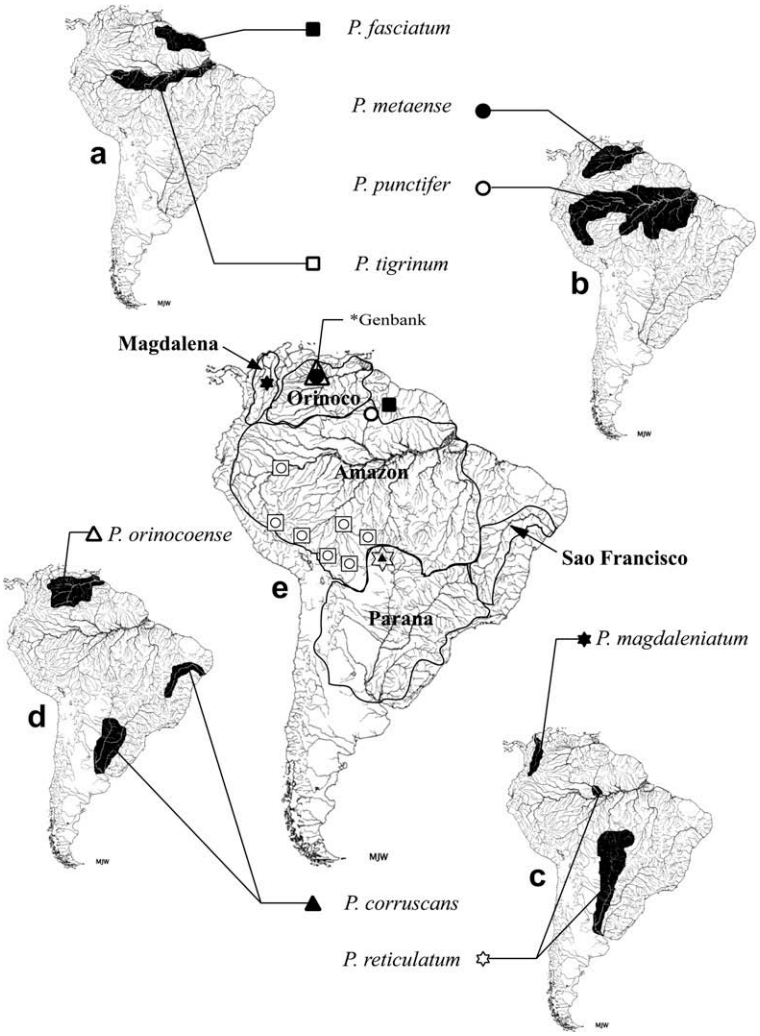

Fig. 1. Distribution areas for the eight recognized species of the Neotropical catfish genus *Pseudoplatystoma* according to Buitrago-Suarez and Burr, 2007 (a–d) and sampling localities (e). Major hydrological basins are illustrated. Each species is represented by a geometric object, while combined objects represent localities where species are found in sympatry.

sympatric in the Amazon Basin, *P. metaense* and *P. orinocense* sympatric in the Orinoco Basin, *P. corruscans* and *P. reticulatum* partially sympatric in the Paraná, *P. magdaleniatum* restricted to the Magdalena basin and *P. fasciatum* to the Guyana shield rivers (Fig 1). However, their sampling lacked the Upper Madera, an important part of the Amazon basin.

The aims of the present study were thus: (1) to define species boundaries within the genus *Pseudoplatystoma* using a molecular phylogenetic approach with new data from the Upper Madera to compare the results with the recent morphology-based revision and (2) to asses the biogeographic patterns within the genus.

