

Molecular identification of a cryptic species in the Amazonian predatory catfish genus *Pseudoplatystoma* (Bleeker, 1962) from Peru

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Abstract *Pseudoplatystoma* species are highly prized South American Pimelodid migratory catfishes. Until recently, their taxonomy was not clearly established, with discrepancies between morphological and molecular analyses. Here, *Pseudoplatystoma* species from the Peruvian Amazon were characterized at the molecular level from a sample representing the observed range of their color pattern variations in the study area. Analyses were performed using seven microsatellite loci for 103 specimens and, for part of them (52), using sequences of two regions of their mitochondrial genome [Cytochrome Oxidase subunit I (COI) and Control Region (CR)]. Factorial correspondence analysis and assignment tests based on microsatellite polymorphism showed that the specimens originally identified as *P. punctifer* belonged to two different gene pools highly differentiated from *P. tigrinum*. Morphological examination identified two different morphotypes (with

and without black stripes), suggesting the existence of two distinct taxa within *P. punctifer*. This result was corroborated by the ML tree based on CR sequences, where all individuals but four clustered in a similar way as in the FCA and Bayesian assignment tests. For these four individuals, mitochondrial introgression or retention of ancestral polymorphism was likely. In contrast, the ML tree based on COI sequences showed that reciprocal monophyly was not yet achieved for this marker for the two *P. punctifer* taxa. The existence of three sympatric species of *Pseudoplatystoma* in the Peruvian Amazon is discussed in relation to their molecular characteristics, color patterns and ecology. Evolutionary scenarios regarding their divergence are hypothesized.

Keywords Microsatellites · Control Region · Barcoding · Migratory catfish

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Introduction

Amazonia covers approximately two-thirds (747,282 km²) of the Peruvian territory. In the Peruvian Amazon, catches are dominated by Characiformes and Siluriformes including Pimelodidae family, among which species of the genus *Pseudoplatystoma* Bleeker 1862, are the most abundant (Cañas 2000; Tello and Bayley 2001; Garcia et al. 2009). These are large predatory species of high economic value and are widely distributed in the main basins of South America (Orinoco, Guyana, Magdalena, Parana-Paraguay and Amazon basins), from the lowland to the Andean piedmont, in the main channels, floodplains and small creeks of the rainforest, but infrequent or absent in the estuary (Barthem and Goulding 1997, 2007).

Until recently, the specific diversity of this genus was underestimated and poorly documented, owing to the lack of clear taxonomical studies to define morphological boundaries among species. Only three *Pseudoplatystoma* species were recognized: *P. corruscans* (Spix and Agassiz 1829) in the Parana-Paraguay basin; *P. tigrinum* (Valenciennes 1840) in the Amazon basin; and *P. fasciatum* (Linnaeus 1766) in the Amazon basin. In a recent systematic review based on morphological characters, Buitrago-Suárez and Burr (2007) defined eight species. Three species were already described: *P. fasciatum*, which is now restricted to the Guayana shield basins; *P. corruscans*, in the Paraná basin; and *P. tigrinum*, which is widely distributed in the Amazon basin. Two species were rehabilitated: *P. punctifer* (Castelnaud 1855), which is widely distributed in the Amazon basin, and *P. reticulatum* (Eigenmann and Eigenmann 1889) in the Paraná-Paraguay basin. Three species were newly described: *P. orinocoense* (Buitrago-Suárez and Burr), *P. metaense* (Buitrago-Suárez and Burr), both from the Orinoco basin, and *P. magdaleniatum* (Buitrago-Suárez and Burr) from the Magdalena basin. In a subsequent molecular phylogeny of the genus *Pseudoplatystoma* using mtDNA analyses (CR), however, Torrico et al. (2009) could not separate *P. fasciatum* from *P. punctifer*, or *P. orinocoense* from *P. metaense*, and suggested synonymy for these species. More recently, a new phylogenetic revision of the genus based, on mitochondrial (cytochrome b) and nuclear (Rag1 intron 1, and S7 intron 1) markers, recognized only four species within the genus: *P. magdaleniatum*, *P. corruscans*, *P. tigrinum* and *P. punctifer* (Carvalho-Costa et al. 2011).

Morphological identification in *Pseudoplatystoma* is essentially based on the number of vertebrae, body shape and color patterns. In the Loreto (Peruvian Amazon) striped catfish belonging to *Pseudoplatystoma* are called “doncella” (*P. punctifer*) and “tigre zungaro” (*P. tigrinum*). In this Amazonian region, color patterns of *P. punctifer* are strongly variable, creating confusion in their identification (Agudelo

et al. 2000). Indeed, this species presents a wide range of pigmentation patterns, ranging from the typical patterns with black and white vertical stripes laterally distributed over the body, to the absence of vertical black lines. These variations could represent the expression of an intraspecific polymorphism or indicate the presence of new species inside *P. punctifer*. This study characterized the genetic variability of the *Pseudoplatystoma* species in the Peruvian Amazon around Iquitos, using both nDNA (microsatellite loci) and mtDNA (CR and COI) from a sample representing the widest possible range of their color pattern variations.

Materials and methods

Sampling

During the period from August 2009 to August 2011, 103 adults specimens of *P. punctifer* (83) and *P. tigrinum* (20) presenting the widest possible range of variation in color pattern were collected at the Belen market of Iquitos (Loreto, Peru). We collected all the specimens found with uncommon color pattern but only some specimens presenting the common pattern (see Buitrago-Suárez and Burr 2007). In doing so, we gave a higher representation to specimens with rare patterns in the sampling. This intentional bias in term of natural relative abundance was necessary to get enough rare specimens for proper comparisons with common specimens. Iquitos is the largest fishing port in the Peruvian Amazon. According to the Regional Office of Production (DIREPRO) catfishes sold in this market come from the vicinity of Iquitos in an area including the lower Ucayali River, the lower Marañon River, and the middle Amazon River (Fig. 1).

Each specimen sampled was measured, photographed and approximately 1 cm³ of muscle was collected and preserved in ethanol for later DNA extraction. To be conserved as reference specimens, 24 *P. punctifer* and 5 *P. tigrinum* individuals were chosen to cover the best range of color pattern variation observed in the Belen market. These vouchers are deposited at the ichthyological collection of the “Instituto de Investigaciones de la Amazonía Peruana” (IIAP, Iquitos, Peru) and the fish collection of the Naturhistorisches Museum Dresden (MTD F, Dresden, Germany).

