DNA barcodes of the suckermouth sailfin catfish *Pterygoplichthys* (Siluriformes: Loricariidae) in the Marikina River system, Philippines: Molecular perspective of an invasive alien fish species

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DNA barcoding for accurate identification of invasive alien fish species is relatively unexplored. In this study, partial sequence (655 bp) of the mitochondrial gene COI (cytochrome c oxidase subunit I) was used to delineate between two species of exotic suckermouth sailfin catfish *Pterygoplichthys* – *P. pardalis* and *P. disjunctivus* and their intergrades, which dominate the ichthyofauna of the Marikina River system, Philippines. Individuals were assigned to groups using an abdominal pattern scheme, and COI gene sequence divergence analysis was determined using Kimura 2-parameter distances. Results revealed two major clusters which were inconsistent with the abdominal pattern categories and were characterized by low genetic divergence (mean 0.2%); one cluster having shared genealogy of individuals pre-identified as *P. pardalis* and the intergrades, and another consisting mostly of *P. disjunctivus*. From the samples, six haplotypes with low genetic divergence (mean 0.5%) were identified, suggesting that the haplotypes belong to a single species despite abdominal pattern variations. Overall, the DNA barcodes do not complement the morphology-based identification of the two species in the river system. The results support the possibility of introgressive hybridization between *P. pardalis* and *P. disjunctivus* and the need to reassess taxonomic assignment of the two species using abdominal patterns as basis for species distinction. It is recommended that multiple molecular tools be used in future studies and that native *Pterygoplichthys* species and other hypostomine loricariids be subjected to DNA

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barcoding to fully classify this genus that deserves taxonomic attention.

KEYWORDS

Janitor fish, invasive alien species, hybridization

INTRODUCTION

Invasive alien species (IAS) are one of the major causes of biodiversity loss with serious economic impacts, particularly in freshwater systems (Clavero and Garcia-Berthou 2005; Sato et al. 2010). Establishment of invasive exotics in a new ecosystem has caused alteration of habitats and has resulted in predation, resource competition with the native species and biotic homogenization (Carol et al. 2009, Mooney and Cleland 2001, Mckinney and Lockwood 1999). Consequently, the introduction of exotics may be exacerbated by the potential of hybridization (Huxel 1999) that not only occurs between introduced and native species (Rhymer and Simberloff 1996) but also between two introduced species (Hayden et al. 2010, Taylor et al. 1986). In scenarios where related taxa are able to interbreed, introductions of IAS may lead to coexistence with native fishes (Closs and Lake 1996, Leprieur et al. 2006), form new hybrid taxa (Childs et al. 1996, Salzburger et al. 2002), or result ultimately in the extinction of the native taxa (Huxel 1999, Allendorf et al. 2001).

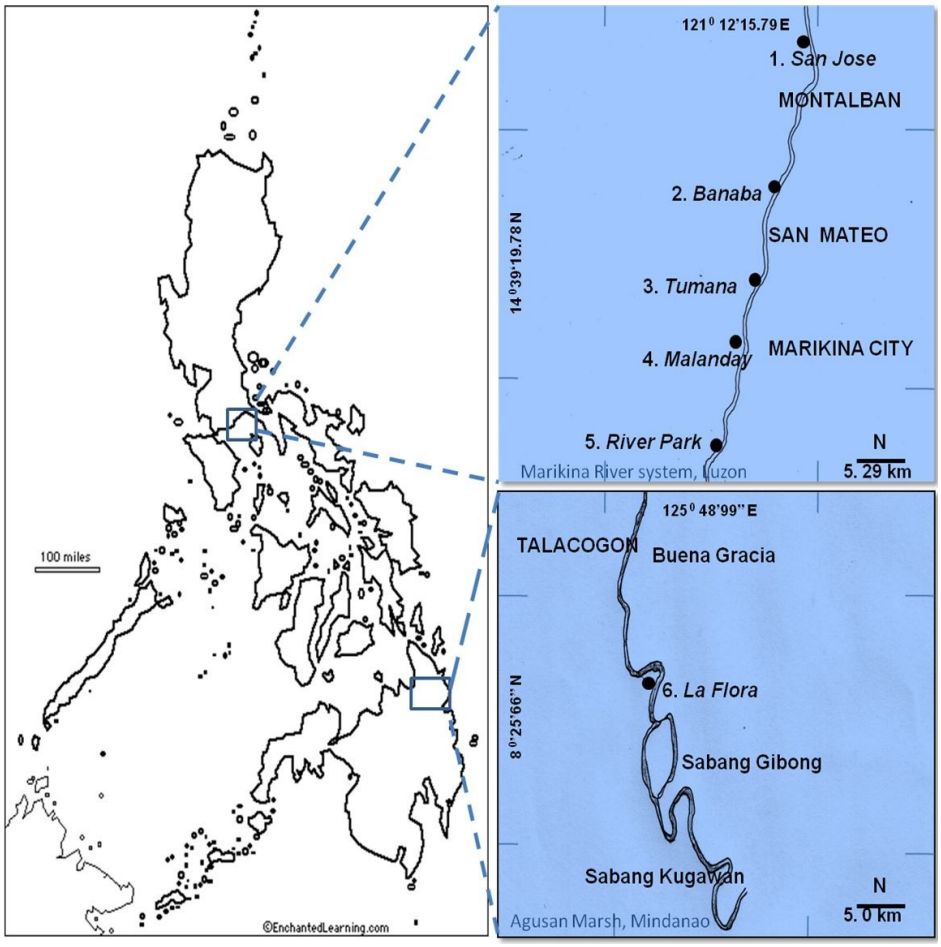

Figure 1. Sampling stations for *Pterygoplichthys* in Marikina River system in Central Luzon, and Agusan Marsh in Mindanao