2. Materials and methods

2.1. Sampling

A total of 212 *Pseudoplatystoma* were sampled between 2000 and 2005 in the Upper Madera basin in the Madre de Dios-Beni, Mamoré, Iténez Rivers and in the Ucayali-Upper Amazon basin. Outside the Amazon basin, sampling was performed in the upper portion of the Paraná basin (Laguna Cáceres), in the Corantijn and in the Ireng River (a tributary of the Amazon system from the Guyana shield) and in the Magdalena River from the Magdalena basin (Table 1). Individuals were identified in the field according to previous identification key (Lauzanne and Loubens, 1985). The diagnostic characters taken into consideration were: (1) the head morphology, *P. fasciatum* is characterized by a robust head of homogeneous width; whereas *P. tigrinum* has a slimmer head, slightly compressed in the middle; (2) the coloration pattern, *P. fasciatum* presents a striped pattern where a white stripe is always observed beside each conspicuous black stripe; *P. tigrinum* color pattern consists of reticulated dark lines; (3) The middle frontal bone fontanel is larger and wider in *P. tigrinum* than in *P. fasciatum*. According to Buitrago-Suarez and Burr (2007)'s revision, those species correspond to the following: in the Guyana Shield *P. fasciatum* stays unchanged (*P. fasciatum sensu stricto*), in the Upper Madera and Ucayali-Upper Amazon Basins *P. fasciatum* becomes *P. punctifer* and *P. tigrinum* remains unchanged, in the Paraná-Paraguay Basin *P. fasciatum* becomes *P. reticulatum*. *P. corruscans* is unchanged. Finally in the Magdalena Basin *P. fasciatum* becomes *P. magdaleniatum*. *Pseudoplatystoma* specimens were not sampled in the Orinoco but five *control region* (*CR*) partial sequences for *P. metaense* (EU082463 and EU082462) and *P. orinocoense* (EU082461, EU082460, EU040286) were recovered from Genbank. Phylogenetic outgroups included three *Brachyplatystoma rousseauxi* (Pimelodidae) collected in the region of Iquitos (Upper Amazon) and *B. vaillanti* (DQ779047) for the *CR*, while the *Cytochrome-b* (*Cyt-b*) data set included also thirteen species (10 genera) of related Siluriformes (Sullivan and Lundberg, 2006). Vouchers were preserved

Table 1
Sampling point location and number of sequenced individuals for both molecular markers (*Cyt-b* and *CR*).

			Voucher[a]	Number of samples per species											
				P. punctifer		*P. fasciatum*		*P. magdaleniatum*		*P. reticulatum*		*P. tigrinum*		*P. corruscans*	
				Cyt-b	*CR*	*Cyt-b*	*CR*	*Cyt-b*	*CR*	*Cyt-b*	*CR*	*Cyt-b*	*CR*	*Cyt-b*	*CR*
Upper Madera															
Madre de Dios	Puerto Maldonado	12°35′14″S–69°10′13″W	PF30422 to PF30427 PT30418 to PT30422	0	9							2	7		
	Manuripi	11°16′44″S–67°40′07″ W	PF3799, PF3817, PF3878,PF3879, PF3880, PF3881, PT4062	7	11							1	4		
Beni	Puerto Salinas	14°20′00″S–67°32′00″W	PF3633, PF3634, PF3635, PF4943, PF4944, PT3631	7	13							2	7		
Mamoré	Securé	15°22′53″S–65°01′15″W		7	10							10	17		
	Ichilo	17°04′55″S–64°37′22″W		3	7							11	9		
	Yata	10°58′01″S–65°36′38″W	PF5094,PF5321,PF5323, PF5324, PF5326, PFPF5328,PF5331	3	7							3			
Iténez	San Martin	13°44′11″S–63°55′02″W	PF3303	7	13							7	6		
	Paraguay	13°31′19″S–61°40′28″W	PF5363, PF5406, PF5463, PF5464, PF5504, PF5549, PT5364, PT5551, PT5551, PT5838	11	17							11	5		
Paraná															
Paraguay	Laguna Caceres	19°03′05″S–57°49′19″W	Unregistered							5	4			5	5
Northern South America															
Branco	Ireng	03°53′35″N–59°41′09″W	GUY04-218	1	1										
Corantijn	Wonotobo falls	04°38′05″S–54°24′14″ W	SU05-522			1§	1§								
Magdalena		07°44′22″S–76°20′39″W	MHN-UC 004 to 0044					7	5						
Ucayali-Upper Amazon															
Ucayali	Pucallpa	08°23′18″S–74°30′16″W	IIAP-30360 to 30364 IIAP-455 IIAP-30283 to 30286 IIAP-30293	0	9							0	12		
Upper Amazon	Iquitos	03°45′19″S–73°12′35″W	IIAP-30185 to 30189 IIAP-30113 to 30117	1	10							2	10		

