SHORT COMMUNICATION

Identification of prohibited red piranha, *Pygocentrus nattereri* (Kner 1858) from confiscated juvenile fish in Manila, Philippines

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Abstract—Trade has significantly influenced marine and freshwater biodiversity because of overexploitation, habitat degradation, and invasive species introduction. Despite the developed international control measures, cases of illegal trade were proven to be rampant by reported cases of wildlife trafficking. In this regard, species authentication in traded fishery products through DNA barcoding is considered to be a more efficient technique in the confirmation of species identity since morphological characterization is difficult in juvenile species. In this study, confiscated live fish fingerlings, declared as "pacu", were brought to the Genetic Fingerprinting Laboratory (GFL) by the Manila Police District - District Special Operations Unit (MPD-DSOU) for species identification. Three random samples were subjected to DNA barcoding by the amplification of cytochrome oxidase I (COI) gene and alignment of generated sequence with reference sequences for *Pygocentrus nattereri* and *Piaractus brachypomus* in the GenBank database. The Kimura 2-parameter (K2P) distance model was used to compute genetic distances and to generate Neighbour-Joining (NJ) tree. The results confirm that the three samples are *P. nattereri* as shown by a monophyletic cluster of sample sequences with reference sequences for *P. nattereri*. The mean genetic distance between the samples and *P. nattereri* was 1.1%. This report shows that the trade of prohibited red piranha is still happening in spite of the ban. This study aims to emphasize the significance of species authentication in strengthening law implementation and the capability of DNA barcoding to support it.

Keywords—Trade, Juvenile, Red piranha, DNA barcoding, Cytochrome oxidase I

INTRODUCTION

Trading of various aquatic species serves as a major threat in biodiversity conservation due to endangering of wildlife species, threatening of wildlife stocks, and introduction of invasive species (Ferrier 2009). Trading was proven to be extensive through the reported cases of wildlife trafficking, disregarding the control measures generated for it (Lavorgna 2014). Illegal trade is fueled by the high demand of wildlife products used for various purposes such as food, commercial products, traditional Chinese medicine, or as personal pets (Hakken 2011). This high demand is directed at Southeast Asia, which is one of the key suppliers of the international market in wildlife trade, making it one of the world's "wildlife trade hotspots" (Felbab-Brown 2013).

The Philippines prohibits the importation or possession of any live piranha under FAO No. 126 series of 1979. In the mentioned order, piranha is defined as: "fishes with lacerating teeth and strongly set on well- developed mandibles with which to take big bites out of the flesh of its victims, usually found in northern South America. They are strictly, freshwater species, sturdy and could adapt easily to new environment even in confinement under aquarium conditions". This ban is supported by FAO No. 221 series of 2003 which is subjected to regulate the importation of live fish and fishery/aquatic products by the evaluation of its risk factors and potential occurrence of unfavorable consequences wherein piranhas are placed under prohibited species, defined as exotic species with known adverse effect on local fauna, human health and the environment.

There have been numerous reports on trading and selling of piranha in the Philippines signifying that importation of piranha is still functional despite the prohibition of the Philippine law. In fact, piranha was recently seen in an aquarium of an establishment in Pasig City (Guerrero 2014). Also, trading through the means of internet selling sites appears to be active up to date since there are advertisement posts marketing red piranhas (KP Sarmiento, personal observation).

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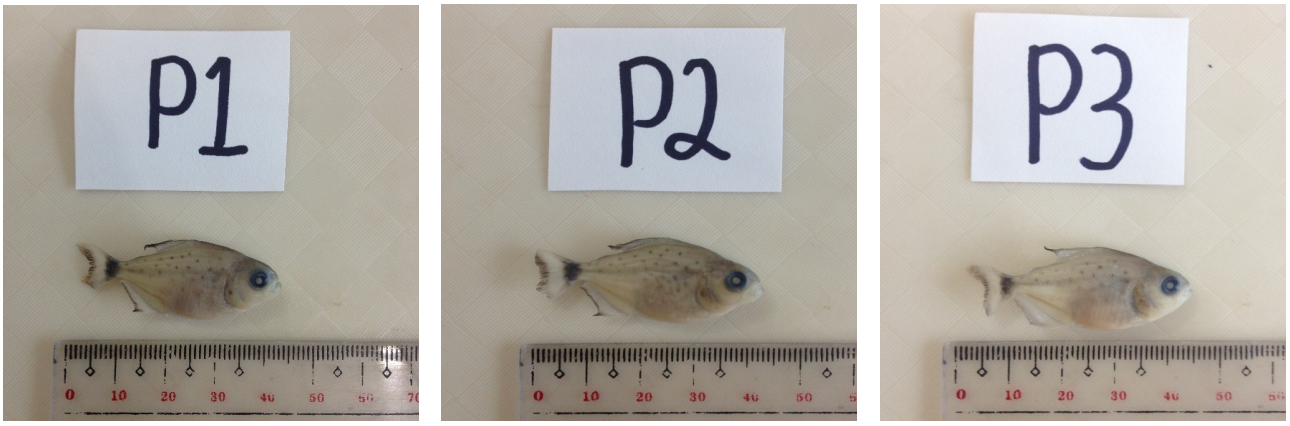