DNA extraction, amplification and genotyping of microsatellite loci

DNA extraction was performed on 100 ng of muscle tissue preserved in 96 % alcohol following the CTAB extraction protocol modified from Doyle and Doyle (1987). DNA amplification for 7 microsatellite loci was done using primers designed for *P. corruscans* (Revaldaves et al.

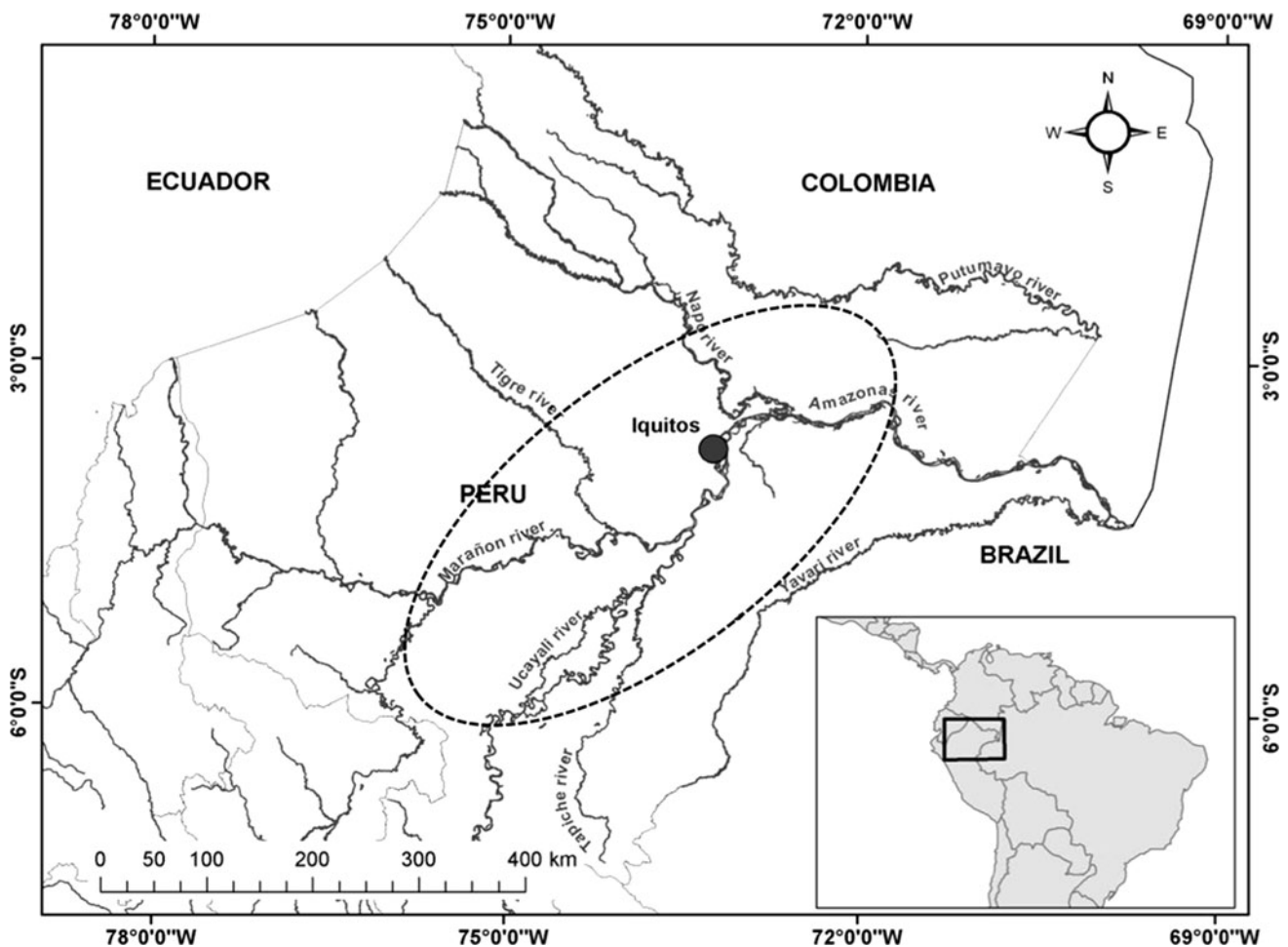


Fig. 1 Map of the Loreto region, indicating the area of influence (broken line ellipse) of the Pimelodid catfish fisheries landing their products in the Iquitos market (source PRODUCE)

2005) in a total volume of 10 μ l with native DNA (0.5 μ l), Taq polymerase (5U/ μ l), Buffer (5X), $MgCl_2$ (25 mM), dNTPs (10 mM), primer (10 μ M). PCR program was a first denaturing cycle at 92 $^{\circ}C$ for 2 min, then 30 cycles (denaturing at 95 $^{\circ}C$ during 1 min, one annealing cycle at optimal temperature for each primer during 1 min and elongation at 72 $^{\circ}C$ during 1 min) and a final extension cycle at 72 $^{\circ}C$ during 40 min.

The amplified segments were denatured and separated by capillary electrophoresis on a sequencer (Applied Biosystems 3130XL). For each capillary a solution of 1 μ l of the PCR product, 8.6 μ l of formamide and 0.4 μ l of GeneScan 500 ROX (fragment length marker) was used. The molecular weight of each microsatellite allele was obtained using Peak Scanner version 1.0 (<https://products.appliedbiosystems.com>).

Amplification and sequencing of mtDNA fragments

Sequencing of Cytochrome Oxidase subunit I gene (COI) and of Control Region (CR) was performed on 52

specimens (10 *P. tigrinum* and 42 *P. punctifer*) presenting the widest possible range of color pattern variation. The COI and CR fragments were amplified using FishF1 5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3' and FishR1 5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3' primers (Hubert et al. 2008), and DL20F: 5'-TTA GCA AGG CGT CTT GGG CT-3' (Agnès et al. 2006) and DL20R: 5'-ACC CCT AGC TCC CAA AGC TA-3' primers (Agnès, courtesy), respectively. For the two fragments, PCR amplifications were performed in 15 μ l of mix solution containing: ultra pure water (6.78 μ l), PCR buffer 5 \times (3 μ l), $MgCl_2$ 25 mM (0.9 μ l), each primer 10 μ M (0.6 μ l), mix dNTP 2 mM (1.5 μ l), polymerase Taq 5 U/ μ l (0.12 μ l), native DNA 100 ng/ μ l (1.5 μ l). For CR, PCR program was a first denaturing cycle at 94 $^{\circ}C$ for 2 min, then 35 cycles (denaturing at 94 $^{\circ}C$ during 30 s, hybridizing at 54 $^{\circ}C$ for 40 s and elongation at 72 $^{\circ}C$ during 1 min) and a final extension cycle at 72 $^{\circ}C$ during 10 min. For COI, PCR program was a first denaturing cycle at 92 $^{\circ}C$ during 2 min, then 30 cycles (denaturing at 95 $^{\circ}C$ during 1 min, hybridizing at 50 $^{\circ}C$ during 1 min

and elongation at 72 °C during 1 min) and a final extension cycle at 72 °C during 5 min. Sequencing reactions were performed in both directions using the same primers used for PCR amplification, and sequences were read on a 3130XL Applied Biosystems analyzer.