[a] Vouchers from Bolivia, French Guyana, Colombia and Peru, are conserved in the Limnology Unit of the Instituto de Ecología (UL-IE-La Paz-Bolivia), in the Museum National d' Histoire Naturelle (MNHN, Genève – Switzerland), in the Muséo de Historia Natural at the Universidad de Caldas (MHN-UC, Caldas-Colombia), and the Instituto de Investigación de la Amazonía Peruana (IIAP, Iquitos – Peru), respectively.

for posterior study (Table 1) in the "Unidad de Limnología" from the "Instituto de Ecología" (UL - IE, La Paz - Bolivia), the "Museum National d'Histoire Naturelle" (MNHN, Genève – Suisse), "Museo de Historia Natural de la Universidad de Caldas" (MHN-UC, Colombia) and in the "Instituto de Investigacion de la Amazonia Peruna" (IIAP, Iquitos – Péru).

2.2. Molecular markers

Among Neotropical Siluriformes, the *Cyt-b* has been used recently to infer deep phylogenetic relationships (Perdices et al., 2002; Hardman and Lundberg, 2006). As the *Cyt-b* gene contains both slowly and rapidly evolving codon positions, as well as conservative and variable regions, it constitutes a suitable marker for phylogenetic purposes at various divergence levels (Farias et al., 2001). Alternatively, the mitochondrial *CR* is a non-coding stretch of DNA that usually exhibits higher rates of molecular evolution. The mtDNA has proved a reliable indicator of species boundaries and geographical population structure (Zink and Barrowclough, 2008), moreover, recent barcoding studies support the view that lineage sorting is challenging the interpretation of patterns of mtDNA variability in a limited number of cases in the wild, at least for fishes (Hubert et al., 2008). In consequence, we used concomitantly the *Cyt-b* and *CR* to assess species boundaries and phylogenetic relationships within the genus *Pseudoplatystoma*.

DNA was extracted using the DNeasy Tissue Kit (Qiagen). Primers for *Cyt-b* amplification were L15162: 5′-GCAAGCTTCTACCATG AGGACAA-3′ (Taberlet et al., 1992) and H15915: 5′-AACTG CAGTCATCTCCGGTTTACAAGAC-3′ (Irwin et al., 1991), and for *CR*, DL20F: 5′-ACCCCTAGCTCCCAAAGCTA-3′; and DL20R: 5′-CCTGAAG TAGGAACCAGATGA-3′ (Agnèse et al., 2006). PCR reactions were run in a total volume of 50 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.3 mM of each dNTP, 20 pmol of each primer, 5 units of Taq DNA polymerase and 5 μl of the Qiagen extract. They were carried out following a touchdown procedure including a first 2 min denaturation step at 95 °C, then 10 standard cycles with an annealing temperature starting at 64 °C for *Cyt-b* or 66 for *CR*, with a 1 °C temperature decrement; finally 25 cycles with 1 min at 92 °C, 1 min at 54 °C for *Cyt-b* or 56 °C for *CR* and 1 min 30 s at 72 °C. Post-PCR extension was carried out for 5 min at 72 °C. PCR products were sequenced on both directions by automatic sequencing. Sequences alignment was optimized by eye using BIOEDIT (Hall, 1999). Sequences were deposited in GenBank (Accession Nos. FJ889681 to FJ889882 for *CR* and Nos. FJ889883 to FJ889986 for *Cyt-b*).