Figure 1. Juvenile finfish samples submitted by MPD-DSOU last January 09, 2015. P1, P2 and P3 had weight of 1.79, 1.59 g and 1.51 g, respectively, and length of 4.5, 4.4, and 4.2 cm, respectively. Morphological measurements were provided by Ms. Eunice Bognot and Mr. Francisco Torres Jr. of the Vertebrate Section at NFRDI.

Pygocentrus nattereri (Kner 1858), also known as "red piranha", is one of the most common piranhas utilized in aquarium trade (Fuller et al. 1999). Red piranhas are high valued pets amounting to P600 (14USD) per piece of 1 inch-size piranha (Idolph 2009). It is considered as potentially invasive species with the score of 19, giving it a medium risk to become an invasive species according to the Fish Invasiveness Scoring Kit (FISK) (Almeida et al. 2013). Red piranhas have been observed to breed on flooded pasture with a depth of ~35 cm and water temperature of 27-28°C, in South-western Brazil near the Rio Vermelho stream (Uetanabaro et al. 1993). This has been supported by more recent studies suggesting that the reproductive season of red piranhas occur when there is high water level and increased flooding rates on grounds with good vegetation (Queiroz et al. 2010; Vicentin et al. 2013). Establishment of piranhas in its non-native grounds may create a great impact in the existing natural fauna because it may act as a voracious piscivore that may outcompete the other fishes in the area (Winemiller 1990).

Red piranha is often interchanged with *Piaractus brachypomus* (Cuvier 1818), also known as "pirapitinga or red-bellied pacu", when morphologically identified due to their similar appearance (Nico et al. 2015). Dentition serves as a primary eminent characteristic in distinguishing the two, red piranha is notable by having a single-row of serrated incisor-like teeth, while the red-bellied pacu has two rows of molariform teeth (Nico et al. 2015). However, at earlier life stages of the two species, dentition characteristics are not very distinct. Moreover, red-bellied pacu, at juvenile stage, often mimics red piranha by displaying dark-grey to black spots in its body, a distinguishing characteristic of piranhas (Hintz 2012) hence making differentiation more difficult.

Accurate identification of species serves as an important tool for the establishment of marine protected areas, resource conservation, compensation of fishery, prevention of illegal trade, and ecological monitoring (Asis et al. 2014; Valdez-Moreno et al. 2010; Moura et al. 2008). Identification based on morphological characteristics becomes problematic when applied during early life stages of fishes, particularly in larval stage and juvenile stage (Graves et al. 1989). In fact, traditional morphological characterization was reported to result to different identification of fish larvae among different taxonomists and various laboratories (Ko et al. 2013). Because of this, DNA-based techniques, including DNA barcoding, are widely applied for more reliable species identification (Shen et al. 2013; Cawthorn et al. 2011).

The use of DNA barcoding was proven to be an effective tool in the assessment of marine biodiversity, conservation efforts, and fisheries management (Trivedi et al. 2015). Predominantly, DNA barcodes are applied in species authentication to prevent mislabeling of fishery products in various countries including the Philippines, South Africa, North America, and United States (Maralit et al. 2013; Cawthorn et al. 2011; Wong et al. 2011, Wong and Hanner 2008). In addition, DNA barcodes are utilized in the determination of specific predator-prey relationships (Jo et al. 2014).

In this study, confiscated live fish fingerlings, declared as "pacu", were identified through the use of DNA barcoding. Four separate plastic bags, each containing twenty-five pieces of suspected piranha fingerlings, were confiscated by the Manila Police District - District Special Operations Unit (MPD-DSOU) while being sold along the sidewalk of Tayuman Street near the corner of Rizal Avenue at about 7:45 p.m. on January 08, 2015. The items were said to cost approximately P13,000 (285USD). Proper identification of aquatic species is highlighted as an important tool as a preventive measure to monitor and manage banned species, and this is achieved through the application of DNA barcoding.