Pterygoplichthys pardalis Castelnau, 1855 and *Pterygoplicthys disjunctivus* Weber, 1991 are armoured suckermouth loricariid catfishes distributed naturally in the Amazon River basin of Brazil and Peru (Weber 2003) and Rio Madeira drainage of Brazil and Bolivia (Page and Robins 2006), respectively. However, reports of their invasion in tropical and semi-tropical regions of North America, Puerto Rico, Malaysia, Indonesia, Vietnam and Taiwan have been documented (Samat et al. 2008, Wakida-Kusonoki et al. 2007, Wu et al. 2011). *P. disjunctivus* and *P. pardalis* are popularly known as "janitor fish" in the Philippines, and they have since invaded many rivers in Luzon and Mindanao. Their establishment in Marikina River, Pasig River and Laguna de Bay in Luzon was first reported in 2006, although reports of introduced suckermouth catfish caught in Laguna de Bay date back to 2002 (Chavez et al. 2006). These species were first imported to the Philippines as aquarium fish; however, many propagules were believed to have accidentally escaped from fish farms into Laguna de Bay when super typhoon Rosing caused flooding in 1995 (Guerrero 2006). The definite means of introduction of *Pterygoplichthys* in Agusan Marsh in Mindanao is unknown.

Pterygoplichthys pardalis and *P. disjunctivus* are largely distinguished by patterns of spots and vermiculations in the abdomen (Armbruster and Page 2006, Chavez et al. 2006, Page and Robins 2006). Meristic and morphometric indices show very little variation between them (Armbruster 2003, Armbruster and Page 2006, Chavez et al. 2006). Observations of these fishes in Marikina River and Agusan Marsh, Philippines show a large bulk of individuals with intermediate coalesced abdominal markings between the two species, suggesting that color pattern character may not be reliable in differentiating the species as there exists a continuous variation in patterns. Recent mtDNA (cytochrome b) studies of *Pterygoplichthys* in Taiwan (Wu et al. 2011) hypothesized the synonymity of *P. disjunctivus* to *P. pardalis* and the hybrid origin of the introduced *Pterygoplichthys* exhibiting "hybrid superiority," which might

have helped increase their fitness during invasions in Taiwanese waters.

Comprehensive species identification is a critical aspect of monitoring, management and better understanding of invasion strategies of exotics in a novel ecosystem (Armstrong and Ball 2005); hence, the need to utilize the standard DNA barcode approach in an attempt to discriminate between invasive fish species with very similar morphologies. DNA barcoding—the sequencing of a short standardized region of DNA—has been proposed as a new tool for animal species identification (Hebert et al. 2003), wherein each species will be delineated by a particular sequence, or a cluster of similar sequences (Ward and Holmes 2007). The barcode region widely adopted is a 650 bp region of the 5'end of cytochrome c oxidase 1 (CO1), a mitochondrial DNA locus currently used for cataloguing animal biodiversity (Hebert et al. 2004, Ward et al. 2009). CO1 in fish, as with other vertebrate mitochondrial DNA, lacks introns, has limited exposure to recombination (Saccone et al. 1999), but is more likely to reveal deeper phylogenetic insights than other mitochondrial genes because changes in its amino acid sequence occur more slowly (Lynch and Jaryl 1993). DNA barcoding helps taxonomy in discovering cryptic species (Hebert et al. 2004) and is regarded as a promising approach for rapid and accurate identification of invasive species, which could be adapted globally for biosecurity (Armstrong and Ball 2005).

In this study, we utilized standard DNA barcode methodology to delineate between the two invasive species—*P. disjunctivus* and *P. pardalis* from the Marikina River system, Philippines along with individuals having intergrade abdominal patterns between them. This study provides an update on the scanty but vital barcode records to identify invasive species with conflicting and inconsistent morphological features. The study also contributes to the scarce mtDNA barcode of the genus *Pterygoplichthys* in the Barcode of Life Database (BOLD). The necessity for more DNA barcode studies for invasive fish species and subsequent submission to public databases for more reliable species match and inference are discussed.

MATERIALS AND METHODS

Collection and sorting of *Pterygoplichthys*

A total of thirty six (36) *Pterygoplichthys* specimens were collected using cast nets from the following five key sampling stations in the Marikina River system, Luzon, Philippines: (1) San Jose, Rodriguez Rizal; (2) Brgy Banaba, San Mateo; (3) Brgy Tumana; (4) Brgy Malanday, Marikina; and (5) River Park, Marikina City. We also included seven *Pterygoplichthys* specimens from La Flora, Talacogon, Agusan del Sur, a section of Agusan Marsh in northern Mindanao (6) as a geographic outgroup in genetic distance comparisons (Figure 1). Fish samples were transported live to the laboratory, or directly preserved in 95% ethanol. Species identification of individuals was carried out using morphological descriptions (Weber 1991, Armbruster and Page 2006, Armbruster 2003).