2.2.1. Phylogenetic construction

Both maximum likelihood (ML) and Bayesian methods were applied to the *Cyt-b* (708 bp) and *CR* (974 bp) data sets using the programs PhyML (Guindon and Gascuel, 2003) and BEAST v1.4.8 (Drummond and Rambaut, 2008), respectively. The best fit concerning nucleotide substitution model, existence of invariable sites (I) and rate heterogeneity across variable sites (Γ) was selected among 28 alternative evolutionary models according to the Akaike Information Criterion (AIC) using the R-based Package Ape (Paradis and Strimmer, 2004; Paradis, 2006). Confidence in the estimated relationships of the ML tree topologies was evaluated by a bootstrap analysis with 1000 replicates (Felsenstein, 1985). Multiple independent runs of BEAST were performed for each mtDNA region. Each run consisted of 10^7 chains sampled at intervals of 1000 generations with a burn-in of 10^6 for the *Cyt-b* and 2×10^6 chains, sampled at 1000 generations intervals and a burn-in of 2×10^5 for the *CR*. Independent runs were merged with LogCombiner v1.4.8 (Drummond and Rambaut, 2008). Convergence of chains to the stationary distribution was checked by visual inspection of plotted posterior estimates using the program Tracer v1.4 (Rambaut and Drummond, 2007) and until the effective sample size for each parameter sampled from the Markov chain Monte Carlo (MCMC) analysis was found to exceed 200.

Using the estimated ML tree, molecular dating was performed using the penalized likelihood (PL) method (Sanderson, 2002). This method assumes that rates of substitution change smoothly along contiguous branches to result in the branch lengths estimated by ML. The trade-off established between a parametric component which assumes that the tree is clock-like, and a non-parametric component where rates vary according to the ML tree, is controlled by a smoothness parameter denoted λ. The optimal value of λ was selected by cross-validation according to Sanderson (2002) using λ values ranging from 0.1 to 10^6. The geographic distribution of *P. magdaleniatum* and the remaining *Pseudoplatystoma* species seems to fit a vicariance event that is likely to illustrate the establishment of the Magdalena basin through an orogenic rise at 11.8 million years ago—Mya—(Hoorn et al., 1995; Lundberg, 1998) thus, this event was used as a calibration point in agreement with previous studies (Sivasundar et al., 2001). This method was performed with the R-based Package Ape (Paradis and Strimmer, 2004; Paradis, 2006).

Haplotype diversity and mean nucleotide divergences (Nei, 1987) were calculated for each DNA region with Arlequin v.3.0 (Excoffier et al., 2005).

3. Results

Maximum likelihood and Bayesian trees were built for the *Cyt-b* and *CR* sequences, according to the selected GTR + I + Γ model (Log-Likelihood = −5020.1408; I = 0.404; γ = 0.889) and TN93 + Γ model (Log-Likelihood = −4177.4349; γ = 0.420), respectively. The combined information (ML and Bayesian) of the *Cyt-b* and the *CR* phylogenies was largely concordant (Fig. 2a and b). In further sections, the Bayesian method will not be considered given the uncertainty associated with posterior probabilities interpretation (Suzuki et al., 2002; Mossel and Vigoda, 2005; Steel and Matsen, 2007). The phylogenetic inference corroborates, partially, the classification of Buitrago-Suarez and Burr (2007): *P. tigrinum*, *P. reticulatum*, *P. corruscans* and *P. magdaleniatum* are indeed differentiated into monophyletic groups; while *P. fasciatum* and *P. punctifer* are found in admixture and so are *P. orinocoense* and *P. metaense*.