MATERIALS AND METHODS

Sample collection

Three random samples (Figure 1) were forwarded to the Genetic Fingerprinting Laboratory from the Vertebrate Section of NFRDI. The samples were obtained from one plastic bag of twenty-five live fish fingerlings submitted by the MPD-DSOU, from United Nations Avenue, Ermita, Manila. The samples were submitted last January 09, 2015 for genetic identification. Approximately 150 mg of muscle tissue, obtained from the ventral side near the posterior end in the body of the fish, was preserved in 95% ethanol and stored in -20ºC.

DNA extraction and COI amplification

DNA was extracted using 10% Chelex based on the protocol of Walsh et al. (1991). DNA extractions were carried out for each sample. A region of cytochrome oxidase I (~600 bp) was amplified using the primers as follows: VF2_t1 (5'TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC3'), FishF2_t1 (5'TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC3'), FishR2_t1 (5'CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA3'), and Fr1d_t1 (5'CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA3') (Ward et

al. 2005; Ivanova et al. 2007). The 25 μl PCR reactions consisted of water, 0.5x PCR buffer, 0.08 mM dNTP's, 1.4 mM $MgCl_2$, 0.6 μM of each primers, 10 μl of 5x BSA, 0.1 unit Taq polymerase and 2 μl of template. The PCR cocktails were subjected to the following conditions: 94°C for 2 min, 38 cycles of 94°C for 30 sec, 52°C for 40 sec, 72°C for 60 sec, and a final extension of 72°C for 10 min. Product amplicons were electrophoresed in 1% agarose gel stained with ethidium bromide and submerged in 1x TAE buffer. Standard sequencing and DNA purification were outsourced to Macrogen Inc., Korea.

Genetic Analysis

BLAST analysis was carried out to identify sequences which are similar to the generated sample sequences (Altschul et al. 1990). Similar sequences were downloaded from GenBank together with their accession numbers (http://www.ncbi.nlm.nih.gov/genbank/) and were used as reference sequences for the generation of phylogenetic tree. MEGA 6.0 (Tamura et al. 2013) was used for aligning DNA sequences together with the reference sequences. Species identification was inferred using Neighbor-Joining (NJ) tree based on the Kimura 2-parameter (K2P) model with 500 bootstrap replications (Tamura et al. 2013). Genetic distance was computed between the sample and reference sequences using K2P model (Kimura 1980).

RESULTS AND DISCUSSION

Mitochondrial DNA CO1 sequences of the three juvenile fish samples were sequenced and analyzed. BLAST analysis revealed that the sample sequences (GenBank: KX396057, KX396058, KX396059) have >99% similarity with *Pygocentrus nattereri* (GenBank: AP012000). Other sequences which have 85-97% similarity with the sample sequences were detected from *Pygocentrus piraya* (Cuvier 1819), *Serrasalmus marginatus* (Valenciennes 1837), *Piaractus mesopotamicus* (Holmberg 1887), *Leporinus friderici* (Bloch 1794), and *Trichomycterus giganteus* (Lima and Costa 2004). CO1 sequences from the mentioned species and from the believed species "Red pacu" or *Piaractus brachypomus* (Cuvier 1818) was used for phylogenetic analysis. CO1 sequence from *Apareiodon affinis* (Steindachner 1879) was used as an outgroup because it belongs to the same order (Characiformes) as *P. nattereri*. Figure 2 shows the result of genetic analysis using Neighbour Joining (NJ) method and Kimura 2-parameter model. The phylogenetic tree suggested that the CO1 sequences of the samples belonged to the same monophyletic clade with *P. nattereri*, supported by a bootstrap value of 100%, confirming that the samples are from the same lineage as that of *P. nattereri*. The resulting tree also confirms that the fish are not *P. brachypomus* or "pacu", as declared. Complete multiple alignments of the sample sequences with reference sequences is shown in Figure 3.

Further analysis was carried out by computing genetic distances of the samples with the reference species (Table 1). Threshold value was set a 3.0% - 3.5% for species delineation among the included sequences (Ward et al. 2009). Genetic distances within the three CO1 sequences from the samples were observed at 0.0% - 1.1% (Table 1). Similar genetic distance range was also observed between the sample sequences and CO1 sequence from *P. nattereri*. These distances fall below the threshold value and are very low as compared to the genetic distance computed for the other reference samples, suggesting that they are the same species. In addition, the distance between the samples and *P. brachypomus*, were observed at 19.0% - 20.7% (Table 1) which are above the threshold value of 3.5% suggesting that the samples and Red pacu are two distinct species. The result of genetic distance analysis strongly suggests that the samples are *P. nattereri*. There were 482 positions in the final dataset for both the K2P genetic distance computation and generation of NJ tree. All positions containing gaps and missing data were eliminated for the generation of NJ tree and K2P genetic distance computation.