To facilitate sorting and molecular analysis of *P. pardalis*, *P. disjunctivus* and their intergrades, individuals were sorted according to abdominal pattern variations (Figure 2) as follows: category *P0*- dark spots on white abdominal background with no coalescence of spots; *P1*- dark spots on white abdominal background with coalescence limited to two spots but not predominant; *P2*- coalescence of 2 spots predominant; *P3*- coalescence of three spots predominant; *P4*- coalescence of four or more spots; and *P5*- continuous curve lines where spots can no longer be identified appearing in vermiculated pattern. Representative *Pterygoplichthys* individuals for each abdominal pattern category were identified by Dr Jonathan W. Armbruster, Curator of Fishes, Auburn University. Category extremes (*P0* and *P5*) were initially identified as *P. pardalis* and *P. disjunctivus,* respectively. However, representative individuals from intergrade categories (*P1-P4*) were assigned as within the range of *P. pardalis*. The thirty six individuals from Marikina River were evenly distributed among the six categories; a limited number of seven (7) *Pterygoplichthys* was obtained from Agusan Marsh and these were utilized as biogeographical outgroups. Individuals were preserved in 95% ethanol as voucher specimens.

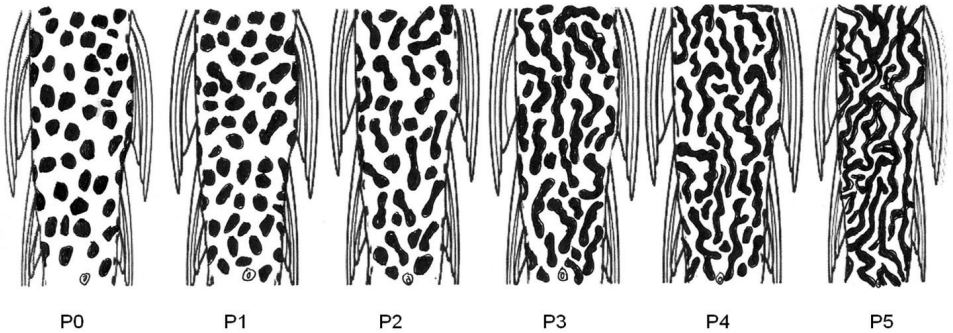

Figure 2. Appearance of categories *(P0-P5)* of abdominal patterns based on coalescence and spots designed to sort *Pterygoplichthys* and intergrades found in Marikina River, Philippines (*PO*- pre-identified *P. pardalis*; *P1-P4*-Intergrades of the *P. pardalis* range*; P5*- pre-identified *P. disjunctivus.*

Genomic DNA extraction, PCR amplification and direct sequencing of PCR products

DNA from white muscle tissue (20 mg) was extracted following the procedure of Wizard® Genomic DNA Purification Kit, (Promega Corp., Madison, USA) for isolating DNA from animal tissue. A 655 bp segment containing 214 codons was amplified from the 5' region of the CO1 gene using the following primers: Fish F2-5' TCGACTAATCATAAAGATATCG GCAC3'; and FishR1-5' TAGACTTCTGGGTGGCCAAA GAATCA3' (Ward et al. 2005).

Polymerase chain reactions (PCR) were performed in 50 µL volumes having the following components: 2 µL dNTP, 5 µL 10x PCR buffer, 0.5µl (0.05 u/µl) *Taq* polymerase (i*Taq*TM DNA polymerase kit, iNtRON® Biotechnology, USA), 32.5 µl ultrapure water, 2.5 µl (0.5 µM) of each primer and 5 µl of DNA template. PCR amplifications were performed using a thermocycler (PE9700, Applied Biosystems Inc., Warrington, UK) with the thermal cycling profile as follows: 95ºC initial denaturation for 2 min, followed by 35 cycles of 94^0C of denaturation at 30 s, 54^0C annealing at 30 s, 72^0C of extension for 1 min, and then held at 72^0C for final extension for 10 min. PCR products were then run in a 1% agarose gel electrophoresis and confirmed PCR products extracted using Qiagen® Qiaquick Gel Extraction Kit (GmbH, Germany). Bidirectional sequencing of the amplified CO1 fragments was performed by 1st Base Asia, Malaysia using ABI® 3730*xl* analyzer (AB, USA) with BigDye v3.1 (AB,USA).

Sequence comparison and phylogenetic analysis

Forward and reverse sequences were aligned and edited using the STADEN package version 1.5.3 (Staden et al. 2000). Edited consensus sequences were then aligned using ClustalW through Bioedit sequence alignment editor version 7.0.9 (Hall 2008). Sequence divergence between individuals was calculated by average pairwise comparisons using MEGA5 (Tamura et al. 2007). Pairwise genetic distances among sequences from the Marikina River and Agusan Marsh samples were calculated using Kimura-2-parameter (K2P) model (Kimura 1980) and a neighbor-joining tree (Saitou and Nei 1987) was constructed to provide a graphic representation of species divergence.

