DNA Barcoding of Birds in the University of the Philippines Diliman Campus, with Emphasis on Striated Grassbirds Megalurus palustris

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DNA barcoding is increasingly being used by researchers across the globe to aid in the identification of species. Using this taxonomic tool on bird species in an urban green space within Manila, i.e. the University of the Philippines Diliman campus, Luzon, Philippines, DNA barcodes of eleven species were generated. Different haplotypes for some of the species were observed. Using BLAST, the cytochrome oxidase subunit 1 (COI) sequence of every species from this study was correctly matched with the corresponding species having a COI record in Genbank, with the exception of the Striated Grassbird Megalurus palustris, which is a new COI record. The three distinct haplotypes for M. palustris were then compared with COI sequences from other members of the sylviid “Old World Warblers” to determine the effectiveness of the DNA barcode in discriminating it with other species. Results show that COI was successful in placing M. palustris as a distinct taxon.

Key Words: cytochrome oxidase I gene, DNA barcoding, Locustellidae, Megalurus, Philippines

INTRODUCTION

Out of the approximately 10,000 identified bird species, 607 can be found in the Philippines. Of the global total, about 2% is found nowhere else in the world (Dickinson et al. 1991; WBCP 2010). Due to the Philippines' highly diverse, endemism-rich, but highly threatened biodiversity, the country is considered a top priority in terms of global conservation (Myers et al. 2000). Yet, Lohman et al. (2010) concluded that these values for Philippine avian endemism might be gravely underestimated.

Taxonomy plays a key role in any biological study. Any experiment addressing scientific problems involving a taxon must first ascertain the correct identification of that taxon. Even today when there is an explosion in the number of species being discovered, morphology alone sometimes is unable to discriminate between species (Packer et al. 2009). DNA barcode profiles were developed as a taxonomic tool to address this limitation.

DNA barcoding employs a universally accepted standard sequence from a short region of the mitochondrial genome that consistently distinguishes between any two given species to their taxonomic status based on a library of sequences linked to vouchered specimens from all over the world (Hebert et al. 2003a). The use of the 648-bp region of the mitochondrial cytochrome c oxidase subunit I (COI) gene has been suggested in previous studies as a DNA barcode for the identification of animal species (Hebert et al. 2003b). Today, several consortia and projects have been established to collectively barcode the DNA of all the identified species of the world as well as new species that are continuously being discovered. The Consortium for the Barcode of Life (CBOL) is an initiative joined by many research organizations, natural history museums and herbaria, and private assemblies.
that intends to develop DNA barcoding as a global standard in the identification of species. The All Birds Barcoding Initiative (ABBI), a flagship program of CBOL, aims to establish an archive of DNA barcodes for all bird species that are linked to museum specimens (ABBI 2005). Currently, the Barcode of Life Data (BoLD) Systems functions as a repository of these DNA barcode sequences as well as a public database in which barcode sequences can be accessed. In the Philippines, the University of the Philippines Diliman Institute of Biology (UPD IB) established the DNA Barcoding of Life-Philippine Network (DOLPhiN) in 2008 and is an ongoing program that aims to apply DNA barcoding to Philippine biodiversity, particularly to its native and endemic species. Luczon et al. (2010) produced the first set of COI DNA barcodes for DOLPhiN from the White Collared Kingfisher, Todiramphus chloris, from UP Diliman, followed by Ong et al. (2011) for the Philippine accipitrids found in the Philippine Eagle Center in Davao City and Aquilino et al. (2011) for the ichthyofauna of Taal Lake.