For both, *Cyt-b* and *CR* sequences, molecular divergence was partitioned in three discrete categories (Table 2). Higher levels of divergence were observed in comparisons involving either *P. magdaleniatum* or *P. corruscans* against all the remaining species (from 0.056 ± 0.014 to 0.076 ± 0.016 for *Cyt-b* and from 0.064 ± 0.016 to 0.082 ± 0.005 for *CR*), *P. magdaleniatum* being the most divergent. On the other hand, pairwise comparison involving *P. fasciatum*, *P. punctifer* and *P. reticulatum* yielded low divergence values ranging from 0.006 ± 0.001 to 0.011 ± 0.005 for *Cyt-b* and from 0.013 ± 0.001 to 0.023 ± 0.010 for *CR*. Finally, intermediate values (ranging from 0.029 ± 0.001 to 0.033 ± 0.002 for *Cyt-b* and from 0.047 ± 0.001 to 0.049 ± 0.005 for *CR*) were obtained whenever *P. tigrinum* sequences were confronted with any of the former three species (*P. fasciatum*, *P. punctifer* and *P. reticulatum*).

Molecular dating was performed by PL. The cross-validation analysis yielded a λ value of 1×10^7 for the *Cyt-b* and 10 for the *CR*. For increasing values of λ, the variations are smoother tending to a clock-like model. Accordingly, the estimated substitution rates (in expected number of substitution per million years—Myrs—and per site) was found to be nearly constant for the *Cyt-b* (mean = 2.5 - 10^{-3}, SD = 2.2×10^{-6}), while the estimated substitution rates for *CR* varied between 3.6×10^{-2} and 9.4×10^{-5} (mean = 6.2×10^{-3}, SD = 6.1×10^{-3}). The divergence between *P. magdaleniatum* and *P. corruscans* was found to be synchronous at around 11.8 million years ago (Mya). The ancestor of the Orinoco's species (*P. orinoco-*