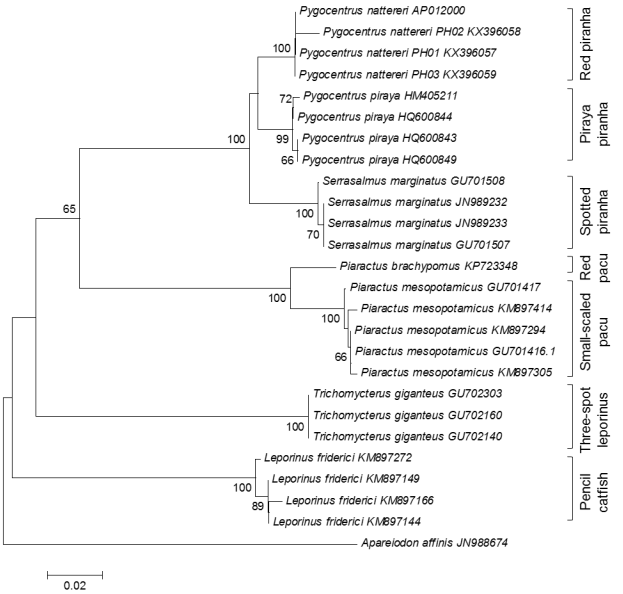

Figure 2. Neighbour-Joining Tree of juvenile fish CO1 sequences using Kimura 2-parameter model. Bootstrap values from 500 bootstrap samples are indicated beside the nodes. The scale bar represents 2% genetic distance and GenBank accession numbers are shown next to species names.

Red piranha or *P. nattereri* is primarily utilized as an aquarium pet fish (Singh and Lakra 2011; Guerrero 2014). It is known to prey on smaller fish, aquatic insects, mollusks and crustaceans; hence, it is considered as opportunistic and omnivorous feeders. These characids usually thrive in moving freshwater, such as rivers and streams, in tropical areas (Saint-Paul et al. 2000). Breeding season of red piranhas was observed during the wet season, particularly, when the water level is high and when flooding occurs (Uetanabaro et al. 1993). It is considered as the most aggressive species of piranha because it tends to compete for food during foraging with other fish species in its area (Nico et al. 2015). Red piranhas are considered as potential invasive species receiving a medium risk score of 19 according to Fish Invasiveness Scoring Kit (FISK) (Almeida et al. 2013). In this regard, *P. nattereri* has been banned in various countries, including the Philippines.

Breeding of red piranhas in captivity was reported in 1994 at the Dallas Aquarium wherein red piranhas are placed in a 2,000-gallon (7,600 L) aquarium (Schleser 1997). The capability of piranhas to breed in a controlled environment suggests its possible adaptation in aquariums in the Philippines. In this regard, juvenile piranhas are more preferred to be imported because of the difficulty in immediate identification and often interchangeable with pacu.

The current case of the presence of red piranha in the country calls out the attention for strengthening measures of assuring species identification since the Philippines have a wide marine and freshwater area to protect. Particularly, there are numerous rivers and streams present within the country, therefore release of these piranhas in the Philippine waters may cause chaos in our ecological biodiversity.