Some resident birds of the Philippines belong to the Old World Warbler group from the family Sylviidae, comprising approximately 400 species. Sylviidae is recognized as one of the main families in the order Passeriformes, albeit its composition remains unsettled among different authors. Based on DNA-DNA hybridization studies, Sibley et al. (1990) proposed that Cisticolidae should be split from Sylviidae, while Sylviidae should be divided into the subfamilies Megalurinae, Acrocephalinae, Garrulacinae and Sylviinae. However, recent work involving direct DNA evidence (Cibois 2003; Barker 2004; Beresford et al. 2005) showed that the Old World Warblers should be reorganized as different families. Based on sequence data of the nuclear intron II of the myoglobin gene and the mitochondrial cytochrome b gene, it was proposed that Cisticolidae should be split from Sylviidae, while Sylviidae should be divided into the subfamilies Megalurinae, Acrocephalinae, Garrulacinae and Sylviinae. However, recent work involving direct DNA evidence (Cibois 2003; Barker 2004; Beresford et al. 2005) showed that the Old World Warblers should be reorganized as different families. Based on sequence data of the nuclear intron II of the myoglobin gene and the mitochondrial cytochrome b gene, it was proposed that the sylvids be divided into the true Sylviidae, Timaliidae, Cisticolidae, Acrocephalidae, Megaluridae, Cettidae, Aegithalidae, Phylloscopidae, the Malagasy warblers and some unclassified sylvids (Alström et al. 2006). Alström et al. (2011) later changed Megaluridae to Locustellidae as the name Locustellinae (Bonaparte, 1854) has priority over Megalurinae (Blyth, 1875) (Bock, 1994). This study, which is a continuation of that of Luczon et al. (2010), aims to produce a barcode profile for Philippine birds found within the UP Diliman campus. Vallejo et al. (2008) identified 36 species, of which 14% are endemic, interspersed in various habitats in UP Diliman such as the campus’s green spaces and buildings. Notable among these birds is the Striated Grassbird, Megalurus palaustris (Figure 1), an Old World Warbler from the sylvid family Locustellidae, which has not been barcoded previously.

**MATERIALS AND METHODS**

**Bird Sampling**

Birds were sampled by setting up mist nets within the University of the Philippines campus in Diliman, Quezon City, Philippines (N 14° 39’, E 121° 04’). Nine sampling sites were established for sampling birds (see Figure 2). Biological samples used for DNA extraction were derived primarily from blood feathers, although blood were also drawn from the jugular vein of live samples or pectoral muscle of specimens that died during capture if blood feathers were not available. In lieu of actual voucher specimens, high quality pictures were taken for each bird in different orientations (lateral, dorsal and ventral). Standard morphometric data such as bill length, bill depth, tarsus length, wing length, tail length, and body weight were also taken. Before releasing, each live bird was clipped on its tail feathers or tagged with aluminum rings on one of its legs to avoid processing recaptured specimens.

**DNA Extraction, PCR Amplification, and Sequencing**

The total genomic DNA was obtained from the blood, blood feathers or pectoral muscle samples using the Promega™ Wizard® Genomic DNA Purification Kit following the manufacturer’s protocols. The 5’ 650 bp fragment of the cytochrome c oxidase subunit I (COI) was amplified using the primers developed by Hebert et al. (2004) - BirdF1 (TTCTCACCACCACAAAGACATTGGCAC) and BirdR1 (ACGTGGGAGATATCCAAATCCTG). Fifty μL amplification reactions were done in 0.2 mL tubes, using Taq DNA Polymerase and dNTPack from Roche® (Roche, USA). The volumes and final concentrations used for each component were the following: 5 μL PCR buffer with MgCl2 (10 mM Tris–HCl, 1.5 mM MgCl2, 50 mM KCl, pH 8.3 [20°C]), 2.5 μL (0.5 mM) each of the primers BirdF1 and BirdR1, 1.0 μL (0.2 mM) dNTPs, 34.75 μL distilled water, 0.25 μL of 5 units/μL Taq polymerase, and 4 μL of the DNA sample. The polymerase chain reaction conditions included a two-stage profile based on the method of Kerr et al. (2007). The first stage involved an initial start of 60 seconds at 94°C followed by six cycles of 60 seconds at 94°C, 90 seconds at 45°C, and 90 seconds at 72°C. It was then followed by another 35 cycles of 60 seconds at 94°C, 90 seconds at 55°C and 90 seconds at 72°C. The final run involved a final extension of five minutes at 72°C.