Data analysis

Nuclear DNA (microsatellites) polymorphism

Microsatellite data were used to characterize the extent of interbreeding or introgression between *P. punctifer* and *P. tigrinum* as well as to investigate population structure within each taxa. Two types of analyses were performed independently with the same data set, without a priori consideration for morphological information (color pattern): a Factorial Correspondence Analysis (FCA) and assignment tests aiming at 1—determining the optimal number of genetic units that minimizes deviation from Hardy–Weinberg equilibrium within each units; 2—assign individuals among these units.

The FCA is not a statistical test *sensu stricto*, as it does not give probability, but it provides visual information, identifying the responsible variables. It was carried out to search for consistent genetic clusters, each of them being constituted of genetically close individuals, as defined by their allelic combinations or by alleles with relatively high frequency. 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 the weight of the heterozygous one.

The Maximum Likelihood (ML) approach is similar to the genetic mixture analysis example proposed by Smouse, Waples and Tworek (1990) on predefined stocks and wild populations of Pacific salmon. Without any a priori knowledge about the source populations, the software Partition ML (Belkhir and Bonhomme 2002) implements a “simulated annealing” method (Kirkpatrick et al. 1983). This method tests for a varying number of underlying source populations contributing to a sample (while assigning individuals to each of them). The most probable partition is structured in various reproductive units, each of which provides the minimum departure from panmixia. The number of clusters and the distribution of individuals among clusters were also evaluated by Bayesian analyses using the software Structure 2.1 (Pritchard et al. 2000). We carried out MCMC simulations using no prior information on the origin of samples and the no admixture model given that samples were expected to come from at least two distinct species. The maximum number of populations (K) was assumed to vary between 1 and 6. For each potential value of K, ten replications were performed, with a number of steps equal to 10,000

for the burning process and 100,000 for the simulations, which was sufficient to achieve convergence according to the criteria indicated by the authors. The probability that a given value of K was the best one was calculated based on mean $\ln \Pr(X/K)$, following the recommendations of the authors. Within each traditionally recognized species, the genetic clusters and units revealed by the FCA and the assignment tests were then compared and tested by θ the estimator of F_{st} and by the estimator of F_{is} . Genetic variability was analyzed using Genetix 4.05.2, (Belkhir et al. 2004). Null hypotheses were rejected at $P \leq 0.05$ %.

Finally, in order to find a morphological marker to differentiate the reproductive units revealed by the allelic variation, the spot and stripe pattern variation of fishes was reviewed considering the limits of the taxa as defined by the FCA and assignment analyses.

Mitochondrial DNA, COI and CR sequencing polymorphism

Partial sequences of COI and CR were obtained for a subset of the individuals analyzed with the microsatellite loci (42 *P. punctifer* and 10 *P. tigrinum*). Genbank sequences from taxa phylogenetically close to *P. punctifer* and *P. tigrinum* were used as taxonomic out-groups to root the COI and CR trees: *P. magdaleniatum* (FJ889882), *P. corruscans* (FJ889877 and FJ889876) and *P. fasciatum* (FJ889869 from Guyana) for CR fragment, and *P. magdaleniatum* (GU570861 and GU570860), *P. corruscans* (HQ689335), for COI fragment. Additionally, Genbank sequences of *P. tigrinum* (FJ889689), *P. punctifer* (FJ889866), for CR, and *P. tigrinum* (GU570938), *P. fasciatum* (GU570852 y GU570851) for COI were added as reference.

For each individual a consensus sequence was obtained using BioEdit 7.0.9.0 software (Hall 1999). For the global alignments no gap was needed in COI, while seven gaps were required for the CR. Number of haplotypes, haplotypic distribution among clusters, and haplotype and nucleotide diversity were estimated by DnaSP software 5.10.01 (Librado and Rozas 2009).

To infer phylogenetic relationships among *Pseudoplattystoma*, we performed a Maximum Likelihood (ML) search, using the algorithm developed by Guindon and Gascuel (2003). The best evolutionary model and its parameters were selected using the Akaike Information Criterion values (Akaike 1973) determined using the Analysis of Phylogenetics and Evolution (APE) R program (Paradis et al. 2004; Paradis 2006). Bootstrap proportions (BP) for the maximum likelihood tree were determined with 1000 pseudoreplicates to estimate each node's robustness. The evolutionary model for ML was also used to calculate the genetic divergence among genetic clusters.

The treatment was implemented using PhyML version 3.2 (<http://atge.lirmm.fr/phyml>) (Guindon and Gascuel 2003).

Results

Genetic variability in the nuclear genome

The factorial plan defined by the first two axes of the FCA represented only 9.67 and 5.66 % of the total variance respectively, but three genetic clusters were clearly separated: two in *P. punctifer* and one in *P. tigrinum* (Fig. 2). The number of individuals in each clade was 20 for *P. tigrinum*, 41 for *P. punctifer* cluster-1 and 42 for *P. punctifer* cluster-2. The variables separating the clusters are private alleles or unique combinations of alleles. In the plane defined by the axes 1 and 2, the alleles with highest absolute contributions (>50), were: Pcor2-228, Pcor8-174, Pcor10-138 characterizing *P. punctifer* cluster-1; Pcor2-220, Pcor8-16, characterizing *P. punctifer* cluster-2 and Pcor5-149, Pcor7-231, Pcor8-216, Pcor1-86, Pcor21-110 characterizing *P. tigrinum*.