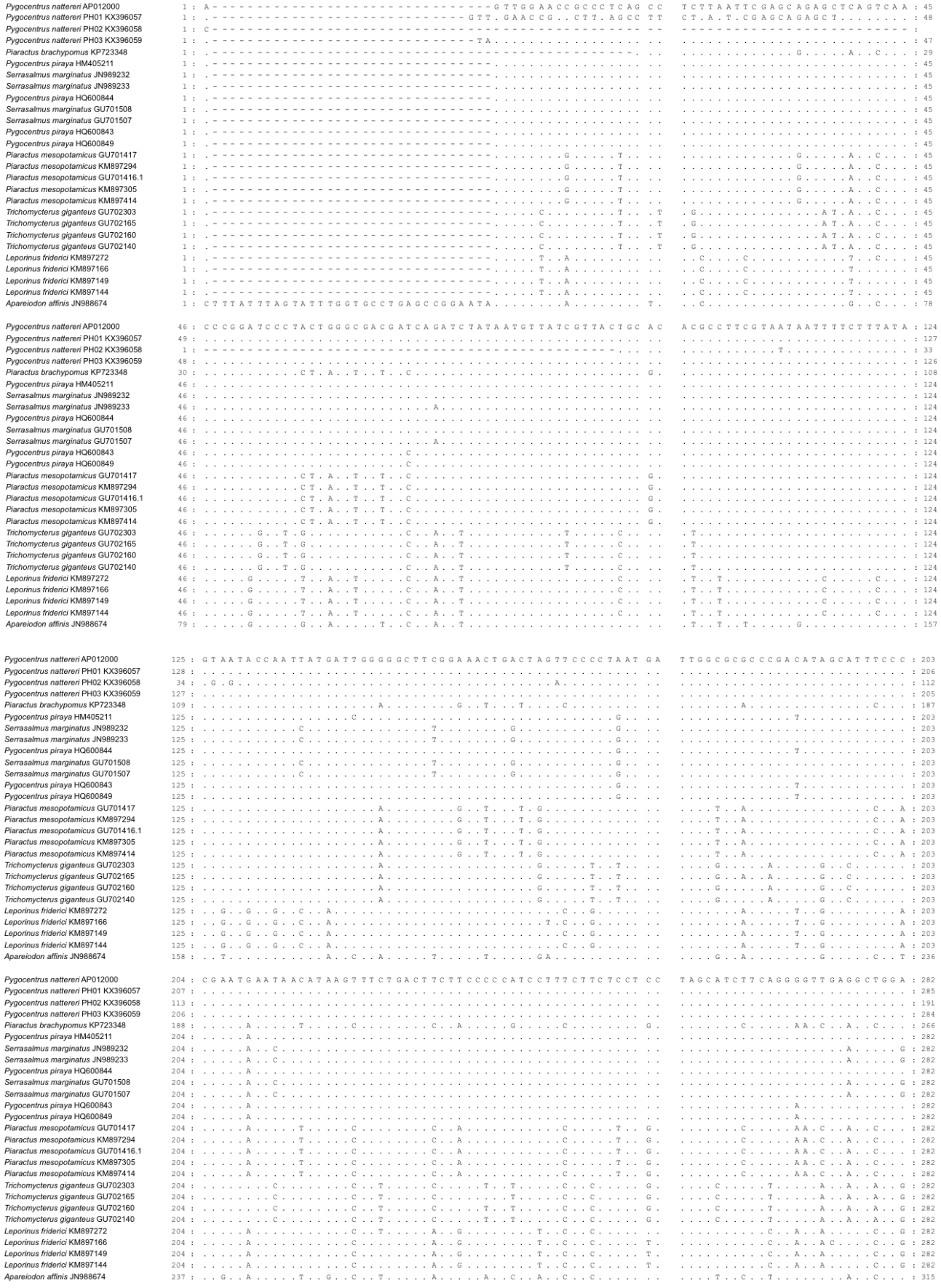

Figure 3. Complete multiple alignments of juvenile fish CO1 sequences together with reference sequences. GenBank accession numbers are shown next to species names. Similar nucleotide sites are illustrated by *dots* and gaps or missing data are denoted by *dashes*.