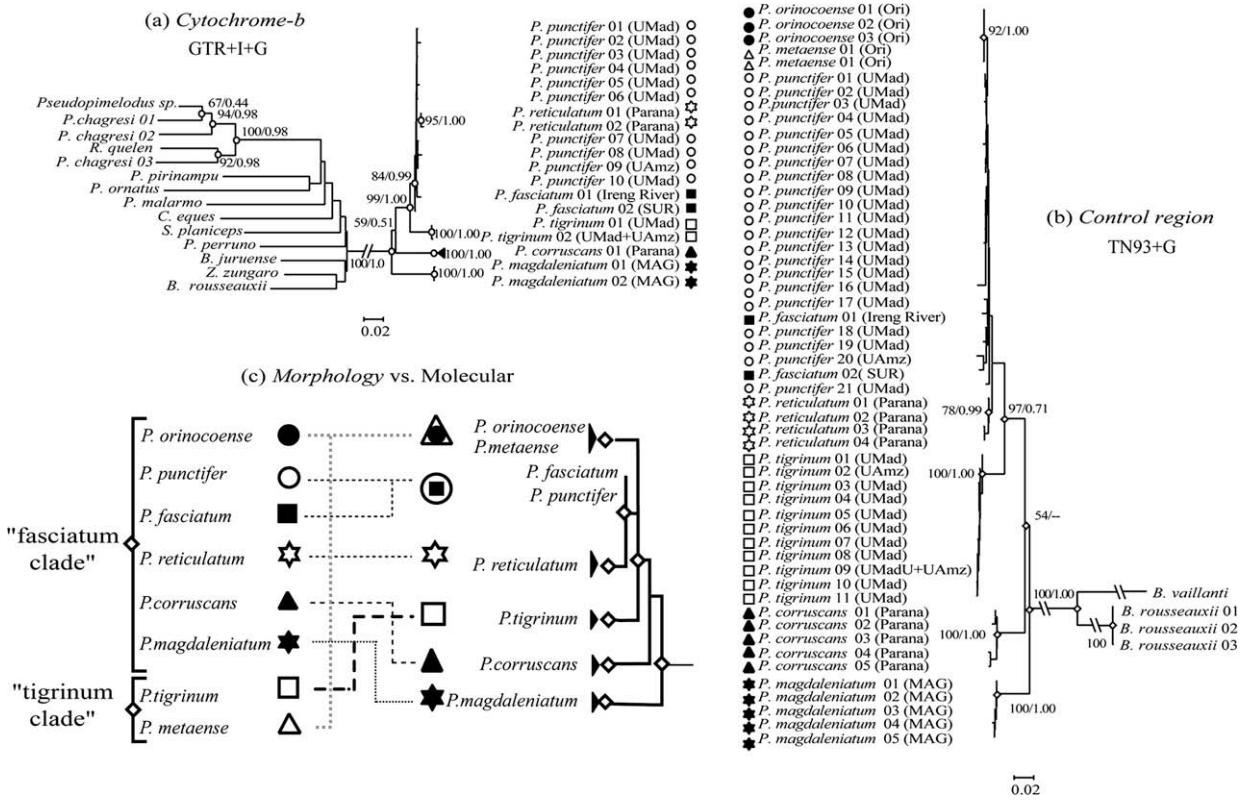

Fig. 2. Maximum likelihood phylogenies for the genus *Pseudoplatystoma* and comparison with morphology-based systematics (Buitrago-Suarez and Burr, 2007): (a) *Cytochrome-b* (GTR + *I* + Γ, I = 0.404, γ = 0.889); (b) *CR* (TN93 + Γ, γ = 0,420); Bootstrap values above 51% and posterior probabilities above 0.5 are indicated in each node; (c) congruence between morphology and molecular data. Different geometric symbols correspond to different species; diamonds are bootstrap supported nodes (*Cyt-b*|*CR*); UMad stands for Upper Madera, UAmz for Ucayali-Upper Amazon, MAG for Magdalena basin, and SUR for Surinam.

Table 2
Divergence among *Cyt-b* and *CR* between six different *Pseudoplatystoma* species.

	P. magdaleniatum Divergence (TN93 +G)	*P. corruscans*	*P. tigrinum*	*P. punctifer*	*P. reticulatum*	*P. fasciatum*
Cytochrome-b						
P. magdaleniatum	–					
P. corruscans	0.076 ± 0.016	–				
P. tigrinum	0.072 ± 0.006	0.070 ± 0.007	–			
P. punctifer	0.065 ± 0.005	0.056 ± 0.005	0.029 ± 0.001	–		
P. reticulatum	0.064 ± 0.014	0.055 ± 0.014	0.033 ± 0.002	0.006 ± 0.001	–	
P. fasciatum	0.065 ± 0.022	0.058 ± 0.023	0.031 ± 0.005	0.007 ± 0.001	0.011 ± 0.004	–
Control region						
P. magdaleniatum	–					
P. corruscans	0.064 ± 0.016	–				
P. tigrinum	0.083 ± 0.006	0.071 ± 0.005	–			
P. punctifer	0.074 ± 0.004	0.071 ± 0;004	0.047 ± 0.001	–		
P. reticulatum	0.077 ± 0.021	0.067 ± 0.002	0.048 ± 0.004	0.014 ± 0.001	–	
P. fasciatum	0.081 ± 0.032	0.073 ± 0.003	0.049 ± 0.006	0.013 ± 0.001	0.023 ± 0.009	–

ense and *P. metaense*) diverged from the remaining species at around 8.2 Mya. The divergence of *P. tigrinum* from the ancestor of *P. fasciatum*, *P. punctifer* and *P. reticulatum* yielded an interval ranging from 6 Mya for the *Cyt-b* and 10.4 Mya for the *CR*. The divergence between *P. reticulatum* and *P. punctifer* yielded an interval ranging from 1.5 Mya (*Cyt-b*) to 0.8 Mya (*CR*).