The most likely assignment by ML Partition was the distribution of the 103 individuals among 3 units ($P < 0.001$, $df = 79$, Log likelihood = $-1,721$) corresponding to the same clusters illustrated by the FCA, except for 3 individuals (2 individuals from *P. punctifer* cluster-1 were assigned to unit-2 and 1 individual from *P. punctifer* cluster-2 was assigned to unit-1). Structure analyses revealed that the optimal number of reproductive units (K) was three, given that $\ln P$ reached a plateau at $K = 3$ and $P(K = 3) = 0.831$. Individuals were partitioned among the three units exactly in the same way as among the three clusters identified by the FCA. Assignment indexes were close to one for all individuals, ranging from 0.95 to 1 with an average value of 0.99. Among the 3 clusters defined by the FCA, the expected non-biased heterozygosity varied from zero (0), for Pcor10 loci (*P. tigrinum*) and Pcor21 (*P. tigrinum* and *P. punctifer*

cluster2), to 0.91 for Pcor2 locus in *P. tigrinum*. The f estimator of F_{IS} , evaluating the departure from within-group panmixia, showed significant negative values in two loci of *P. punctifer* cluster-1 (Pcor10 and Pcor21), indicating an excess of heterozygotes for these loci, whereas the multiloci f estimator indicated a departure from panmixia in *P. tigrinum* and *P. punctifer* cluster-2 (Table 1). The θ (estimator of F_{ST}) values confirmed the strong genetic differentiation ($\theta = 0.46$, $P < 0.0001$) between the two clusters observed in *P. punctifer* by the FCA and assignment analyses. Subsequently the three clusters, identified as sympatric reproductive units, will be considered as probable biological species according to the Mayr concept (1942).

Preliminary approach of the relationship between genetic variation and pigmentation

In order to find distinguishing phenotypic characters, the 103 individuals collected were separated according to the three clusters defined by the FCA analysis and corroborated by the assignment analyses. The three clusters totally matched the following morphological delimitations. Specimens in *P. punctifer* cluster-1 were characterized by a relatively short and superficial fontanel ending about midway between the eyes and the posterior edge of the operculum, a head with almost straight lateral edges, and close black and white vertical lateral stripes that terminate before the dorsal region (Fig. 3a). The most noticeable feature distinguishing specimens of *P. punctifer* clusters 1 and 2 was the absence of lateral vertical black stripes in specimens belonging to cluster-2 (Fig. 3b). Every specimens assigned to cluster-2 by the FCA had no black stripes and, once this particular character was singled out, were easily and unambiguously distinguished from the specimens belonging to cluster-1 (common *P. punctifer*). Although less evident than the stripe pattern, specimens of *P. punctifer* cluster-2 also tended to have flatter dorso-ventral body and head profile. *P. tigrinum* specimens differed very clearly from specimens of both *P. punctifer* cluster 1 (black-and-white-striped species) and cluster 2 (white-striped species) by their typical characteristics (Buitrago-Suarez and Burr 2007): a head with concave lateral edges, a large fontanel reaching the operculum and straight or loop-like black stripes continuing to the dorsal part of the body without vertical white lines (Fig. 3c).

Genetic variability in the mitochondrial genome

Polymorphism

The Maximum Likelihood trees were built according to the selected HKY + G model (Log likelihood = -2113.26 , Gamma shape parameter = 0.642, transition/transversion

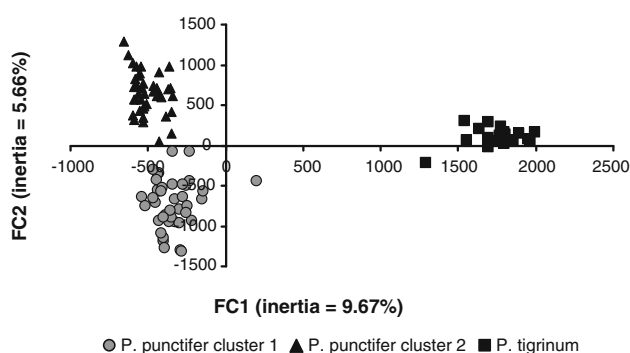


Fig. 2 Projection on the factorial plan defined by the first two axes of the FCA of *Pseudoplatystoma* individuals from the Loreto region (Peruvian Amazon) in function of their allelic frequencies

Table 1 Allelic frequencies, observed and expected heterozygosity, Fis-values and significance among seven microsatellite Pcor loci analyzed for 103 *Pseudoplatystoma* individuals in the Loreto region (Peruvian Amazon), separately for each clade evidence by the FCA analyses

