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Short Communication

DNA Barcoding of Confiscated Endangered Philippine Sailfin Lizard *Hydrosaurus pustulatus* (Eschsholtz, 1829)

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Abstract: The Philippines, a known biodiversity hotspot, faces the threat of higher extinction rates due to disruptive human activities that include illegal wildlife trade. Among those species affected by these activities is the endemic Philippine Sailfin Lizard (*Hydrosaurus pustulatus*), which is currently listed as 'Vulnerable' by the International Conservation of Natural Resources (IUCN). Proper identification of illegally traded samples could be addressed using DNA barcoding that uses small segments of DNA in confirming species identity. *Cox1* gene has been used in DNA barcoding *H. pustulatus*. However, the 16S rRNA gene region, which is also commonly used as a marker for reptiles, is lacking in the species. Here, we demonstrated the use of DNA barcodes, specifically *cytochrome oxidase subunit I* (*cox1*) genes, in the confirmation of identity of illegally traded *H. pustulatus* and its possible geographic origin. We also provided novel 16S gene sequences together with *cox1 gene* to infer its placement within the Agamidae. BLAST results show that the generated *cox1* barcodes matched with *H. pustulatus* (100%) found in GenBank. Furthermore, haplotype network analysis revealed that the confiscated samples were similar to the *H. pustulatus* haplotype found in Polillo Island. Phylogenetic analysis using concatenated 16S rRNA and *cox1* genes showed that *H. pustulatus* clustered with a congeneric, *H. amboinensis* (ML Bootstrap=100; NJ Bootstrap=100). In this study, we demonstrated that DNA barcodes could aid not only in the proper identification of the species but also their possible geographic origin. This could be useful in providing data on hotspot areas of wildlife trafficking. In addition, the use of the 16S gene can potentially be used together with *cox1* in discriminating between *H. pustulatus* and *H. amboinensis*.

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Keywords: DNA Barcoding, Wildlife Forensics, Philippine Sailfin Lizard, *cox1*, 16S

1. INTRODUCTION

The Philippines is considered as one of the biodiversity hotspots in the world with its high levels of endemism coupled with higher risk of extinction due to human activities such as energy use and land conversion which leads to habitat loss (Ehrlich 1994; Cincotta et al. 2000; Myers et al. 2000). In addition, wildlife trafficking has been a major challenge in Asia with millions of animals being illegally exported in the region alone (Nijman 2010).

Among those species facing illegal wildlife trade is the endemic Philippine sailfin lizard, *Hydrosaurus pustulatus* (Eschsholtz 1829), which is categorized as 'Vulnerable' by the International Conservation of Natural Resources (IUCN) (Ledesma 2009). This omnivorous reptile is found in several islands of the Philippines including Polillo, Mindoro, Negros, Guimaras, Panay, Masbate, Tablas, Romblon, Sibuyan, Catandauanes, Bicol, and other small isolated islands where its identification is confused with its congeneric species, *H. amboinensis* (Ledesma 2009; Siler et al. 2011).

H. pustulatus is a squamate reptile classified under subfamily Hydrosaurinae, along with its congeneric *H. amboinensis*, of family Agamidae (Pyron et al. 2013). Agamidae is a monophyletic family considered as the Old World counterpart of the New World Iguanidae and is a sister clade to Chamaelonidae based on their 16S and 12S mitochondrial rRNA genes (Honda et al. 2000). However, *H. pustulatus* is not represented in this

phylogenetic analysis due to lack of available molecular data.

DNA barcoding could be utilized in the rapid identification of known and novel species by using combinations of nucleotides found in DNA to produce unique barcodes that could discriminate different species (Hebert et al. 2003; von Crautlein et al. 2011). The use of mitochondrial genes has been demonstrated to allow the identification of cryptic species especially among animals (Hebert et al. 2003; Feng et al. 2011; Luo et al. 2011). However, DNA barcoding efforts in the Philippines has not been significantly documented, with some taxa lacking representation in the genetic database (Fontanilla et al. 2014). In addition, reptiles lack efficient universal primers that target the commonly used animal mitochondrial gene, the cytochrome oxidase subunit I (*cox1*) (Vences et al. 2012). One solution is the use of the 16S rRNA gene, which currently serves as the suitable mitochondrial DNA marker for the taxon (Vences et al. 2012). However, no 16S rRNA gene has been published in the genetic database for the identification of *H. pustulatus*.