To confirm the amplification of the COI gene and the absence of any contamination to the DNA sample, the PCR products were run on a 1% agarose gel in 0.5X TBE buffer and visualized using EtBr-UV illumination. The bands on the gel were excised and purified using...
QIAquick® DNA Extraction Kit (Qiagen, USA) following the manufacturer’s protocols. Purified PCR products were then sent to Macrogen, Inc. in Korea or 1stBase in Singapore for sequencing.

**Sequence Assembly**
The COI sequences acquired from the sequencing companies were assembled using the STADEN package (Staden et. 2000) and then aligned using BioEdit Sequence Alignment Editor Version 7.0.5.3 (Hall 1999). All sequences were submitted to BoLD under the project Barcoding of UP Diliman Birds (Project code: BUPD). The sequences were also submitted to GenBank and were given the accession numbers JF957001 – JF957040.

**Figure 1.** Representative image of *Megalurus palustris*. A - Lateral view, B - Dorsal view, C - Ventral view.
Basic Local Alignment Search Tool (BLAST)
Each sequence was queried in BLAST (Altschul et al. 1990) in order to score its similarity with other DNA sequences available in GenBank. The validity of each bird species sequence was evaluated based on the sequences that scored the highest (i.e. highest scorers should be at least confamilial with the species of query). Kimura-2-Parameter (Kimura 1981) distances were then calculated using PAUP*4.0b10 (Swofford 2002) for the sequences in this study and those from the BLAST results.

Sequence Analysis
The barcodes of *M. palustris* are new records and were subjected to further sequence analysis that would determine their placement with other COI sequences of sylvid birds (Old World Warblers) from the BoLD and GenBank databases. Two outgroup sequences were added to the alignment and were taken from families Zosteropidae and Timaliidae, which are closely related to the Old World Warblers (Ericson & Johansson 2003). Analysis of the dataset was limited to 600 nucleotides,
while identical sequences were removed from the alignment using DAMBE (Xia et al. 2001), leaving only distinct sequences.

K2P pairwise distances of the dataset were then calculated using PAUP*4.0b10. A COI gene tree for the Old World warblers was then constructed to check if the COI gene would demonstrate clustering that reflects species delineation. The tree was derived from the K2P distances of the aligned sequences and then constructed using Neighbor Joining (NJ) (Saitou & Nei 1987) method.

RESULTS

Although the COI region cannot always elucidate basal relationships among taxa, as a barcode region, it has the ability to identify species almost unambiguously. A total of 40 cytochrome c oxidase subunit I (COI) sequences from 11 species belonging to 10 families and four orders were obtained (Table 1). All species are native and resident species in the Philippines, with the exception of the Eurasian Tree Sparrow *Passer montanus*, which is an introduced species. Another species, the White-collared Kingfisher *Todiramphus chloris*, was already sequenced and analyzed by Luczon et al. (2010). All sequences were checked for gaps by comparing the translated amino acid sequences with *Gallus gallus* complete cytochrome c oxidase subunit 1 gene (GenBank AP003580). No gaps or premature stop codons were found, indicating no pseudogenes were inadvertently obtained.