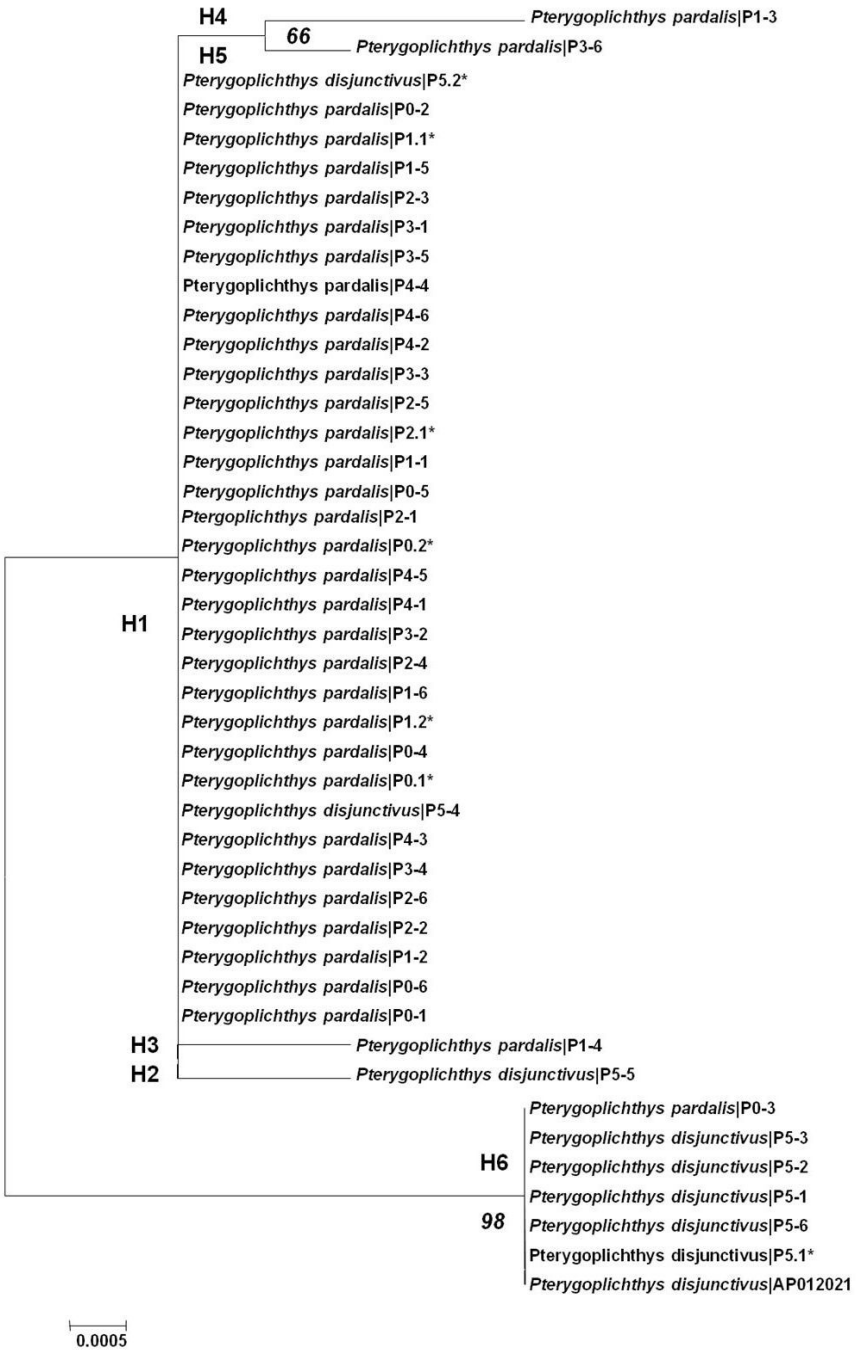

Figure 3. Unrooted K2P distance neighbor joining tree of *Pterygoplichthys* COI sequences from Marikina River, Luzon (36 sequences) and Agusan Marsh, Mindanao (* ; 7 sequences) based on abdominal patterns. *P. disjunctivus* (AP012021) was used as reference. Bootstrap values were shown next to the branches. Scale bar represents five nucleotide substitutions for every 1000 nucleotides.

A gene tree for the family Loricariidae was constructed using the unique haplotypes of *Pterygoplichthys* from this study, COI sequences of loricariids obtained from GenBank, the BOLD Systems, as well as sequences from unpublished sources. The optimal model that best fits the data set was first identified using jModelTest version 0.1 (Posada 2008), where the Bayesian

Inference Criterion (BIC) method of model selection was used. The model-based neighbor joining (NJ) and the non-model based maximum parsimony (MP) (Fitch 1977) methods were built using PAUP* version 4.0b10 (Swofford 2002) while the model-based maximum likelihood (ML) (Felsenstein 1981) method was undertaken in PhyML version 2.4.4 (Guindon and Gasquel 2003). Multiple optimal topologies were summarized through consensus methods. Nodal support was evaluated with 1000 nonparametric bootstrap pseudoreplicates.

Specimens were deposited in the Zoological Museum, Institute of Biology, University of the Philippines-Diliman with voucher labels (P0 1-6; PI 1-6; P2 1-6; P3 1-6; P4 1-6 and P5 1-6) containing necessary specimen data. Specimen data, trace files, images and metadata were submitted to the BOLD systems (Process ID: BMP001-11 to BMP036-11 for Marikina River specimens and BPMP044-11 to BPMP037-11 for Agusan Marsh samples. COI sequences submitted to GenBank were given accession numbers JF498719-JF498754; and JF769355-JF769361.

RESULTS

A 655-bp COI fragment was successfully amplified for all 36 *Pterygoplichthys* individuals representing six abdominal pattern categories from Marikina River, Luzon and seven individuals from Agusan Marsh, Mindanao. Prior to this study, no public records were available in GenBank and BOLD system databases for either *P. pardalis* or *P. disjunctivus*. The mean K2P nucleotide divergence for all abdominal pattern categories in *Pterygoplichthys* from Marikina River shows extremely low levels of genetic differentiation between the two putative species and their intergrades at 0.2%**.** Combined COI sequences of the Marikina River and Agusan Marsh samples produced two clusters, one cluster exhibiting shared genealogy of *P. pardalis* and the intergrades (*P0-P4*) while the other cluster, though not consistent, was mostly composed of pre-defined *P. disjunctivus* (*P5*) (Figure 3).