In this study, we aimed to utilize the *cox1* gene to confirm the identity of confiscated *H. pustulatus* specimens and determine their possible geographic origin. Using sequences from the *cox1* and 16S genes, the placement of *H. pustulatus* within the Agamidae is elucidated.

2. METHODOLOGY

2.1 Acquisition of Samples

Seven vials of *H. pustulatus* tail clippings were used as the source of DNA. These came from samples that were confiscated by the Department of Environment and Natural Resources-Biodiversity Management Bureau (DENR-BMB) Wildlife Rescue Center (WRC) from an illegal consignment on 28 January 2016 at the Philippine Airlines (PAL) cargo terminal. This consignment was to be exported to Japan along with other confiscated endangered and endemic species including tarsiers, watersnakes, ratsnakes, and the Philippine scops owl.

2.2 DNA Extraction and Amplification

Tissue samples from the tail clippings were subjected to DNA extraction using the GeneJET Genomic DNA Purification Kit (Thermo Scientific) following the manufacturer's protocol.

Extracted DNA was subjected to PCR amplification using VF1: 5'TTCTCAACCAACCACAAAGACATTGG-3' (Ivanova et al. 2006) and VR1: 5'TAGACTTCTGGGTGGCCAAAGAATCA-3' (Ward et al. 2005) as the forward and reverse primers, respectively, in order to amplify the *cox1* gene. PCR was utilized using 1.0 µL of 10x PCR buffer, 36.25 µL distilled water, 0.25 µL (5units/ µL) Taq polymerase, 1.5 µL (1mM) forward and reverse primers, 2.5 µL (0.2mM) DMSO, 2.0 µL of (50mM) $MgCl_2$, and 5.0 µL (3-40 ng/µL) DNA template from the sample. The PCR thermal regime for the *cox1* gene consisted of an initial denaturation at 94°C for 2 mins, followed by 5 cycles of denaturation at 94°C for 40 secs, annealing at 45°C for 40 secs, and extension at 72°C for 1 min; the annealing temperature was then changed to 51°C and the thermal cycling ran for another 35 cycles before the final extension at 72°C for 5 cycles.

The 16S rRNA was amplified using 16S Ar: 5'-CGCCTGTTTATCAAAAACAT 3' and 16S Br: 5'-CCGGTCTGAACTCAGATCACGT-3' (Palumbi 1996) as the forward and reverse primers, respectively. The same PCR components followed the concentrations used in amplification of *cox1* except those of the DNA template and distilled water were adjusted to 1.0 µL and 40.25 µL, respectively. The PCR regime consisted of an initial denaturation at 96°C for 5 mins, followed by 43 cycles of denaturation at 96°C for 30 secs, annealing at 45°C for 30 secs, extension at 65°C for 1 min, and a final extension at 72°C for 5 mins.

2.3 Agarose Gel Electrophoresis and Sequencing

A 1% Agarose gel was prepared by dissolving one gram of agarose powder (Vivantis) in 100mL of 0.5X tris borate EDTA(TBE) buffer. One µL of 10mg/ml ethidium bromide (Invitrogen™) was added to the mixture for gel visualization. The gel mixture was allowed to solidify in a cassette and was submerged in 0.5X TBE buffer inside the AGE setup.

Each PCR product was loaded into the wells using 2 µL of Blue Juice as the loading dye. One µL of KAPPA Express DNA Ladder (KAPABiosystems) was used as the molecular weight marker. The setup was run for 30 minutes at 100 volts and was visualized under a UV illuminator after the run. Bands indicating the presence of the gene were cut given that they corresponded to their expected sizes (*cox1*=800bp; 16S=500bp). These bands were subjected to gel extraction and PCR clean-up using the QIAquick® Gel Extraction Kit (QIAGEN®), after which they were sent to 1st Base in Singapore for Sequencing.

2.4 Molecular Identification of confiscated *H. pustulatus* specimens

The consensus sequences for *cox1* and 16S genes were determined from generated forward and reverse reads using STADEN package (Staden et al. 2000). The consensus sequences were subjected to Basic Local Alignment Search Tool (BLAST) to identify the samples' closest match.

2.5 Determination of geographic location

To determine the possible geographic location of the confiscated samples, *H. pustulatus cox1* sequences were also downloaded from GenBank. These sequences represent different clades of *H. pustulatus* haplotypes with known location based on the data published by Siler et al. (2014). Sequences were

aligned using BioEdit v7.2.5 (Hall 2005) via the ClustalW (Thompson et al. 2003) function. Aligned sequences were trimmed using the program GBlocks v.0.91b (Castresana 2002). A median joining network was constructed using the R program packages pegas (Paradis 2010) and ape (Paradis 2018).