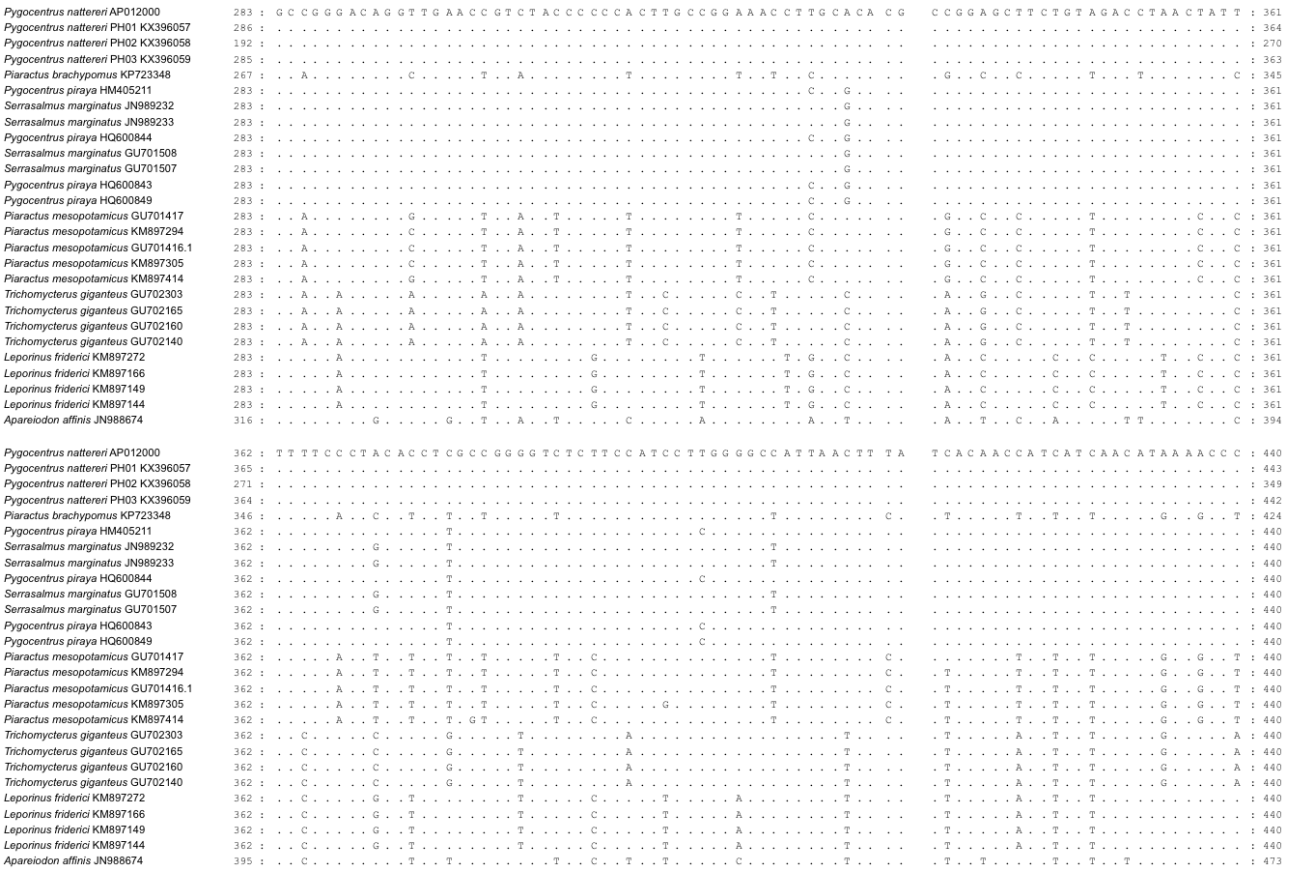

Figure 3 *continued.* Complete multiple alignments of juvenile fish CO1 sequences together with reference sequences. GenBank accession numbers are shown next to species names. Similar nucleotide sites are illustrated by *dots* and gaps or missing data are denoted by *dashes*.

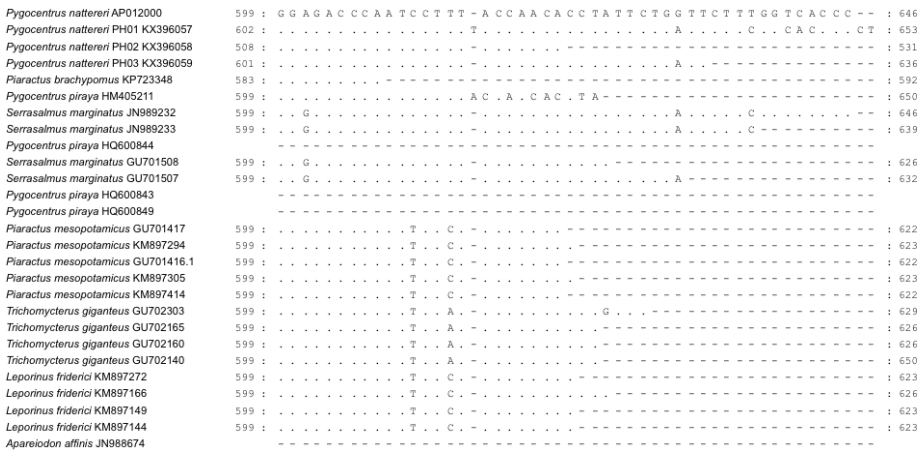

Figure 3 ***continued.*** Complete multiple alignments of juvenile fish CO1 sequences together with reference sequences. GenBank accession numbers are shown next to species names. Similar nucleotide sites are illustrated by *dots* and gaps or missing data are denoted by *dashes*.

Species transfer from one country to another is considered as a rising international concern not only due to the high probability of diseases introduction, but also its possible disruptive effects on aquatic communities (de Silva 1989). In line with this, policies are carried out to be able to control the entrance of possible invasive species that may harm the natural fauna in the Philippine environment. DNA barcoding allows rapid and more accurate method for species identification, thereby providing reliable data regarding species to monitor and hold. The confirmation of the identity of fish samples in this study supports the prevention measures regarding the introduction of exotic fishes in the Philippines. DNA barcoding serves to be an effective tool in monitoring entrance of alien species contributing to increased efforts in wildlife conservation.