Shared genealogy and inconclusive relationships between morphological and COI data

From the thirty six *Pterygoplichthys* CO1 sequences from Marikina River, six haplotypes were identified (*H1, H2, H3, H4, H5* and *H6*). Shared genealogy among the pre-identified specimens was inconsistent with the abdominal pattern categories (Figure 3, Figure 4). Of the six specimens pre-identified as *P. pardalis* (*P0*) and 24 intergrades of the *P. pardalis* range (*P1-P4*) analyzed, 28 individuals possessed identical COI haplotypes (*H1*). Of the six pre-identified *P. disjunctivus* samples (*P5*), four individuals possessed identical COI haplotypes (*H6*) while the other 2 individuals possessed *H1* haplotypes. There are eight variable positions across the six different haplotypes . Three of these variable positions are non-synonymous substitutions, giving rise to a different amino acid each (see Table 1). Integration of the six haplotypes produced two clusters of low genetic distances (mean 0.5%, range 0.2-0.9%). No new haplotype was found from Agusan Marsh samples. *Pterygoplichthys* H6 clustered with a non-native *P. disjunctivus* sequence with high bootstrap support and 0% genetic divergence. All haplotypes were clearly distinguished from two other congeners *P. etentaculatus* and *P. gibbiceps* (Figure 4).

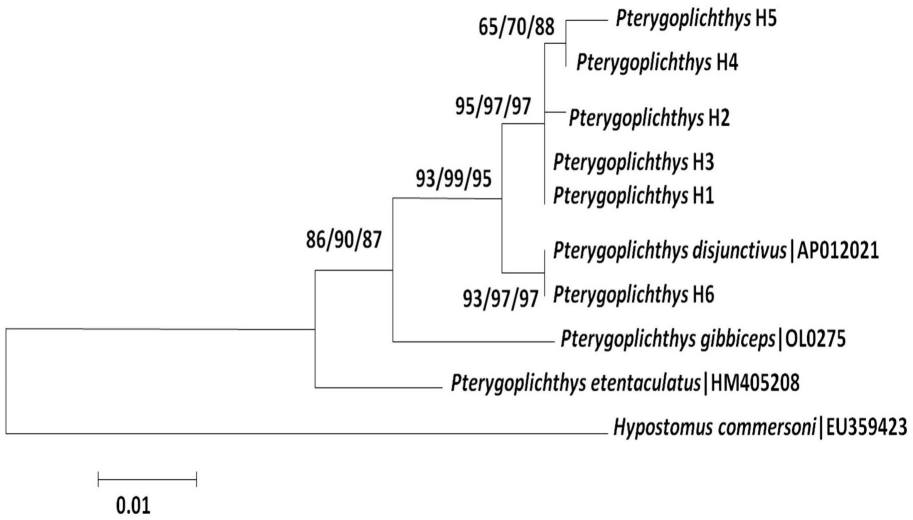

Figure 4. Maximum likelihood tree of six *Pterygoplichthys* haplotypes from the Marikina River system, Philippines (H1-H6) compared with two congeners (*P. etentaculatus*, *P. gibbiceps*) and 1 conspecific sequence *P. disjunctivus* (AP012021). (*H. commersoni*: subfamily Hypostominae as outgroup). Tree was based on 642 nucleotides of the CO1 gene (GenBank accession numbers shown) using the HKY+I+G as optimal model. Values on nodes indicate percentage bootstrap support based on ML/NJ/MP analyses; Scale bar represents one nucleotide substitution for every 100 nucleotides.

COI genetic distance comparisons on loricariids

K2P genetic divergences of the *Pterygoplichthys* haplotypes were shown to have low divergence at the species level (0.5%). To place this level of genetic differentiation into context, these haplotypes differ from their congeneric counterparts (*P. etentaculatus* and *P. gibbiceps*) at 1.5% (Table 2) indicating that the COI divergence between the haplotypes was smaller than their congeners with available DNA barcode.

Based on the ML and NJ trees of the COI gene using the HKY+G+I as

optimal model determined by the jModeltest (Figure 5), pairwise comparisons of our sequences with representative genera within the subfamily Hypostominae show defined congeneric differences. Defined consubfamilial differences with our haplotypes were also seen from representative Ancistriinae, Hypoptopomatinae, Neoplecostominae, Delturinae, and Loricariinae genera. Nonetheless, it is noteworthy to point out an inconsistency in the cluster association of the genus *Otocinclus—O. cocama* and *O. vittatus,* which were separated from other representative members of the subfamily Hypoptopomatinae (Figure 5; broken lines) with high bootstrap support (100%). In summary, the average congeneric distance was approximately three-fold the conspecific distances observed in this study, giving low resolution between the *P. pardalis* and *P. disjunctivus* cluster but with a more defined distinction compared to other genera of the other loricariid subfamilies in the NJ tree.

Conflicting taxonomical designations and database matches

Species level match queries using some of our *Pterygoplichthys* CO1 sequences in the BOLD systems website showed conflicting matches with other loricariids and were not able to accurately identify our *Pterygoplichthys* sequences at the species level. For example, *Pterygoplichthys* haplotype *H6* sequence was shown to have high specimen similarity to *P. pardalis* (100%), *P. anisitsi* (99.69%), *P. disjunctivus* (99.40%), *Glyptoperichthys* sp (99.39%), *Hypostomus plecostomus* (99.39%) and *P. joselimaianus* (98.58%) (www.boldsystems.org).

DISCUSSION

Our study represents the first molecular assessment of the DNA barcodes of *Pterygoplichthys* in the Marikina River system Philippines, where difficulty in distinguishing between *P. pardalis* and *P. disjunctivus* morphologically is apparent. This study provides an update on the morphology-based distinction of this invasive fish, which is largely proliferating in many freshwater systems in the country.