2.6 Determination of the placement of *H. pustulatus* within the Agamidae

Gene sequences of 16S and *cox1* from the whole mitochondrial genome of representatives of family Agamidae were downloaded in GenBank. These gene regions were aligned with the 16S and *cox1* gene sequences obtained from the confiscated *H. pustulatus* samples using the program BioEdit (Hall 2005). The aligned datasets were trimmed using the program GBlocks (Castresana 2002) for the sequences to be suitable for gene tree construction. Trimmed *cox1* and 16S gene sequences were concatenated using the program DAMBE (Xia & Xie 2001).

The model of substitution for the concatenated dataset for the ML tree construction was determined using jModelTest v2.1.10 (Guindon & Gascuel 2003; Darriba et al. 2012). Construction of maximum likelihood tree was carried out using IQTree (Nguyen et al. 2004). Bootstrap support of 1000 replicates were generated for neighbor-joining (Saitou & Nei 1987) and maximum likelihood (Felsenstein 1981) methods using PAUP 4.0 (Swofford 2002) and IQTree (Nguyen et al. 2004), respectively.

3. RESULTS AND DISCUSSION

3.1 Molecular confirmation of confiscated *H. pustulatus* specimens

All sequences generated from this study were submitted to the Barcode of Life Data (BOLD) with the following accessions: MN228919-MN228925 (*cox1*) and MN322560-MN322566 (16S). BLAST results using the *cox1* gene region showed that the confiscated specimens were identified as *H. pustulatus* with 100% identity. Its congeneric, *H. amboinensis*, was only 95.41%-96.18% identical with the samples (Table 1). Since there are currently no available *16S* gene region sequences, BLAST results of the 16S gene region from the confiscated samples were most similar (99.1%) with its congeneric, *H. amboinensis* (Table 1). Furthermore, the haplotype network (Figure 1) based on the *cox1* gene of the confiscated *H. pustulatus* specimens suggest that the samples were identical to *H. pustulatus* found in Polillo Island and most similar to Aurora and suggests the possible origin of these samples from these areas.

H. pustulatus is one of the endemic species in the Philippines frequently targeted for illegal wildlife trade due to their striking dorsal crests, conspicuous caudal sail-like structure and ornate coloration (Siler et al. 2014). In fact, pet traders use different media including online sites in advertising the sale of Philippine Sailfin Lizards as pets. Fortunately, with the advent of DNA barcoding effort in the Philippines, identification of illegally traded wildlife became more effective and efficient. For example, in the Philippines, the use of *cox1* gene was proven to be useful in confirming the identity of some frozen dressed pangolins, which were confiscated from a Chinese fishing vessel in Tubbataha, to be that of the critically endangered Sunda pangolin *Manis javanica* (Luczon et al. 2016). This shows that the use of DNA barcoding could be useful when morphological identification becomes impossible due to circumstances such as sample decomposition or alteration.

Table 1. Basic local alignment search tool (BLAST) results for confiscated samples of *Hydrosaurus pustulatus*.

Voucher Specimen	Accession Number	Gene	BLAST Identified organism	% Identity	Accession Number
1A	MN228925	*cox1*	*Hydrosaurus pustulatus*	100.0%	KTO75334
			Hydrosaurus amboinensis	95.4%	AB475096
	MN322566	16S	*Hydrosaurus amboinensis*	99.1%	AB475096
1B	MN228923	*cox1*	*Hydrosaurus pustulatus*	100.0%	KTO75334
			Hydrosaurus cf. *amboinensis*	96.1%	FJ952249
	MN322564	16S	*Hydrosaurus amboinensis*	99.1%	AB475096
1C	MN228922	*cox1*	*Hydrosaurus pustulatus*	100.0%	KTO75334
			Hydrosaurus cf. *amboinensis*	96.0%	FJ952249
	MN322563	16S	*Hydrosaurus amboinensis*	99.1%	AB475096
2B	MN228924	*cox1*	*Hydrosaurus pustulatus*	100.0%	KTO75334
			Hydrosaurus cf. *amboinensis*	96.0%	FJ952249
	MN322565	16S	*Hydrosaurus amboinensis*	99.1%	AB475096
4A	MN228921	*cox1*	*Hydrosaurus pustulatus*	100.0%	KTO75334
			Hydrosaurus cf. *amboinensis*	96.2%	FJ952249
	MN322562	16S	*Hydrosaurus amboinensis*	99.1%	AB475096
4B	MN228920	*cox1*	*Hydrosaurus pustulatus*	100.0%	KTO75334
			Hydrosaurus amboinensis	95.5%	AB475096
	MN322561	16S	*Hydrosaurus amboinensis*	99.1%	AB475096
4C	MN228919	*cox1*	*Hydrosaurus pustulatus*	100.%	KTO75334
			Hydrosaurus amboinensis	95.8%	FJ952255
	MN322560	16S	*Hydrosaurus amboinensis*	99.1%	AB475096