Several haplotypes were detected within species: three for the Pied Fantail *Rhipidura javanica*, four for the Brown Shrike *Lanius cristatus*, two for the Grey Wagtail *Motacilla cinerea*, two for *Passer montanus*, two for the Long-tailed Shrike *Lanius schach*, and three for the Striated Grassbird *Megalurus palustris*. Only one haplotype was found in the Yellow-vented Bulbul *Pycnonotus goiavier*, the Pacific Swallow *Hirundo tahitica*, the Common Kingfisher *Alcedo atthis*, the White-breasted Waterhen *Amaurornis phoenicurus*, and the Zebra dove *Geopelia striata*; however, only one individual each was sequenced for *A. phoenicurus*, *H. tahitica* and *A. atthis*. Most COI sequences generated from this study have similar COI sequences that correspond to the same species in the records of Genbank with a K2P distance range of 0 – 2.041%, lower than the threshold set by Hebert et al. (2004) at 2.7%. Many of these matches were derived from specimens outside the Philippines; for instance, *L. cristatus* haplotype 1 was most similar with *L. cristatus* from Russia (GenBank GQ482014) at 2.041% K2P distance; *L. cristatus* haplotype 3 with that from China (GenBank EF621603) at 0.153%, *Passer montanus* haplotype 2 with that from South Korea (GenBank EF515797) at 0.312%, and *Lanus schach* haplotype 1 with that from China (GenBank EF621605) at 0.611%. On the other hand, COI sequences for *A. phoenicurus* and *M. palustris* are not in the records of Genbank. The species that are most similar to them are the Giant Woodrail *Aramides ypecaha* (12.179% K2P distance) and the Middendorf’s Warbler *Locustella ochotensis* (10.677 – 11.864%), respectively. *A. phoenicurus*, however, does have records in BoLD with 99.86-100% similarity to the

### Table 1. BLAST results and K2P distances of the COI sequences of birds in University of the Philippines Diliman.

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th># of indiv.</th>
<th>BLAST Results</th>
<th>K2P distance (% difference)</th>
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</thead>
<tbody>
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<td>1</td>
<td><em>Pycnonotus goiavier</em></td>
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<td><em>Pycnonotus goiavier</em> (FJ473200)</td>
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<tr>
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<td><em>Rhipidura javanica</em> (FJ473207)</td>
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<td></td>
<td></td>
<td>0/655</td>
</tr>
<tr>
<td></td>
<td><em>Rhipidura javanica</em></td>
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<td><em>Rhipidura javanica</em> (FJ473100)</td>
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<td></td>
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<td>2</td>
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<td>0/655</td>
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<tr>
<td></td>
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<td></td>
<td></td>
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<td>0/695</td>
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Table 1 continued next page...
**Table 2**. Minimum, maximum, and mean Kimura-2-Parameter pairwise values of all 169 unique Old World Warbler haplotypes.

<table>
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<tr>
<th>Comparisons</th>
<th># of comparisons</th>
<th>minimum</th>
<th>average</th>
<th>maximum</th>
<th>standard error</th>
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<tr>
<td>within species</td>
<td>331</td>
<td>0</td>
<td>0.0094</td>
<td>0.1126</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

COI sequence from UP Diliman; these BoLD sequences for *A. phoenicurus* were derived from individuals from Australia and Japan.

**Analysis of Molecular Data for Megalurus palustris**

A total of 169 unique sequences were used for the study. This consists of COI sequences of Old World Warblers (*Sylviidae*) downloaded from Genbank and BoLD databases, the three sequence haplotypes of *M. palustris* generated in this study, and two outgroup representatives each from Zosteropidae (*Zosterops erythropleurus*) and Timaliidae (*Garrulax lunulatus*). As seen in table 2, the average K2P distance decreases as the taxonomic level being evaluated becomes more exclusive. It should be noted, however, that the maximum K2P distance for the conspecific comparison is high (0.1126). This high distance came from a pairwise comparison between two *Sylvia mystacaea* individuals.