4. Discussion

4.1. Molecular systematic and biogeography

The analyses of molecular data support several aspects of the morphology-based classification proposed by Buitrago-Suarez and Burr (2007), but some other aspects remain contentious. First, morphology and molecular data support the monophyly of the genus *Pseudoplatystoma* and show a complex distribution array, encompassing also the Bolivian Amazon for *P. punctifer* and *P. tigrinum*, a region that was not sampled by Buitrago-Suarez and Burr (2007). The molecular data also show that *P. tigrinum*, *P. corruscans*, *P. reticulatum* and the novel *P. magdaleniatum* are highly supported clades, validating their taxonomic status. On the other hand, a major discrepancy exists concerning the relationships between the different species that has important repercussions for their biogeography. According to Buitrago-Suarez and Burr (2007), morphological characters suggested a two clade partition for the genus (Fig. 2c): a "*P. fasciatum* clade" including *P. fasciatum*, *P. punctifer*, *P. reticulatum*, *P. orinocoense*, *P. magdaleniatum* and *P. corruscans*; and a "*P. tigrinum* clade" grouping *P. tigrinum* and *P. metaense*. This

dichotomy is not consistent with the molecular data which instead support five main clades: *P. mataense* grouped with *P. orinocoense* (Bootstrap, 92% for the *CR*), *P. reticulatum* (Bootstrap value 95% and 78% for *Cyt-b* and *CR*, respectively), *P. tigrinum* (100% for both *Cyt-b* and *CR*), *P. corruscans* (100% for both *Cyt-b* and *CR*) and *P. magdaleniatum* (100% for both *Cyt-b* and *CR*). Strikingly *CR* sequences corresponding to *P. metaense* (formerly *P. tigrinum*) and *P. orinocoense* (formerly *P. fasciatum*) from the Orinoco basin resulted in a single supported clade with no differentiation between these two morphologically distinct species. This surprising result which suggests either a mtDNA introgression between the two species or a misidentification of the samples requires further investigation. Likewise, it was not possible to differentiate *P. punctifer* (Amazon) from *P. fasciatum* (Guyanas) at the molecular level. This could result from translocation of individuals or introgression events or inadequate species identification. However, as no haplotypes were shared between the Guianan and the Amazonian samples the translocation or introgression scenarios seem unlikely. On the other hand, inadequate external identification can not be invoked as no significant morphological difference were found between both species (Buitrago-Suarez and Burr, 2007) and as they can only be distinguished by their geographical location and minor osteological features. The absence of phylogenetic and clear morphological differentiation between *P. fasciatum* and *P. punctifer* suggests that Buitrago-Suarez and Burr, 2007 erroneously separated *P. fasciatum* into two distinct species and therefore invalidates the taxonomic status of *P. punctifer*.

Three main geographical groups were observed within the genus *Pseudoplatystoma*: Magdalena (*P. magdaleniatum*), Paraná (*P. corruscans*) and Amazon (*P. tigrinum*). Although the general features of South America's geological evolution are still under discussion (Garzione et al., 2008; Lundberg, 1998) our dating of the differentiation between the main Amazonian basins are consistent with Hoorn et al. (1995) and Lundberg (1998), who dated the establishment of the Magdalena, Paraná and Amazon basins during the Late Miocene between 11.8 and 10 Mya and the primary isolation of the Orinoco between 8.0 and 5.0 Mya.