Introduced populations often typify defined lower genetic divergence than the populations to which they are derived due to founder effects, or population bottlenecks (Allendorf and Lundquist 2003, Dlugosch and Parker 2008). However, as in the

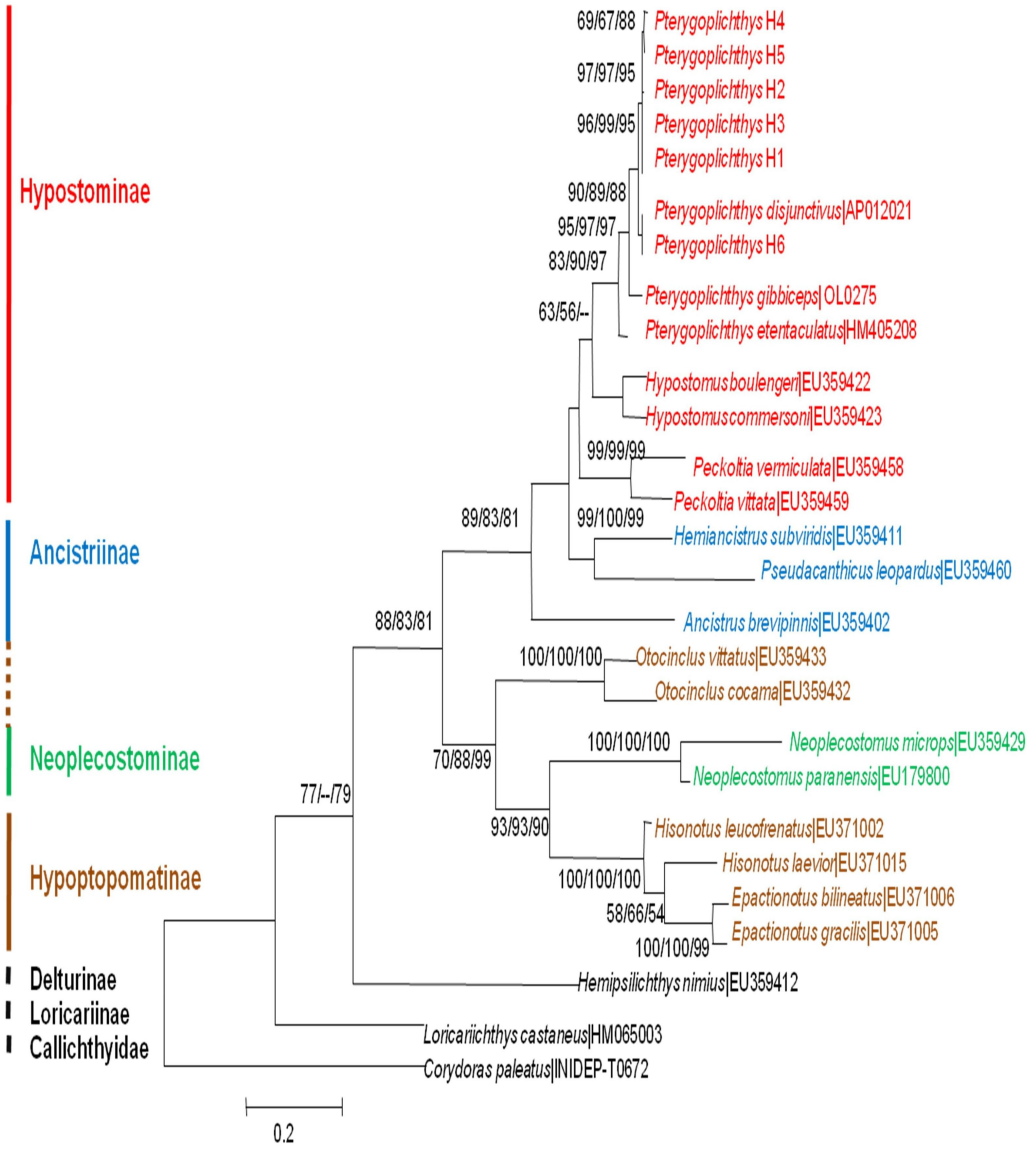

Figure 5. Maximum likelihood tree of six *Pterygoplichthys* haplotypes from the Marikina Riversystem, Philippines (H1-H6) compared with representative species from other loricariid subfamilies (*Corydoras paleatus* : Callichthyidae utilized as outgroup). Tree was based on 642 nucleotides of the CO1 gene (GenBank accessession numbers shown) using the HKY+I+G as optimal model. Values on nodes indicate percentage bootstrap support based on ML/NJ/MP analyses; values less than 50% are not shown. Scale bar represents two nucleotide substitutions for every ten nucleotides.

case of the introduced *Pterygoplichthys* in Philippine waters, where DNA barcode sequences from the parent population are not available, low genetic divergence due to presumed repeated inbreeding of exotics in a new environment cannot be concluded at present. The close genetic divergence of *Pterygoplichthys* from Marikina River to those in Agusan Marsh, a large catchbasin in southern Mindanao, with some sequences having even zero % divergence suggests that the *Pterygoplichthys* from the two sites are of the same species. The population variability of *Pterygoplichthys* from these two very distant locations may be explored using mitochondrial DNA (mtDNA) control-region sequence in future studies. The *P. disjunctivus* sequence used lacked genetic divergence with our H6 haplotype, which most likely indicates that H6 is a *P. disjunctivus* CO1 sequence. However, it should be noted that the *P. disjunctivus* COI sequence was derived from a non-native source and was not evaluated for barcode compliance under the BOLD systems, so a definitive conclusion about the identity of H6 cannot be made as of this time.