**Gene Tree Construction**

Prior to tree construction, the dataset was checked for substitution saturation using Xia test (Xia & Lemey 2009). Since no saturation was observed, we proceeded with the construction of a Neighbor-Joining (NJ) tree based on the K2P model (see Figure 3). Bootstrap values for the NJ tree generated were included in the topology.
Figure 3. Neighbor-joining phylogenetic tree of some old world warblers including outgroup taxa from Zosterpoidae (white-eyes) and Timaliidae (babblers), based on a 600-bp fragment of the COI gene. Bootstrap support values are indicated at the nodes of the tree; bootstrap values less than 50% are not shown. Scale bar represents corrected genetic distance based on K2P model of substitution (0.02 = 1 nucleotide change for every 50 nucleotides). Classifications are based on Alström et al. (2006), Alström et al. (2011), and Sibley et al. (1990).
The gene tree was rooted on the outgroup taxon, Z. erythropneurus, revealing relationships within the Old World Warbler group with nine genera (containing 51 ingroup species) and their relationship with the second outgroup taxon, G. lunulatus.

The three distinct haplotypes of M. palustris clustered together in the NJ tree. M. palustris haplotype 3 (JF957021) seemed to have diverged from the other two haplotypes (JF957022 and JF957020). This topology received 100% NJ bootstrap support. The M. palustris clade is then shown as a sister clade of several species of Locustella with bootstrap support of 96%. Most congeneric and conspecific sequences were grouped together and were supported with high bootstrap values.

Observations regarding the placements of other sylviids based on the COI gene tree are worth mentioning. Groupings of some recently recognized sylviid families were evident in the gene tree such as the Phylloscopidae (Phylloscopus spp.), Acrocephalidae (Acrocephalus and Hippolais spp.), and Locustellidae (Megalurus, Locustella, and Bradypterus spp.). The cettid family was consistently grouped with the sylvid Urosphena squameiceps with 74% bootstrap support. The family Sylviidae sensu stricto did not cluster together, with the genus Sylvia being monophyletic but is not joined by Chamaea fasciata and Urosphena squameiceps. Chamaea fasciata clustered with the Phylloscopidae clade in the NJ tree; however, this relationship is not well supported.

The relationship of the second outgroup taxon, G. lunulatus, with the rest of the taxa is also quite problematic. Instead of diverging from the Old World Warblers, it consistently clustered with members of the Old World Warblers. However, its placement is also not supported by bootstrap.

**DISCUSSION**

Eleven species of birds from UP Diliman were successfully barcoded. This study was able to sample about 29% of the species observed by the previous wildlife survey of Ong et al. (1999) or 31% of the species observed by Vallejo et al. (2008).

Except for P. goiavier, H. tahitica, A. phoenicurus, A. atthis and G. striata, the species sequenced in this study have several haplotypes associated with them even if the study site was just within the UP Diliman campus. Additional haplotypes for H. tahitica and A. atthis could potentially be found with additional sampling.

Hebert et al. (2004) set a threshold to delineate species and discover new ones based on pairwise sequence differences. The threshold, though later criticized, is set by multiplying by ten the average intraspecific distance of a particular group studied. If two individuals have a genetic distance that exceeds this threshold, then these individuals most likely belong to different unrecognized species. Empirically, their study derived a 2.7% threshold for birds. From the BLAST results in this study and the K2P distances generated, sequence homology was not always 100% for intraspecific comparison between the individuals and the gene sequences from Genbank. However, it still falls within the threshold set by Hebert et al. (2004) at 2.7% or less for species demarcation. This demonstrates the effectiveness of the COI DNA barcodes as markers for accurately identifying species. In addition, these differences in sequences might indicate geographic variations between populations of each species. For example, Kerr et al. (2009) found 44 Palearctic bird species exhibiting divergent COI sequences that follow known phylogeographic patterns of distribution.

Currently, three subspecies of the Striated Grassbird are recognized: M. palustris palustris (Horsfield, 1821) distributed across the Philippines and throughout the insular south-east Asia; M. palustris toklao (Blyth, 1843) from Pakistan, India, China, and throughout the peninsula of Myanmar, Thailand, Laos, and Cambodia; and M. palustris forbesi (Bangs, 1919) from northern Luzon and Borneo. Another race described as M. palustris andrewsi has been synonymized with M. palustris toklao (Deignan 1946; Internet Bird Collection 2011). Aside from these, no other subspecies has been distinguished, though studies on M. palustris have been limited.