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CONFLICTS OF INTEREST

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CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Sarmiento wrote the manuscript. Sarmiento and Ventolero conducted laboratory experiments, particularly DNA extraction, PCR amplification, and analysis of DNA sequences. Santos provided overall concept of the paper, supervised the experiments and edited the manuscript.

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Table 1. Computed K2P pairwise genetic distances of piranha samples with reference sequences. GenBank accession numbers are shown next to species names.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	*Pygocentrus nattereri* AP012000		0.000	0.005	0.000	0.027	0.008	0.010	0.010	0.008	0.010	0.010	0.008	0.008	0.028	0.028	0.028	0.028	0.028	0.025	0.025	0.025	0.025	0.029	0.031	0.030	0.030	0.036
2	*Pygocentrus nattereri* PH01 KX396057	0.000		0.005	0.000	0.027	0.008	0.010	0.010	0.008	0.010	0.010	0.008	0.008	0.028	0.028	0.028	0.028	0.028	0.025	0.025	0.025	0.025	0.029	0.031	0.030	0.030	0.036
3	*Pygocentrus nattereri* PH02 KX396058	0.011	0.011		0.005	0.027	0.009	0.012	0.012	0.009	0.011	0.012	0.010	0.010	0.029	0.029	0.029	0.029	0.029	0.026	0.027	0.026	0.026	0.030	0.032	0.031	0.031	0.037
4	*Pygocentrus nattereri* PH03 KX396059	0.000	0.000	0.011		0.027	0.008	0.010	0.010	0.008	0.010	0.010	0.008	0.008	0.028	0.028	0.028	0.028	0.028	0.025	0.025	0.025	0.025	0.029	0.031	0.030	0.030	0.036
5	*Piaractus brachypomus* KP723348	0.190	0.190	0.207	0.190		0.027	0.028	0.028	0.027	0.029	0.028	0.026	0.026	0.010	0.009	0.009	0.009	0.010	0.030	0.030	0.030	0.030	0.036	0.036	0.036	0.036	0.036
6	*Pygocentrus piraya* HM405211	0.029	0.029	0.040	0.029	0.190		0.010	0.010	0.002	0.010	0.010	0.003	0.003	0.028	0.029	0.029	0.029	0.029	0.029	0.029	0.029	0.029	0.030	0.029	0.029	0.029	0.036
7	*Serrasalmus marginatus* JN989232	0.045	0.045	0.057	0.045	0.205	0.045		0.000	0.010	0.002	0.000	0.010	0.010	0.029	0.030	0.030	0.030	0.030	0.027	0.027	0.027	0.027	0.029	0.031	0.030	0.030	0.038
8	*Serrasalmus marginatus* JN989233	0.045	0.045	0.057	0.045	0.205	0.045	0.000		0.010	0.002	0.000	0.010	0.010	0.029	0.030	0.030	0.030	0.030	0.027	0.027	0.027	0.027	0.029	0.031	0.030	0.030	0.038
9	*Pygocentrus piraya* HQ600844	0.026	0.026	0.037	0.026	0.187	0.002	0.043	0.043		0.009	0.010	0.002	0.002	0.028	0.028	0.028	0.028	0.028	0.029	0.029	0.029	0.029	0.028	0.029	0.029	0.029	0.037
10	*Serrasalmus marginatus* GU701508	0.043	0.043	0.054	0.043	0.209	0.043	0.002	0.002	0.040		0.002	0.010	0.010	0.030	0.030	0.030	0.030	0.030	0.027	0.027	0.027	0.027	0.029	0.030	0.030	0.030	0.038
11	*Serrasalmus marginatus* GU701507	0.045	0.045	0.057	0.045	0.205	0.045	0.000	0.000	0.043	0.002		0.010	0.010	0.029	0.030	0.030	0.030	0.030	0.027	0.027	0.027	0.027	0.029	0.031	0.030	0.030	0.038
12	*Pygocentrus piraya* HQ600843	0.029	0.029	0.040	0.029	0.183	0.004	0.045	0.045	0.002	0.043	0.045		0.000	0.027	0.027	0.027	0.027	0.027	0.029	0.029	0.029	0.029	0.028	0.029	0.028	0.028	0.037