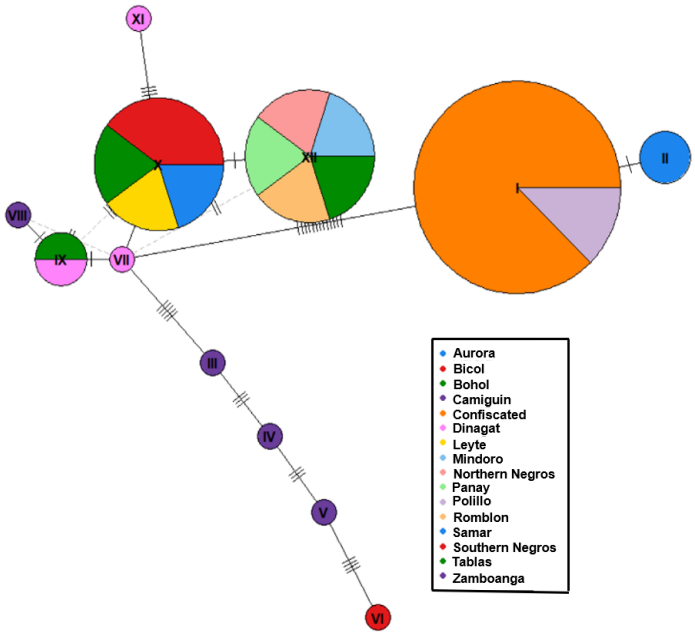

Figure 1. Haplotype Network of *H. pustulatus* haplotypes of the cox1 gene from the confiscated samples and GenBank sequences used in the study. Location of the GenBank sequences are based on data published by Siler et al. (2014). Each bar represents a single nucleotide change.

3.2 Phylogenetic analysis using *cox1* and 16S *rRNA* gene sequences

Concatenation of the 16S rRNA and *cox1* genes resulted to a 796-nucleotide long sequence alignment which was used to infer the phylogenetic relationship in Agamidae using the Transversional model (TVM) (Posada 2003) with invariant sites (+I) and rate variation among sites (+G) as determined by jModelTest as the optimal model. The resulting ML tree supports the monophyly of Agamidae (ML Bootstrap=100; NJ Bootstrap=100). Furthermore, *H. pustulatus* clustered with *H. amboinensis*, supporting the grouping of the two congeneric species into subfamily Hydrosaurinae (ML Bootstrap=100; NJ Bootstrap=100). This tree also supports the clustering of subfamilies Agaminae (ML Bootstrap=99; NJ Bootstrap=99), Amphibolurinae (ML Bootstrap=100; NJ Bootstrap=97), Draconinae (ML Bootstrap=100; NJ Bootstrap=100), and Leiopinae (ML Bootstrap=100; NJ Bootstrap=100) of Agamidae (Figure 2).

Although no 16S rRNA gene region is present in the current genetic database for *H. pustulatus,* we were able to demonstrate the use of the gene, along with the *cox1* gene, in elucidating the relationships within the family. These genes could likewise distinguish *H. pustulatus* and *H. amboinensis* as observed in a distinct divergence between the two species (Figure 2). The inclusion of 16S rRNA gene region could also be useful in elucidating a wider phylogenetic study of the species with other reptiles due to the lack of *cox1* for the said group.

4. CONCLUSION

DNA barcodes are shown to be useful in confirming the identity of *H. pustulatus* as demonstrated by the *cox1* gene. Furthermore, the gene was also able to narrow down the likely origin of the trafficked *H. pustulatus* samples based on the *cox1* barcode data from Siler et al. (2014). In addition, we have also providing novel 16S gene sequences for the species, adding a gene marker for possible identification of the species. Nevertheless, creating a more extensive database that includes all possible localities where the species is located could provide a more comprehensive detection system of wildlife trafficking hotspots in the Philippines provided that there is sufficient variation to distinguish populations from across the different localities. Determination of the most likely areas where illegal wildlife trafficking is rampant could be useful from the perspective of law enforcement.