Species synonymity and the possibility of hybridization

From the literature, there were no fixed K2P distance cut-off values for species distinction. For Canadian fishes, the average conspecific, congeneric and confamilial K2P distance cut-off is within 0.27%, 8.37% and 15.38%, respectively, (Hubert et al. 2008), while these ranges are slighly wider in Australian marine fishes at 0.39%, 9.93% and 15.46%, respectively (Ward et al. 2005). Steinke et al. (2009) employed a 2.0% threshold for intraspecific sequence divergence. The genetic divergences of our *Pterygoplichthys* haplotypes (0.5%), with no defined species distinction, were way below the accepted ranges set at 10X the average within species variation (Hebert et al. 2004) to account for interspecific distinction.

Table 1. Eight variable positions across the six (6) different haplotypes in *Pterygoplichthys* from Marikina River, Philippines

	Nucleotide position								Codon position		
Haplotypes	**10**	**11**	**21**	**347**	**370**	**419**	**502**	**648**	**4**	**7**	**216**
H1	A	G	C	T	A	T	C	A	V	A	H
H2	A	G	G	T	A	T	C	A	V	G	H
H3	A	G	C	T	A	T	C	C	V	A	P
H4	G	T	C	T	A	T	C	A	L	A	H
H5	A	T	C	T	A	T	C	A	L	A	H
H6	A	G	C	C	G	C	T	A	V	A	H

Table 2. Summary of genetic divergences (K2P percentage) of the six *Pterygoplichthys* haplotypes within various taxonomic levels.

Comparisons within	Number of comparisons	Mean (%)	Min (%)	Max (%)	SE
Pterygoplichthys haplotypes	15	0.5%	0.2	0.9	0.2
Within genus*	34	1.5%	0.0	3.1	0.4
Within subfamily (Hypostominae) †	71	5.5%	0.2	10.9	0.9
Among 6 loricariid subfamilies‡	300	15.3%	0.2	24.9	1.7

*The Boldsystems available sequences of the species *P. etentaculatus*, *P. gibbiceps* and *P. disjunctivus* were utilized to estimate congeneric divergence values; †*Hypostomus boulengeri*, *H. commersoni*, *Peckoltia vittata* and *P. vermiculata* were used to estimate divergence values among other members of the subfamily Hypostominae); ‡Representative members of other five loricariid subfamilies (Ancistriinae, Hypoptopomatinae, Neoplecostominae, Delturinae, Loricariinae) were included to estimate divergence values with the *Pterygoplichthys* haplotypes.

We propose that abdominal pattern differences may not be a stable and reliable trait to differentiate between *P. pardalis* and *P. disjunctivus*. This is based on inconsistensies with the composition of haplotypes and the very minimal base pair differences, with genetic values too low to support defined species distinction between the clusters generated. If a specimen differs by only one or two base pairs with a defined species, the *a priori* probability that it is the same species with the identified specimen is very high (Ward et al. 2009). Nonetheless, our conclusions would be impacted by the DNA barcode of the native *P. pardalis* and *P. disjunctivus*, which is not available at present.

Our findings are consistent with the findings of Wu et al. (2011) that suggests a totally fluent gene flow between those with ventral spots and vermiculations in *P. pardalis* and *P. disjunctivus* as inferred using cytochrome b gene. Our results show similar topologies wherein a shared genealogy of the most common haplotypes were also observed. Nonetheless, the similarity of outcomes of our separate experiments is not quite surprising as cytochrome b is also a mitochondrial gene and is

expected to show closer genetic distances compared to nuclear genes. Low COI genetic divergences are often utilized to propose synonymity in species with conflicting and intermediate morphologic features (Byrkjedal et al. 2008, Da Silva et al. 2010, Rodriguez et al. 2008). Our results concurrently agree that to settle the validity of *P. disjunctivus*, pure specimens from their original habitat in South America have to be subjected to DNA barcode analysis along with nuclear DNA gene markers and microsatellite studies.

Cytochrome oxidase, hybridization and the case of invasive alien species

Inferring from the shared genealogy of the pre-identified *Pterygoplichthys* samples and the low genetic divergence without defined distinction between the two species in Marikina River, our results suggest that the *Pterygoplichthys* in Marikina River may be hybrids, congruent with the hypothesis of Wu et al. (2011) that the exotic populations may have originated from hybridization between the two closely related species or allopatric populations. It is very likely that hybrid *P. pardalis* and *P. disjunctivus* through aquarium trade were introduced accidentally to this river, brought into sympatry, and has since interbred. Hybridization and back-crossing between two introduced species of previous allopatric locations, when complemented by poor reproductive separation, high fertility and hybrid viability, make it possible for introgression and gene flow to result in a single merged population (Verspoor and Hammar 1991, Hammar et al. 1989, Sato et al. 2010). Such is the most likely scenario for *Pterygoplichthys* in Marikina River with limited and relatively small geographical range. Nonetheless, the possibility that the *Pterygoplichthys* present in the river are of the same species with variable abdominal patterns, as well as the possibility of incomplete lineage sorting of these fishes, cannot be discounted. The only way to resolve this is to check a nuclear gene and see if two distinct sequences between the two species emerge; hence, the need to test a nuclear gene in future studies.