13	*Pygocentrus piraya* HQ600849	0.029	0.029	0.040	0.029	0.183	0.004	0.045	0.045	0.002	0.043	0.045	0.000		0.027	0.027	0.027	0.027	0.027	0.029	0.029	0.029	0.029	0.028	0.029	0.028	0.028	0.037
14	*Piaractus mesopotamicus* GU701417	0.196	0.196	0.213	0.196	0.040	0.196	0.211	0.211	0.193	0.215	0.211	0.189	0.189		0.003	0.003	0.003	0.003	0.032	0.032	0.032	0.032	0.034	0.035	0.034	0.034	0.032
15	*Piaractus mesopotamicus* KM897294	0.201	0.201	0.218	0.201	0.035	0.201	0.216	0.216	0.197	0.220	0.216	0.193	0.193	0.004		0.000	0.002	0.003	0.032	0.031	0.032	0.032	0.034	0.035	0.034	0.034	0.032
16	*Piaractus mesopotamicus* GU701416.1	0.201	0.201	0.218	0.201	0.035	0.201	0.216	0.216	0.197	0.220	0.216	0.193	0.193	0.004	0.000		0.002	0.003	0.032	0.031	0.032	0.032	0.034	0.035	0.034	0.034	0.032
17	*Piaractus mesopotamicus* KM897305	0.204	0.204	0.221	0.204	0.038	0.204	0.219	0.219	0.200	0.223	0.219	0.196	0.196	0.006	0.002	0.002		0.003	0.032	0.031	0.032	0.032	0.034	0.035	0.034	0.034	0.032
18	*Piaractus mesopotamicus* KM897414	0.203	0.203	0.220	0.203	0.040	0.203	0.218	0.218	0.200	0.222	0.218	0.196	0.196	0.004	0.004	0.004	0.006		0.032	0.032	0.032	0.032	0.034	0.035	0.034	0.034	0.032
19	*Trichomycterus giganteus* GU702303	0.205	0.205	0.222	0.205	0.241	0.235	0.217	0.217	0.231	0.217	0.217	0.235	0.235	0.253	0.249	0.249	0.252	0.253		0.003	0.000	0.000	0.029	0.031	0.030	0.030	0.031
20	*Trichomycterus giganteus* GU702165	0.205	0.205	0.222	0.205	0.233	0.235	0.217	0.217	0.231	0.217	0.217	0.235	0.235	0.245	0.240	0.240	0.244	0.244	0.004		0.003	0.003	0.029	0.031	0.030	0.030	0.031
21	*Trichomycterus giganteus* GU702160	0.205	0.205	0.222	0.205	0.241	0.235	0.217	0.217	0.231	0.217	0.217	0.235	0.235	0.253	0.249	0.249	0.252	0.253	0.000	0.004		0.000	0.029	0.031	0.030	0.030	0.031
22	*Trichomycterus giganteus* GU702140	0.205	0.205	0.222	0.205	0.241	0.235	0.217	0.217	0.231	0.217	0.217	0.235	0.235	0.253	0.249	0.249	0.252	0.253	0.000	0.004	0.000		0.029	0.031	0.030	0.030	0.031
23	*Leporinus friderici* KM897272	0.220	0.220	0.238	0.220	0.270	0.212	0.227	0.227	0.208	0.223	0.227	0.205	0.205	0.257	0.254	0.254	0.253	0.256	0.237	0.230	0.237	0.237		0.005	0.003	0.003	0.033
24	*Leporinus friderici* KM897166	0.231	0.231	0.249	0.231	0.278	0.223	0.238	0.238	0.219	0.234	0.238	0.215	0.215	0.264	0.261	0.261	0.260	0.263	0.252	0.244	0.252	0.252	0.011		0.003	0.003	0.035
25	*Leporinus friderici* KM897149	0.224	0.224	0.242	0.224	0.270	0.216	0.232	0.232	0.212	0.227	0.232	0.208	0.208	0.257	0.254	0.254	0.253	0.256	0.245	0.237	0.245	0.245	0.006	0.004		0.000	0.034
26	*Leporinus friderici* KM897144	0.224	0.224	0.242	0.224	0.270	0.216	0.232	0.232	0.212	0.227	0.232	0.208	0.208	0.257	0.254	0.254	0.253	0.256	0.245	0.237	0.245	0.245	0.006	0.004	0.000		0.034
27	*Apareiodon affinis* JN988674	0.280	0.280	0.292	0.280	0.266	0.276	0.293	0.293	0.280	0.289	0.293	0.285	0.285	0.251	0.248	0.248	0.247	0.251	0.258	0.254	0.258	0.258	0.254	0.269	0.267	0.267	

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