The use of the early isolation of the Magdalena basin as a reference for establishing a molecular calibration (11.8 Mya, Lundberg, 1998) resulted in a low rate of molecular evolution for *Pseudoplatystoma* mitochondrial genome. In fact, the mean substitution rates for both mitochondrial regions (0.25% per Myrs for *Cyt-b* and 0.62% per Myrs for the *CR*) are probably among the lowest published so far for fishes. For instance, Bermingham et al. (1997) proposed a *Cyt-b* substitution rate for marine fishes of 1.0–1.3% per Myrs which has been frequently used for mitochondrial DNA evolution. More recently, Sivasundar et al. (2001) and Hubert et al. (2007) reported estimates ranging from 0.84% to 0.57% per site per Myrs for Characiformes *CR*, while Barluenga and Meyer (2004) proposed a substitution rate between 6% and 7% per Myrs, for Cichlids. On the other hand, Hardman and Lundberg (2006) found low rates of molecular substitution for the *Cyt-b* (0.38–0.53% per Myrs) and the nuclear *rag2* genes (0.075–0.089% per Myrs) for the related 'phactocephalines' Pimelodidae, which suggests, along with the present results, that lower rates of molecular evolution may characterize Neotropical Siluriforms. However, as it has been clearly established recently in another group of Vertebrates, mitochondrial mutation rates are not expected to be uniform across groups of taxa (Nabholz et al., 2008).

According to the PL calibrated molecular clock, the estimated age for the differentiation of *P. corruscans* (11.8 Mya) is consistent with the split of the Paraná-Paraguay and Amazon basins at the low Miocene (Lundberg, 1998) as is the inferred date of vicariance of the Orinoco's species (*P. orinocoense* and *P. metaense*) at around 8.0 Mya (Hoorn et al., 1995). Before the final establishment of the Amazon, between 15 and 5 Mya, the last series of massive marine incursions took place. Those events have been previously suggested to have promoted allopatric speciation in elevated regions such as Brazil and Guyana Shields and Andean foreland (Hubert and Renno, 2006). This vicariant event could be at the origin of the differentiation of *P. tigrinum* and its sister lineage (*P. fasiatum*, *P. punctifer*, *P. reticulatum*) since both distribution data and age estimates are concordant with this scenario. Subsequently, during the last 4 Myrs the marine regression followed by the establishment of the Amazon's main channel would have allowed further colonization of the central Amazon lowlands (Museum hypothesis; Haq et al., 1987; Hoorn, 1993). Nevertheless more data are needed in order to test this hypothesis.

Finally, the relatively young ages inferred for the divergence of *P. reticulatum* (found in the Paraná and the Amazon Basins) between 0.8 and 1.5 Mya was not consistent with the primary establishment of the Paraná at the Late Miocene (Hoorn et al., 1995; Lundberg, 1998). This likely results from recent speciation in relation with unknown vicariant events. Previous authors evidenced that headwater capture events and temporary connections between the headwaters of the Amazon and the Paraná promoted speciation by long-distance dispersal and further allopatric divergence (Lovejoy and Araujo, 2000; Montoya-Burgos, 2003; Hubert et al., 2007). The existence of dispersal routes between the Guyanas, Orinoco, Amazon and Paraná basins (Hydrogeology hypothesis; Montoya-Burgos, 2003; Hubert and Renno, 2006; Hubert et al., 2007) is consistent with the large geographical distributions of *P. reticulatum* in the Paraná and Amazon basins. An historical exchange zone between the Amazon and the Paraná basins has also been evidenced for several fish species (Hubert et al., 2007), and might also explain the extant distribution of *P. reticulatum*. The existence of dispersal routes is also consistent with the extant distribution of *P. punctifer*, differentiated neither morphologically (Buitrago-Suarez and Burr, 2007) nor genetically from *P. fasciatum*, in the Amazon and Guyanas basins. Indeed, a connection currently exists between the Guyana and Amazon basins through the inundated savannah of the Rupununi, which connects the Rio Branco (Amazon) to the Essequibo River (Lowe-McConnell, 1964). This connection was also evidenced through genetic analysis (Lovejoy and Araujo, 2000; Willis et al., 2007), providing an explanation for the extant distribution of *P. fasciatum*. The role of the Rupununi savannah in the evolution of the genus *Pseudoplatystoma* was also recognized by Buitrago-Suarez and Burr (2007). Nevertheless, further complementary sampling and analysis are necessary to define the precise nature of the relationship between the Orinoco species.

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