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Association of *Drd4* gene polymorphisms with range in species of the genus *Copsychus*

by

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Undergraduate honors thesis under the direction of

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Submitted to the LSU Honors College in

April, 2014

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ABSTRACT Polymorphisms in the dopamine receptor D4 (*Drd4*) gene have been implicated to influence exploratory behaviors and, possibly, differences in range sizes among many passerine birds. I found evidence of sequence variation at the *Drd4* gene among species in the genus *Copsychus*, among three species: *C. cebuensis, C. malabaricus,* and *C. saularis*. In sequences that contained 237 codons, 5 were variable among the 3 species. Sequence variation was analyzed using MEGA6 to produce a phylogenetic tree representing the relationships among the 3 species. Given that *C. cebuensis* has the most restricted range, sequence variation at *Drd4* might be expected to be most similar between the two species that exhibit broad distributions, *C. saularis* and *C. malabaricus*. However, the phylogenetic tree grouped *C. cebuensis* with *C. malabaricus*, an arragment observed for trees constructed with different genes. My results suggest that *Drd4* polymorphisms may not have a significant impact on exploratory behaviors and range sizes, however, *Drd4* may be a reliable gene to include in future phylogenetic studies.

KEY WORDS *Behavior, Copsychus cebuensis, Copsychus malabaricus, Copsychus saularis, dopamine receptor, Drd4, exploratory behavior, pairwise distance, phylogeny, polymorphisms, range*

Polymorphisms of specific genes may influence the behaviors of many animals; for instance, recent studies have indicated that variations in the dopamine receptor D4 gene (*Drd4*) could affect risk-taking and exploratory behaviors (Fidler *et al.* 2007 & Korsten *et al*. 2012). Fidler et al. (2007) studied the Great Tit (*Parus major*), and concluded that *Drd4* gene polymorphisms are correlated to differing levels of exploratory and/or novelty-seeking behaviors. Exploratory behaviors can serve as a proxy for related characteristics, such as dispersal potential (Korsten *et al.* 2010). Bowman (2003) found a positive, proportional relationship between median dispersal distance and territory size across bird species, regardless of body mass. The purpose of my study was to take these associations between genetically-driven exploratory behaviors a step further

than individual territory sizes, and determine if there is a genetic basis for variations in species' range sizes within the genus *Copsychus*. Identifying the genes that regulate the expression of certain behaviors is an important step in developing methods to quantify behavior. This study, and related studies, could provide clues as to how various species will be impacted by anthropogenic activities, such as deforestation and habitat fragmentation. If behaviors can be shown to be directly controlled by a select number of genes, there might be evidence for a genetic predisposition for a species to be especially vulnerable to deforestation and/or fragmentation. For this study, I sequenced the *Drd4* gene in three species of passerine birds in the genus Copyschus (family Muscicapidae). *C. malabaricus* (White-rumped Shama) inhabits old-growth forests, and *C. saularis* (Oriental Magpie-Robin) thrives in open woodlands along the coast (Lim *et al*. 2010). Both species have a broad distribution, ranging from India, through China, and into Sundaland; however, *C. saularis* is considered more vagile than *C. malabaricus* (Lim *et al*. 2010). In contrast, *C. cebuensis* (Black Shama) is endemic to the island of Cebu in the Philippines, and is currently endangered due to its restricted range and declining habitat (Lim *et al*. 2010). Since *C. cebuensis* is the least vagile of the three species studied, it is the most vulnerable to habitat loss and, if it is unable to adapt, is likely to become extinct in the wild. Species distribution maps can be found in the appendix, figures 1 – 3.

MATERIALS AND METHODS

The DNA used in this study came from twelve individuals of three species collected by the Louisiana State University Museum of Natural Science (Appendix 1). DNA was extracted from five samples of *Copsychus malabaricus*, and five of *Copsychus saularis*. The *C. cebuensis* sequences were provided by samples that had been previously sequenced. *C. malabaricus* specimens were collected from Tawau, Sabah; Mulu, Sarawak; and Banggi Island, Sabah. *C.*

saularis specimens were obtained from Mulu, Sarawak, and *C. cebuensis* samples were collected from Cebu, Philippines.