Furthermore, inclusion of the 16S gene was able to aid in inferring the relationship of *H. pustulatus* with other agamids by complementing the *cox1* database for the taxon. The concatenated dataset was also able to demonstrate the high support for various subfamilies within the Agamidae.

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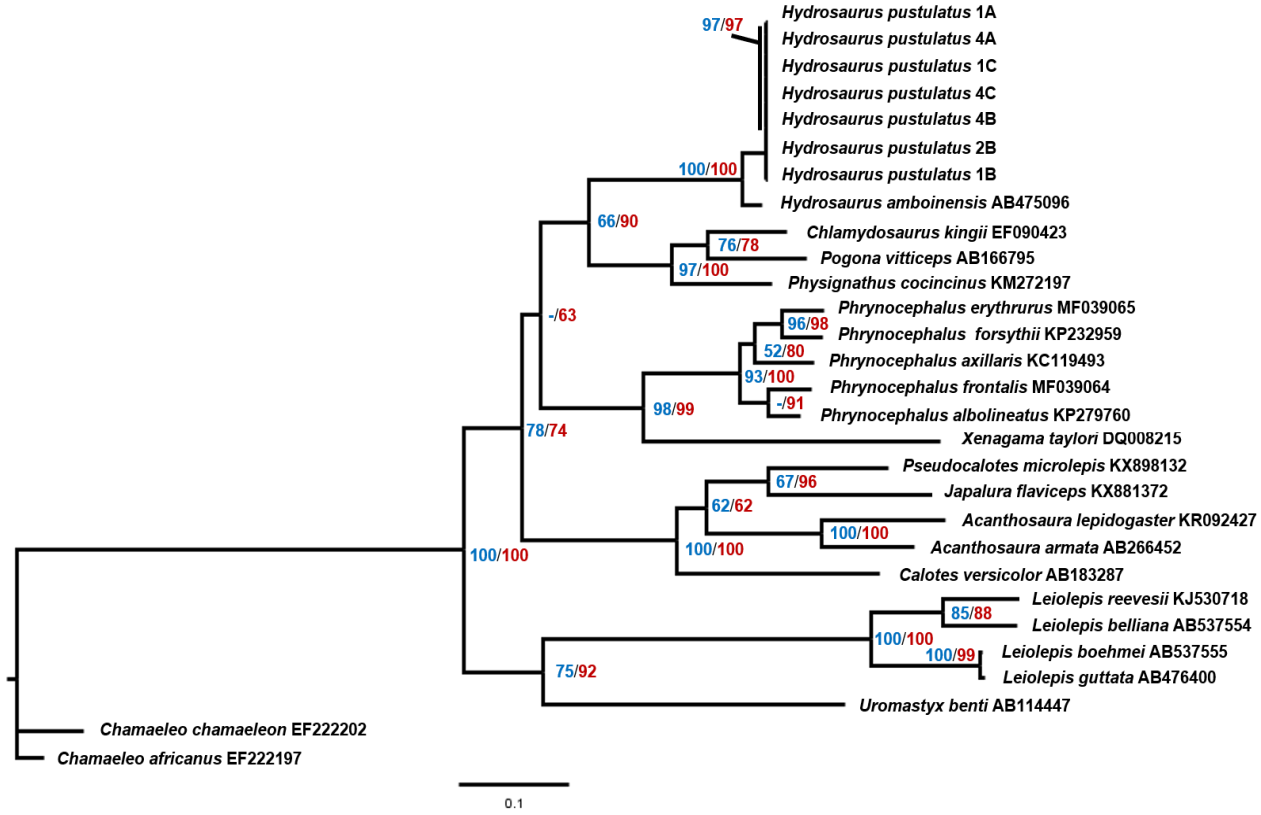

Figure 2. Maximum likelihood tree of Agamidae based on 796 nucleotides of the concatenated cox1 and 16S rRNA genes, with Chamaeleonidae (*Chamaeleo chamaeleon, Chamaeleo africanus*) as the outgroup. Values on the nodes represent percent bootstrap values based on 1000 bootstrap replicates using maximum likelihood (red) and neighbor-joining (blue) methods; values less than 50% are not shown. Scale bar represents one nucleotide substitution for every 10 nucleotides.

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