Pcor 2	Cluster 1		Cluster 2		Pcor 7		Cluster 1		Cluster 2		Pcor 8		Cluster 1		Cluster 2		Pcor 5		Cluster 1		Cluster 2		Pcor 10		Cluster 1		Cluster 2		Pcor 21		Cluster	
	Pt	A	Pt	A	Pt	A	Pt	A	Pt	A	Pt	A	Pt	A	Pt	A	Pt	A	Pt	A	Pt	A	Pt	A	Pt	A	Pt	A	Pt	A		
186	-	0.08°	-	0.08	0.02	-	0.08	166	0.05°	131	0.21	-	0.06	86	-	0.93°	-	136	0.04°	-	-	100	0.01°	-	-	-	-	-	-	-	-	
188	-	0.06°	-	0.15	0.04	-	0.15	168	0.51	0.05	0.14	133	0.05	-	0.08	88	-	0.08°	-	138	-	-	0.61°	102	0.05°	-	-	-	-	-	-	
194	0.18°	-	-	0.06°	0.06°	-	0.06°	174	0.05	-	0.58	135	-	-	0.06°	104	0.29	-	0.48	140	-	-	0.02°	106	0.82	-	-	-	-	-	-	
196	0.01	0.08	0.01	0.70	0.10	0.70	0.15°	178	0.02°	-	-	137	0.09	-	0.04	108	-	0.10°	146	0.32	-	0.30	108	0.12°	-	-	-	-	-	-	-	
198	-	0.10°	-	0.03°	0.03°	-	0.03°	180	0.04°	-	-	139	0.04°	-	-	110	0.05	-	0.25	158	0.57	1.00	0.04	110	-	-	-	-	-	-	-	
200	-	0.03°	-	0.13°	0.13°	-	0.13°	182	0.05	-	0.04	141	0.15	-	0.13	112	0.18°	-	-	160	0.02°	-	-	-	-	-	-	-	-	-	-	
202	-	0.03°	-	0.02	0.02	-	0.02	184	0.02°	-	-	145	0.01°	-	-	114	0.01°	-	-	166	0.04°	-	-	-	-	-	-	-	-	-	-	
204	-	0.03°	-	0.10	0.10	-	0.10	186	0.09°	-	-	147	0.17	-	0.02	116	0.02°	-	-	176	0.01°	-	-	-	-	-	-	-	-	-	-	
208	-	0.05°	-	0.05	0.05	-	0.05	188	0.18	-	0.19	149	0.02	0.93	0.10	118	0.21°	-	-	228	-	-	0.02°	-	-	-	-	-	-	-	-	
216	0.26	-	0.13	0.02°	0.02°	-	0.02°	190	0.01	-	0.01	151	0.04	-	0.01	120	0.12	-	0.18	-	-	-	-	-	-	-	-	-	-	-	-	
218	0.04	-	0.06	0.05°	0.05°	-	0.05°	194	0.01°	-	-	155	0.07	0.08	0.30	122	0.10°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
220	0.30°	-	-	0.02°	0.02°	-	0.02°	196	0.01	0.15	-	157	0.01°	-	-	124	0.01°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
222	0.09	0.05	0.07	0.17	0.17	-	0.05	198	-	0.03°	-	159	0.09	-	0.12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
224	0.07	0.08	0.12	0.20	0.20	-	0.20	200	-	0.03°	-	161	0.06	-	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
226	0.01	0.08	0.07	0.01°	0.01°	-	0.01°	208	-	0.13°	-	163	-	-	0.02°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
228	-	0.03	0.34	0.01°	0.01°	-	0.01°	210	-	0.05°	-	165	-	-	0.02°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
230	-	0.20	0.18	0.18	0.18	-	0.06°	216	-	0.43°	-	169	-	-	0.01°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
232	0.02	0.10	-	0.13°	0.13°	-	0.13°	218	-	0.05°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
234	0.01	0.05	-	0.09	0.09	-	0.09	224	-	0.08°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
236	0.01°	-	-	0.01	0.01	-	0.01	228	-	0.03°	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
He	0.81	0.93	0.81	He	0.90	0.48	0.86	He	0.70	0.79	0.61	He	0.89	0.14	0.86	He	0.82	0.14	0.68	He	0.57	-	0.54	He	0.32	-	-	-	-	-	-	-
Ho	0.78	0.85	0.73	Ho	0.83	0.30	0.90	Ho	0.59	0.65	0.38	Ho	0.90	0.15	0.88	Ho	0.83	0.15	0.57	Ho	0.66	-	0.34	Ho	0.37	-	-	-	-	-	-	-
Fis	0.02	0.14	0.12	Fis	0.07	0.39*	0.15	Fis	0.15	0.18	0.41*	Fis	0.01	0.06	0.06	Fis	0.02	0.06	0.25	Fis	-0.13	-	0.23	Fis	-0.14	-	-	-	-	-	-	-
Multilocus																																
Pp	Pt	Pnsp																														
He	0.72	0.35	0.62																													
Ho	0.71	0.30	0.54																													
Fis	0.01	0.16***	0.13***																													

Pt = *P. tigrinum*, allelic frequency (A), expected heterozygosity (H_e), observed heterozygosity (H_o), panmixia deviation (Fis), * significant at $p < 0.05$; *** significant at $p < 0.001$, °private allele

Fig. 3 Lateral view: **a** *Doncella*, *P. punctifer* cluster-1 (479 mm), **b** *Doncella*, *P. punctifer* cluster-2 (462 mm); **c** Tigre zungaro *P. tigrinum* (535 mm), and **d** *Platystoma punctifer* (Castelnau F. L., 1855) holotype, MNHN 1582, illustration from original description. Ceramic tiles in each picture measured 20 cm

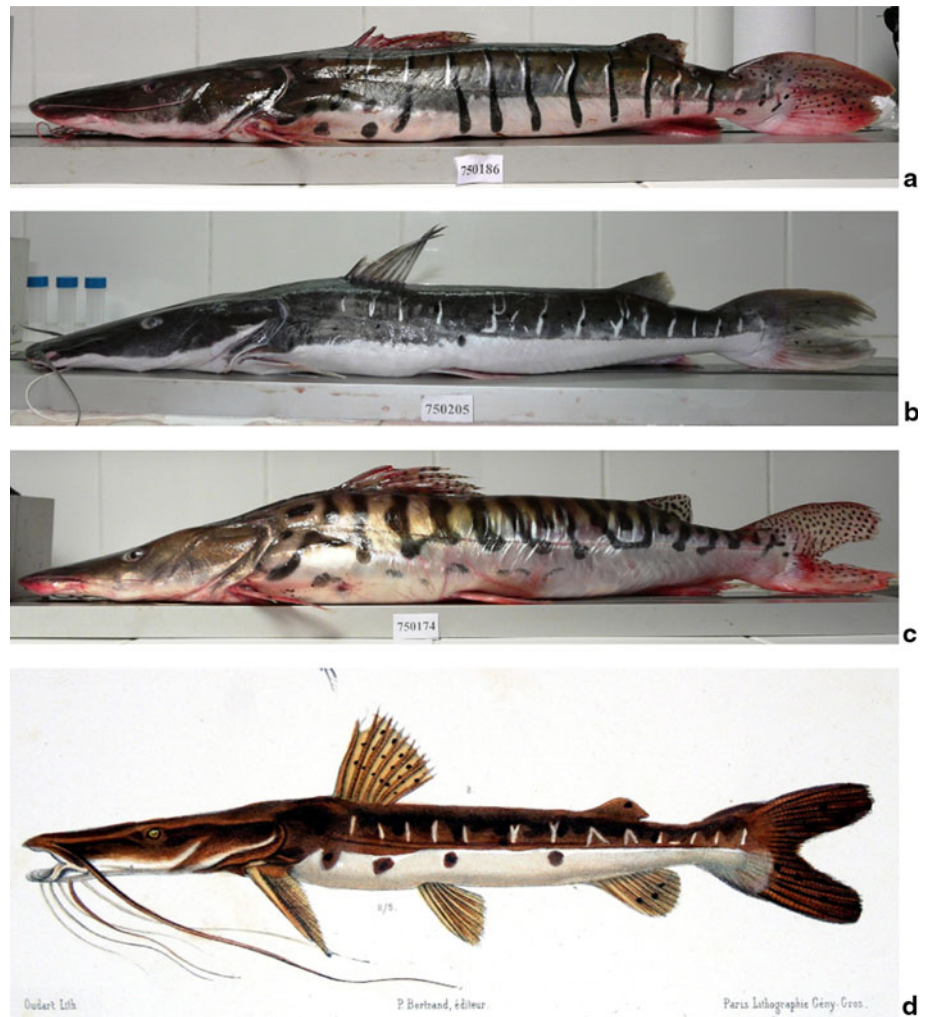


Table 2 Genetic parameters for COI and CR sequences in *P. punctifer* clusters 1 and 2 and *P. tigrinum*