DNA was extracted using the DNeasy Blood and Tissue Kit following the manufacturer's instructions (Appendix 2). Extracted DNA was amplified using Polymerase Chain Reaction (PCR) with LongAmp *Taq* (New England BioLabs 2014). PCR reaction concentrations were 1.5 mM $MgCl_2$, 5X Long amp reaction buffer, 0.24mM dNTPs, 10 μM forward primer, 10 μM reverse primer, 2.5 units of Long amp Taq, 1 uL of DNA, and nuclease-free water to a final volume of 25 uL. The primer sequences used were designed for *P. major* by Fidler *et al*. (2007), which amplified the middle of exon 2 to the 3' end of exon 4:

Forward (F) primer: 5' GAG GAG TGT GGT CCC TCA GC 3'
Reverse (R) primer: 5' CGC AGA AAT AGA CCT TTA ATG AAC TA 3'

Initial denaturation took place at 94˚C for 60 seconds, and was followed by 35 cycles of 94˚C for 30 seconds, 50˚C for 60 seconds, and 65˚C for 150 seconds. Final extension took place over 10 minutes at 65˚C.

Amplified DNA was electrophoresed on agarose gels to confirm amplification (Appendix 3). PCR product was purified using QIAquick PCR Purification Kits according to the manufacturer's instructions (Qiagen, Valencia, CA, 2008) (Appendix 4). Purified PCR product was cycle-sequenced in both directions, using 1.5 μL purified PCR product, 1.75 μL 5x ABI Buffer, 1.0 μL F/R primer, 0.4 μL BigDye Terminator v 3.1, and de-ionized water to a total reaction volume of 7.0 μL. Cycle sequencing consisted of 2 cycles of 96˚C for 20s, 50˚C for 15s, and 60˚C for 4 minutes; followed by 23 cycles of 96˚C for 12s, 50˚C for 15s, and 60˚C for 4 minutes. After cycle sequencing, the samples were purified with Sephadex G50 (Appendix 5).

The purified product was sequenced on an ABI 3130XL Genetic Analyzer at the Louisiana State University Genomics Facility.

The sequences were corrected and aligned using Sequencher v. 5.1 (Gene Codes Corporation 2012), and analyzed with MEGA v. 5.05 and v. 6.05 (Tamura *et al*. 2011 and 2013). Missing data (approx. 100 bp) from one of the *C. cebuensis* sequences was filled in using the nucleotides present in the other *C. cebuensis* sequence.

RESULTS

After several trials, 10 of the 12 samples yielded usable sequences (2 from *C. cebuensis*, 3 from *C. saularis* and 5 from *C. malabaricus*). In most cases, the reverse primer yielded the most complete sequences. Of the 713 nucleotides present in all 10 samples, there were 13 variable sites (Table 1). Of the 13 variable sites, positions 141, 183, 324, 384, and 396 were 4-fold degenerate sites, in which the nucleotide change occurs in the 3rd position and did not result in a change in amino acid coding.

The sequences yielded 237 codons, of which 5 produced variable amino acids among the 3 species (Table 2). Codon 134 (sites 400-402) produced serine in one *C. malabaricus*, proline in another *C. malabaricus*, and threonine in all other samples. Codon 135 (sites 403-405) produced glycine in *C. saularis* and aspartic acid in *C. malabaricus* and *C. cebuensis*. Codon 138 (412-414) produced glycine in *C. saularis* and tryptophan in *C. malabaricus* and *C. cebuensis*. Codon 161 (481-483) produced arginine in 3 of the 5 *C. malabaricus* sequences, and leucine in all other specimens. Codon 194 (580-582) yielded arginine in one *C. saularis* (#74753), lysine in *C. cebuensis*, *C. malabaricus*, and one *C. saularis* (#74754); the identity of the third *C. saularis* (#73476) amino acid was ambiguous. Each sequence contained potential stop codons located at

positions 158, 171, 203, 217, and 235, when using the first base as the start of the sequence. The stop codons were present regardless of which base was used as the start of the sequence, and analyzing the sequences by the first base yielded the fewest stop codons. The most significant variation occurred before any of the stop codons in the sequence. MEGA analysis produced a phylogenetic distance table and a maximum likelihood phylogenetic tree, both of which grouped *C. cebuensis* and *C. malabaricus* together, and left *C. saularis* as an outgroup (Table 3 and Figure 4). The resulting phylogenetic tree resembled the tree produced by Lim *et al*. (2010), which was based on several genetic markers (Figure 5). The maximum phylogenetic distance between any two of the species was 1.3% and was found between *C. saularis* and *C. malabaricus* (Table 3).