This study presents the first barcodes for M. palustris (ABBI 2011). Three unique 600-bp barcode sequences of the mitochondrial COI gene were derived from five individuals sampled within the UP Diliman campus. One of the M. palustris COI haplotypes has a considerably high distance value with respect to the other haplotypes. Since two subspecies are found in the Philippines, this could indicate variation at the subspecies level. However, further studies on populations of the Striated Grassbirds in the Philippines are needed in order to confirm this hypothesis.

The placement of the sequences obtained for M. palustris was evaluated through construction of a gene tree and sequence divergence comparisons among species of the Old World Warblers “Sylviidae” where the current family of the Striated Grassbird, Locustellidae, was previously included. Sequences include genera that were under Sylviidae sensu Sibley et al. (1990) such as Phylloscopus, Acrocephalus, Locustella, Sylvia, Chamaea, Cettia, Urosphena, and Hippolais.

The three unique M. palustris sequences are shown to cluster together with high bootstrap support. The intraspecific distances based on five individuals of this
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species from UP Diliman ranged from 0 - 0.0592, which is lower than the 10x the average intraspecific distance for the Sylviidae (10 x 0.00943 = 0.0943) calculated in this study. This indicates the presence of only one species for the five individuals.

In the *Sylvia* clade, however, intraspecific distances between two Ménétries’s Warbler *Sylvia mystacaea* sequences have reached 0.1126, greater than the screening threshold of the Sylviidae, which therefore warrants further scrutiny to test for misidentification or the presence of a cryptic species. Evaluation and validation for new species other than that from *M. palustris*, however, are beyond the scope of this study.

The COI tree showed that the *M. palustris* sequences clustered together with several *Locustella* species (Figure 3). The relationship within the locustellid family, however, has yet to be re-established as Alström et al. (2011) have recently discovered non-monophyly within this clade based on four nuclear genes and one mitochondrial gene. The phylogeny of Locustellidae they generated strongly disagreed with the current taxonomy at the generic level, and they proposed a revised classification that recognized four genera of the Locustellidae family. One of these, *Megalurus*, is actually non-monophyletic. They stressed, however, that the classification is tentative and took into account the phylogenetic uncertainty that is due to the conflict between their results based on multiple loci on the one hand and morphology and vocalizations on the other. Nevertheless, the obtained *M. palustris* sequences in our study nested together with *Locustella*, the Striated Grassbird’s sister genus.

Analyses using the COI barcode gene for the Old World Warblers returned monophyletic groupings of conspecific, congeneric, and even some confamilial sequences with strong bootstrap support. These observations support the potential of the DNA barcode in differentiating closely related species but destabilize the efficiency of the COI gene in inferring phylogeny of distant taxa. For this reason, morphology, biogeography, ecology, and other taxonomic characters should also be considered when trying to infer species relationships as relying on a single-locus DNA barcode is not enough (Moritz & Cicero 2004).

CONCLUSION AND RECOMMENDATIONS

This study provides COI barcodes for bird species captured within the University of the Philippines Diliman. Using BLAST, the species were correctly identified by comparing it with other COI sequences in Genbank. The COI sequence of the Striated Grassbird *Megalurus palustris* generated in this study is a new record for DNA barcoding. The placement of *M. palustris* within the Old World Warbler group and the utility of the COI gene have also been evaluated by comparing genetic distances with other COI sequences from species belonging to the sylviid taxa. Results show that the COI was able to place *M. palustris* in a distinct taxonomic group. This provides support for the use of COI as a means of species delineation.

DNA barcodes for *M. palustris* have been produced but not for its three subspecies. Barcoding studies for its subspecies are therefore recommended to explore recent species lineage divergences that might have occurred within the taxon as well as for a comprehensive evaluation if DNA barcodes have enough resolution in investigating subspecies relationships. Additional sampling for other species, particularly acquiring a minimum of five individuals per species, is also recommended.

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