Genetic parameters	COI			D-Loop		
	Cluster-1 <i>P. punctifer</i>	Cluster-2 <i>P. punctifer</i>	Cluster-2 <i>P. tigrinum</i>	Cluster-1 <i>P. punctifer</i>	Cluster-2 <i>P. punctifer</i>	Cluster-2 <i>P. tigrinum</i>
Number of sequences (N)	11	31	10	11	31	10
Number of haplotypes (H)	2	4	2	8	10	09
Haplotypic diversity (Hd)	0.182 ± 0.144	0.432 ± 0.099	0.200 ± 0.154	0.93 ± 0.066	0.817 ± 0.055	1.00 ± 0.045
Nucleotidic diversity (π)	0.0003 ± 0.0003	0.0008 ± 0.0002	0.0004 ± 0.0003	0.005 ± 0.0010	0.007 ± 0.055	0.006 ± 0.0010
Total number of sites (excluding gaps)	576	576	576	854	854	854
Total number of polymorphic sites	1	3	1	16	27	16
Mean nucleotidic differentiation between pairwise sequences (K)	0.182	0.469	0.200	4.400	5.991	5.289

ratio = 12.283) and TN93 model (Log likelihood = -1175.58, Gamma shape parameter = 0.008, transition/transversion ratio = 50.168) for the CR and COI

fragments, respectively. The COI fragment is conserved within *Pseudoplatystoma* with a weak polymorphism ($H = 0.57$ and $\pi = 0.008$), only 17 polymorphic sites (out

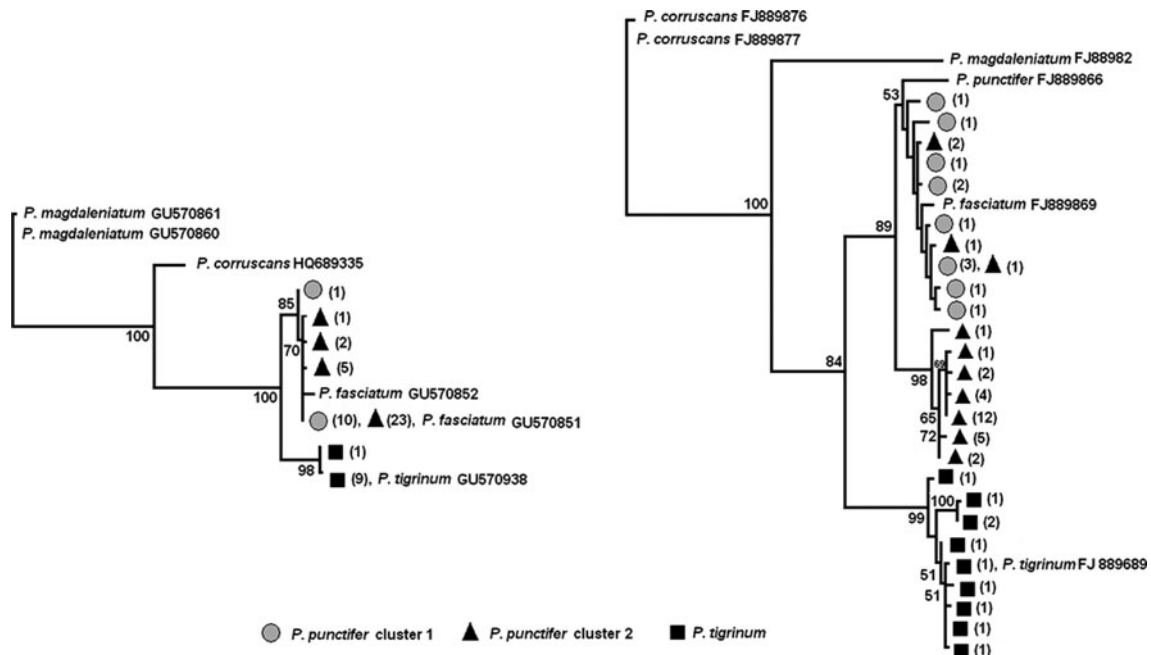


Fig. 4 Maximum likelihood trees including 52 *Pseudoplatystoma* specimens from the Loreto region (Peruvian Amazon). *Left*: COI tree according to the TN93 + G model (LogL = 1175.58), *right*: CR tree according to HKY + G model (LogL = -2113.26). Bootstrap values

of 576 analyzed sites) and only 7 haplotypes among the 52 sequences studied (Table 2). It is worth noting that the 10 individuals of *P. punctifer* cluster-1 had a single haplotype, shared with 23 of the 32 specimens of *P. punctifer* cluster-2 and also shared with the *P. fasciatum* specimen from genbank (Fig. 4a). In contrast, the CR showed a high polymorphism ($H = 0.93$ and $p = 0.022$), with 67 polymorphic sites out of 854. Of the 26 haplotypes, 9 were found only in *P. tigrinum* ($Hd = 1.00$, $p = 0.006$), 7 in *P. punctifer* cluster-1 ($Hd = 0.93$, $p = 0.005$), and 9 in *P. punctifer* cluster-2 ($Hd = 0.82$, $p = 0.007$). One haplotype was shared between 3 individuals of *P. punctifer* cluster-1 and one individual of *P. punctifer* cluster-2.

Phylogenetic relationships

Maximum Likelihood trees constructed according to the best models of evolution, showed contrasted topologies between COI and CR (Fig. 4). In the COI tree *P. punctifer* clusters 1 and 2 and the Guyana sequences of *P. fasciatum* did not reach reciprocal monophyly. They were located in a well supported clade (bootstrap = 85), sister clade of the monophyletic *P. tigrinum* clade (bootstrap = 98), with a nucleotide divergence value of 2.4 %.