DISCUSSION

The phylogenetic tree based on *Drd4* sequences placed *C. cebuensis* and *C. malabaricus* together – despite the large gap in distribution size – and left *C. saularis,* the most vagile species, as an outgroup. My tree, even though it was produced using only one gene, was similar to the tree produced by Lim *et al*. (2010), which used the entire mitochondrial ND2 gene, portions of nuclear myoglobin intron 2 (Myo2), and the transforming growth factor beta 2 intron 5 (TGFb2-5). My phylogenetic tree depicted a recent common ancestor between *C. cebuensis* and *C. malabaricus* that is not shared by *C. saularis*. It was also apparent from phylogenetic distance analysis of my sequences that *C. cebuensis* is slightly more related to *C. saularis* than *C. malabaricus* is to *C. saularis*.

Fidler *et al*. described the *Drd4* coding region of *P. major* as nucleotides 647–947 in their sequence (GenBank: DQ006802). I did not obtain sequence data for the majority of this region in

Copsychus; however, the region that I did obtain contained little variation among species and was missing several of the indels present in *P. major*. It is, of course, expected that the coding region for *Drd4* in *Copsychus* may differ slightly from that in *Parus*, and the variation that is present could still influence behavior. Future studies should investigate whether the proteins encoded by my sequences exhibit variation among species, in order to identify any possible correlation between *Drd4* and range size.

Possible *Drd4* polymorphisms at several sites could be evidence of a genetic basis for variations in range sizes among species. At site 134, the switch from threonine to serine in *C. malabaricus* (sample #74745) would not likely affect the overall function of the encoded protein. However, the switch from threonine to proline in *C. malabaricus* (sample #73498) would likely result in a conformational change in the protein due to the effects of proline as an alpha helix breaker. At site 135, *C. saularis* contains glycine, a small hydrophobic molecule, whereas *C. malabaricus* and *C. cebuensis* contain aspartic acid, a negatively charged hydrophilic molecule. Switching from a hydrophilic molecule to a hydrophobic molecule is very likely to cause a conformational change in the encoded protein, and could affect its function. At codon 138, *C. saularis* produces glycine instead of tryptophan. This likely has only a minor effect on protein structure and function, as both amino acids are nonpolar. However, tryptophan has a significantly larger side chain than does glycine. The presence of positively charged arginine at site 161 in 3 of the 5 *C. malabaricus* sequences (sample #73498, 73499, and 74734) contrasted with the presence of hydrophobic leucine in the other sequences. Site 194 yielded lysine in most samples, arginine in one *C. saularis* (sample #74753), and was heterozygous in another *C. saularis* (sample #73476). While variable, the effects of this change would likely be minimal as both amino acids are positively charged and relatively large. Assuming translation did stop at the stop codon located at

position 158, there would be 3 sites coding for variable amino acids among the three species, most notably at site 135. The variation I found could be due to the phylogenetic distance between the species, and is therefore not conclusive evidence that *Drd4* polymorphisms are directly correlated to exploratory behaviors and range sizes in the species studied. The variation that I discovered was minimal and, even though a single amino acid change could dramatically affect overall protein form and function, I concluded that *Drd4* is likely only one of many genes that possibly influence exploratory behaviors.

In addition to a very small sample size of 10 sequences, I encountered a number of set-backs throughout the study. I found through trial and error that the reverse primers yielded the best sequences, and that the forward primers were largely unreliable. The primers should be redesigned for further replications of this study, and should be designed specifically for *Copsychus*. Another issue encountered over the course of this study was the duration of the lab work associated with DNA extraction, amplification, and purification. There were often several days, up to a week between steps in the process, and it is possible that my PCR product was degreaded by overexposure to light before sequencing. Regardless, my results do not suggest that *Drd4* polymorphisms have a significant impact on exploratory behaviors and range sizes, but could be a reliable gene to use for future phylogenetic studies.