To date, no specific nuclear gene has been used as a diagnostic locus to distinguish variations in abdominal or color patterns. Nonetheless, Costedoat et al. (2007) have indicated that the nuclear intron *Tp1b* gene may be promising for integrated studies on hybrid zones. Other studies have found positive association between intermediate morphological patterns and both nuclear (Rag-1, Rag-2) and mitochondrial genes (CO1, control region, cytochrome b) (Drew et al. 2008, Wang et al. 2010).

The ineffectiveness of species level detection by DNA barcoding of problematic species is often due to introgressive hybridization and is common in birds (Kerr et al. 2007), insects (Whitworth et al. 2007) and fishes (Hubert et al. 2008) except chondrichthyans (Gardner 1997). Mitochondrial inheritance in fishes has been found to be predominantly maternal (Brown 2008), and hybridization among species created taxonomic uncertainty since any hybrid or subsequent generation would have the mtDNA of the maternal lineage only (Dowling and Secor 1997). Hybrid lineages are typically identified by morphological intermediacy and increased heterozygosity at nuclear gene loci (Dowling and Secor 1997, Freyhof et al. 2005). In the case of introduced species into novel environments, recently evolved species complexes will have higher proportions of species that are barcode identical and that could lead to taxonomic confusions at the molecular level (Ward et al. 2009).

Though our own findings show parallel results with Wu et al. (2011), we do not concur with their speculations of "hybrid superiority" of *P. pardalis* x *P. disjunctivus* acquiring increased fitness in novel environments. The *Pterygoplichthys* population in the heavily polluted areas of Marikina River (JC Jumawan, unpublished observations) is largely composed of the intergrade groups (*P1-P4*), but a separate study from our group showed that there were no significant differences in the reproductive phenology patterns, fecundity and gonad charactersistics of the intergrades compared to *P. pardalis* and *P. disjunctivus* (Jumawan et al. 2010). Ongoing embryology studies from our laboratory show that gametes of *P. pardalis*, *P. disjunctivus* and those of the intergrades can readily be induced to fertilize regardless of the ventral-pattern differences without noticeable difference in fertilization and mortality rates.

We speculate that the exotic *Pterygoplichthys*, regardless of the "hybrid" populations, possess very high tolerance to poor water conditions, often dominating the ichthyofauna of polluted, hypoxic waters with rich organic load. Habitat disturbances could serve as significant environmental drivers that may promote proliferation of invasives by providing them with favorable habitat conditions (Sato et al. 2010, McKinney 2006). The absence of predators for the adult sizes also favor their reproduction and proliferation in novel environments. These speculations, however, call for estimation of propagule pressure, long term monitoring on mating biology and physiological studies from several invaded ecosystems in comparison with the native *Pterygoplichthys*.

Phenotypic plasticity of a species is associated with epigenetic mechanisms (Corse et al. 2009). The hybrid zone of the invasive *Chrondrostoma nasus nasus* and the endemic *Chondrostoma toxostoma toxostoma* in the Durance River has shown unidirectional change in body shape, with the tendency towards having a spindle-shaped body and a snout steered upward compared to its allopatric populations, regardless of genomic combination (Costedoat et al. 2007, Corse et al. 2009). Further, the female invasive bighead goby (*Neogobius kesslere*) appears to be altricial compared to the round goby, with the tendency to spawn earlier than the native (Kovac et al. 2009). Defined epigenetic evidence with regards to variation in abdominal patterns of allopatric populations of *Pterygoplichthys* from South America compared to the sympatric hybrid zones have yet to be determined.

Campaign to barcode invasive alien species and hypostomine loricariids

It is apparent that the confident inference of species-specific divergence in this study is limited due to scanty records of other *Pterygoplichthy*s species for comparison. For *Pterygoplichthys,* the only submitted, but non-public, sequence of *P. pardalis* was also sourced from Binangonan Rizal, Philippines. The close morphologic resemblance, close genetic distances or probable misidentifications of some submitted sequences in the BOLD systems may also be the probable factors why species-level match query was not accurate, despite the fact that four other hypostomine species aside from *P. pardalis* and *P. disjunctivus* highly matched (98-100%) our samples. The genus *Pterygoplichthys* has 16 taxonomically classified species; however, the BOLD systems database only has four—*P. anisitsi*, *P. gibbiceps*, *P. etentaculatus* and a problematic *P. joselimaianus* COI sequence. This limitation leads to the problem of comparing intraspecific genetic variation in order to confidently assign individuals to the species level (Darling and Blum 2007) and entail more barcoding initiatives aimed at populating the database with related species to better infer relationships (Schindel and Miller 2005).

Our results contribute to the scanty DNA barcode data of the invasive alien fish species worldwide, which could serve as a reference to better understand the invasive nature of these fishes. *Pterygoplichthys* in many freshwaters in the country may also serve as a good model for future biogeographic studies. Further molecular studies with a native *P. pardalis* and *P. disjunctivus* would have to be explored in future studies. Regarding the question of the appropriate cluster association of the genus *Otocinclus* in this study, it is also recommended that the taxonomic classification of the subfamily Hypoptopomatinae be revisited.

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NO CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest among authors, institutions and individuals mentioned above in the conduct of this study and submission of the manuscript.

CONTRIBUTION OF INDIVIDUAL AUTHORS

Ms. JC Jumawan primarily conducted the sampling, sample processing, manuscript writing, and submission for publication. Dr BM Vallejo contributed in the conceptualization of the study design and writing of the manuscript. Dr CB Buerano and Dr. AA Herrera facilitated in the conduct of the early stages of the experiment and contributed in the writing of the manuscript. Dr. IKC Fontanilla supervised in the conduct of the laboratory experiment; contributed in the analysis of data and writing of the manuscript.

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