The CR tree, however, evidenced three clades supported by significant bootstrap values (53, 98, 99) and strongly congruent with the 3 clusters (FCA analysis) or units (ML Partition and Bayesian analyses) observed with the nuclear

established after 1,000 replicates are indicated at the nodes when they are higher than 50. Number of individuals sharing the same haplotype is indicated between brackets

genome analyses: a *P. tigrinum* clade (bootstrap 99) and a *P. punctifer* cluster-2 clade (bootstrap 98) sister clade (bootstrap 89) of a *P. punctifer* cluster-1 clade (bootstrap 55). This last clade included 4 specimens of *P. punctifer* cluster-2. The nucleotide divergence between *P. punctifer* cluster-1 and *P. punctifer* cluster-2 clades was 1.9 %. The *P. tigrinum* clade, supported by a high bootstrap value (99), was the sister clade of *P. punctifer* clusters 1 and 2 clades. Nucleotide divergences between *P. tigrinum* and *P. punctifer* cluster-1 clade and between *P. tigrinum* and *P. punctifer* cluster-2 were 4.2 and 5.1 %, respectively.

Discussion

Is there a new *pseudoplatystoma* species in the Amazon basin?

Morphological characterization of adult specimens is usually the first step to differentiate species. However, different species may be erroneously clustered within a polymorphic species, or a valid species may be erroneously split into several species (Chong and Khoo 1987; Frankham et al. 2004; Renno et al. 2006; Römer 2006, Römer and Hahn 2008). Indeed, strong phenotypic variation at the intra specific level can be related to sexual dimorphism or geographical populations (Römer 2006). In contrast, individuals of different species can be insufficiently differentiated

morphologically to be easily separated. Then, in the case of cryptic species, the confusion of species may prevent the understanding of ecological and evolutionary processes and lead to the underestimation of species richness (Rocha-Olivares et al. 2001; Römer and Hahn 2008) or to inappropriate valorization of biodiversity and low yields in artificial breeding projects (Bartley et al. 2000; Frankham et al. 2004; Meldgaard et al. 2007).

For the genus *Pseudoplatystoma*, the wide distribution of some species in major river basins of South America might mask the existence of cryptic species. According to Buitrago-Suarez and Burr (2007), only two species of *Pseudoplatystoma* are distributed in the Peruvian Amazon: *P. tigrinum* and *P. punctifer* (formerly *P. fasciatum*, whose geographical distribution would be restricted to the Guyana shield). However, independent molecular analyses using mtDNA (Torrico et al. 2009; Carvalho-Costa et al. 2011) and nDNA (Carvalho-Costa et al. 2011) could not separate *P. fasciatum* and *P. punctifer*. Consistently, here, the GenBank Guyana sequence of *P. fasciatum* was nested inside the *P. punctifer* clade (cluster-1), supporting the hypothesis of conspecific status for these two taxa. The present study revealed the genetic differentiation of two taxa within *P. punctifer*, corroborated by both nDNA and mtDNA analyses. An intra specific structure in *P. punctifer* may be explained by the existence of geographic populations mixed up during sampling at the Iquitos market, or populations isolated by spawning migrations (homing behaviour) but naturally mixed in fishing area. Such hypotheses, however, are unlikely because no scientific evidence ever suggested a homing behaviour in *Pseudoplatystoma* species and because in all previous studies, using both mitochondrial and nuclear markers, their intra specific structure appeared very weak or nonexistent at much larger geographic scales (Torrico et al. 2009; Carvalho-Costa et al. 2011) than in the present study. It must be pointed out that the sampling area, a few hundred kms around Iquitos, actually represents a unified hydrographical network without physical barriers (sympatric area) for migrating species such as *Pseudoplatystoma* spp. A more plausible hypothesis explaining the presence of two *P. punctifer* taxa would be the existence of two species or incipient species within *P. punctifer*. This hypothesis is supported by the differentiation in two clusters within *P. punctifer*, evidenced for the nuclear DNA by 1- the AFC, 2- the maximum likelihood analysis (Partition ML) and 3- by the Bayesian analysis (Structure). It is also partially supported for the mitochondrial DNA (Control Region sequencing) with the haplotypes of the two clusters distributed in two monophyletic clades (excepted for only 3 haplotypes). This differentiation is quantified by high F_{st} values, indicating low or nonexistent gene flow between the two clusters, as expected between species, and by the existence of clear distinctive phenotypic characters (stripe patterns) (see

Harrington and Near 2012). On the other hand, the significant F_{is} values observed within *P. punctifer* cluster-2 and *P. tigrinum* samples could result from a Wahlund effect (Hartl and Clark 1997) caused by sampling, at the Iquitos market, specimens originating from differentiated genetic populations within these taxa.

Interestingly, Castelnau (1855) described a *Platystoma punctifer* from the Amazon basin with a morphology and color pattern (absence of black stripes) extremely similar to the *P. punctifer* cluster-2 identified in the present study (Fig. 3d). It must be pointed out here that although cluster-2 is intentionally over-represented (because of the sampling design) in our sampling, it is much less abundant than cluster-1 (with black stripes) in nature (pers. obs.). In their revision of the genus, Buitrago-Suarez and Burr (2007) nonetheless had access to specimens of both *P. punctifer* cluster-1 (they showed a picture of cluster-1, identified as *P. punctifer* from the Iquitos area: Figure 15) and cluster-2 (they showed the original plate of Castelnau (1855) as illustration of *P. punctifer*: Figure 21). Surprisingly, they analyzed them as a single group designated as *P. punctifer*. This accidental admixture of two species under *P. punctifer* might have led Buitrago-Suarez and Burr (2007) to erroneously consider that *P. fasciatum* is not present in the Amazon but only restricted to the Guyanas, and to consider that only *P. punctifer* is present in the Amazon basin. This could account for why three independent molecular studies (Torrico et al. 2009; Carvalho-Costa et al. 2011; present study) were not able to genetically differentiate between *P. fasciatum* and the *P. punctifer* with black and white stripes (our cluster-1, and the most abundant in nature) described in Buitrago-Suarez and Burr (2007). From the available data, it appears that *P. fasciatum* might correspond to our cluster-1 and that the species described by Castelnau (1855) as *Platystoma punctifer* might correspond to our cluster-2 (Fig. 3). Further morphological analyses by taxonomists are obviously needed to confirm this hypothesis and to try and find consistency between morphological and molecular data. In any case, our results clearly demonstrate that three species of *Pseudoplatystoma* inhabit the Amazon basin.

Evolutionary considerations

Considering a mean nucleotide substitution rate of 0.62 % per site per million years for the CR (Torrico et al. 2009), based on the separation of the Magdalena, Amazon and Parana rivers 11.8 Myrs ago (Hoorn et al. 1995; Lundberg 1998), the divergence time among the three sympatric species corresponding to *P. tigrinum*, *P. punctifer* cluster-1 and cluster-2 would be: 1.5 Myrs between *P. punctifer* cluster 1 and cluster-2, 3.4 Myrs between *P. tigrinum* and *P. punctifer* cluster-1, and 4.1 Myrs between *P. tigrinum* and *P. punctifer* cluster-2. Taking into account this molecular

dating, the three species could have evolved during the Miocene under allopatric speciation scenarios related to fragmentation barriers, such as paleoarchs, aquatic refuges, or marine incursions (reviews in Hubert and Renno 2006; Hubert et al. 2007; Albert and Reis 2011). Alternatively, species may have diverged through ecological speciation, as observed in some piranha species (Hubert et al. 2008a). *P. tigrinum* and the black-striped *P. punctifer* (cluster-1) are known to have different habitat requirements: the former being more frequent in lentic, open environments, whereas the second favors structured (logs, wood sticks, vegetated area) lotic conditions (Reid 1983; Loubens and Panfili 2000). Local fishermen call the species lacking the black-stripes (cluster-2), the doncella “playera” or “beach” doncella, as they usually catch it on open sandy or muddy banks of the white-water river channels. The three sympatric *Pseudoplatystoma* species therefore seem to have specific habitat preferences that might have contributed in driving their speciation. Detailed ecological studies, however, are needed to confirm this hypothesis.

Distribution of the species without black-stripe, corresponding to *P. punctifer* cluster-2 (potentially *Platystoma punctifer* castelnaui 1855)

This species was observed in Central Amazonia in Santarem, Brazil by two independent sources: F. Carvajal (unpublished data) and Barthem and Goulding (1997, the photo on page 21 shows a specimen caught in Santarem, M. Goulding, pers. com.) and Manaus (pers. obs.). It was also observed in several localities from the Peruvian Amazon: in the Loreto region (present study), in the Marañon sub-basin, in the Putumayo River (unpublished data) and in the Ucayali region, in the Ucayali River (picture top right, page 178 in Barthem and Goulding 2007) and in the Pachitea River (Ortega H. pers. com.). If our hypothesis that it corresponds to *Platystoma punctifer* (Castelnaui 1855) is valid, then it must be pointed out that the Holotype (MNHN 1582) comes from an unknown locality in Brazil (Buitrago-Suarez and Burr 2007). Therefore, although less abundant than the black-striped species (cluster-1, potentially *P. fasciatum*), the species without black stripes appears relatively widespread along the main axis of the Amazon River. Despite extensive sampling in the main river basins of the Bolivian Amazon (Upper Madera River), however, it was never observed by the authors of the present study.

Remark about the effectiveness of the COI barcoding marker for species identification

Gene sequences of COI region have been chosen for a fast, standardized and precise labeling or identification of the

living world (DNA barcoding). Many studies support the effectiveness of this mt-DNA fragment in the labeling and identification of fish species (Hebert et al. 2004a, b; Hubert et al. 2008b; Steinke et al. 2009; Ardura et al. 2010; Aquilino et al. 2011; Asgharian et al. 2011; Lakra et al. 2011; Zhang and Hanner 2011) and processed fish products (Ardura et al. 2010; Wong et al. 2011). In some instances, however, closely related species may have diverged too recently to be unambiguously separated by COI barcode sequences (Toffoli et al. 2008; Steinke et al. 2009; Serra-Pereira et al. 2010; Smith et al. 2011). In the case of *P. punctifer* in the Peruvian Amazon, the much lower nucleotide diversity (π) of COI compared to CR (Table 2) probably explains the inability to separate the two species evidenced by the nDNA analysis and the topology of the CR tree. Such difficulties associated with COI illustrate the need to consider more than one region of the mitochondrial genome to discriminate closely related species or cryptic species, as suggested in earlier studies (Ward et al. 2009; Smith et al. 2011). Additionally, morphological, ecological, physiological, environmental and life-history traits in natural or captive populations should also be taken into account, as they may provide valuable information on the taxonomical limits of the species in quest (Römer 2001; Römer and Beisenherz 2006).

The recent speciation suggested in *P. punctifer* could also explain retention of ancestral polymorphism or mtDNA introgression from cluster-1 to cluster-2, explaining that 4 specimens identified as *P. punctifer* cluster-2 by their nDNA and phenotypic characters were nested in 1-cluster *P. punctifer* clade.

Implications for natural resource management (conservation, fishing and fish farming)

Pseudoplatystoma species are among the top predators of the Amazon basin (Barthem and Goulding 1997, 2007) and as such, participate in maintaining equilibrium of the food chains (Winemiller and Jepsen 1998; Lucas and Baras 2001; Winemiller 2005). Modifying the abundance of top predators frequently leads to profound ecosystem alterations through cascading effects (Pace et al. 1999; Myers et al. 2007). Hence, the management and conservation of *Pseudoplatystoma* species and other large Pimelodid catfish are of utmost importance in the Amazonian ecosystems. Indeed, the underestimation of species diversity with the confusion of species can have prejudicial effects, by generating inappropriate decisions. Precise information of life history strategies of species in relation with fishing pressure is necessary to define management and conservation policies. Thus, confusing the life history traits of two or more species hampers a proper management of each of them. In fish farming, misidentified species can be unintentionally hybridized, which may result in strong

efficiency loss in the domestication process, with decreasing reproductive success (infertility), reducing adaptive value (outbreeding depression) and disappearance of unique evolutionary units in the wild in case of restocking (Bartley et al. 2000; Frankham et al. 2004; Meldgaard et al. 2007). Until recently, the joint IRD-IIAP scientific program aiming at developing the aquaculture of doncella in the Peruvian Amazon (Nuñez et al. 2011; Baras et al. 2011a, b), ignored this possible admixture between *Pseudoplatystoma* species. Fortunately the present study has permitted to verify that only *P. punctifer* cluster-1 (the most abundant in nature) was present in our breeding stocks. From now on, aquaculture managers wishing to develop the Doncella production should use the absence or presence of lateral black stripe, a very easily diagnosed character, to limit the risks of mixing the two species.

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