ACKNOWLEDGEMENTS

I would like to thank Christine Bergeon Burns, Kristin Brzeski, Jean Elbers, and Robert Ford, graduate students in the Conservation Genetics Lab at Louisiana State University, for their assistance and advice with lab techniques. I am also grateful to Dr. Frederick Sheldon for his assistance with the organization of data and the interpretation of results. I am especially grateful

to my thesis advisor, Dr. Sabrina Taylor, for her continued support and guidance. Funding for my project was provided by the Conservation Genetics Lab at LSU, Chancellor's Student Aid, and by the Tiger Athletic Foundation Thesis Research Scholarship.

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APPENDIX

FIGURES AND TABLES

Table 1. Representation of variable nucleotide sites, depicting the nucleotide present at each position. CM = *C. malabaricus*, CC = *C. cebuensis*, CS = *C. saularis*.

Site	73499 CM	74734 CM	73487 CM	74745 CM	73498 CM	27309 CC	27354 CC	74753 CS	73476 CS	74754 CS
87	C	C	C	C	T	C	C	T	T	T
141	C	C	C	C	C	C	C	G	G	G
183	C	C	C	C	C	C	C	T	T	T
213	T	T	T	T	T	C	C	C	C	C
324	C	C	C	C	C	C	C	C	T	C
384	C	C	C	C	C	C	C	T	T	T
396	C	C	C	C	C	C	C	T	T	T
400	A	A	A	A	C	A	A	A	A	A
401	C	C	C	G	C	C	C	C	C	C
404	A	A	R	A	A	A	A	G	G	G
412	T	T	T	T	T	T	T	G	G	G
482	G	G	T	T	G	T	T	T	T	T
581	A	A	A	A	A	A	A	G	R	A

Table 2. Variable amino acid codons, representing the amino acid that is produced by the sequence present at each codon. CM = *C. malabaricus*, CC = *C. cebuensis*, CS = *C. saularis*.

Codon	73499 CM	74734 CM	73487 CM	74745 CM	73498 CM	27309 CC	27354 CC	74753 CS	73476 CS	74754 CS
134	T	T	T	S	P	T	T	T	T	T
135	D	D	D	D	D	D	D	G	G	G
138	W	W	W	W	W	W	W	G	G	G
161	R	R	L	L	R	L	L	L	L	L
194	K	K	K	K	K	K	K	R	?	K

Table 3. Pairwise distances among *Copsychus malabaricus*, *C. saularis*, and *C. cebuensis*. *C. malabaricus* and *C. cebuensis* are more closely related to each other than either is to *C. saularis*, which only differs by up to 1.3% from the other two species.

Specimen (Sample # and Species)	73499 C. malabaricus	74734 C. malabaricus	73487 C. malabaricus	74745 C. malabaricus	73498 C. malabaricus	27309 C. cebuensis	27354 C. cebuensis	74753 C. saularis	73476 C. saularis	74754 C. saularis
73499 C. malabaricus										
74734 C. malabaricus	0.000									
73487 C. malabaricus	0.001	0.001								
74745 C. malabaricus	0.003	0.003	0.001							
73498 C. malabaricus	0.003	0.003	0.004	0.006						
27309 C. cebuensis	0.003	0.003	0.001	0.003	0.006					
27354 C. cebuensis	0.003	0.003	0.001	0.003	0.006	0.000				
74753 C. saularis	0.012	0.012	0.010	0.012	0.012	0.009	0.009			
73476 C. saularis	0.013	0.013	0.012	0.013	0.013	0.010	0.010	0.001		
74754 C. saularis	0.012	0.012	0.010	0.012	0.012	0.009	0.009	0.000	0.001	

Figure 1. Map of the Phillipines highlighting the island nation of Cebu, to which *C. cebuensis* is endemic.

Figure 2. Distribution map of *C. malabaricus*. Image from www.hbw.com.

Figure 2. Distribution map of *C. saularis*. Image from www.hbw.com.

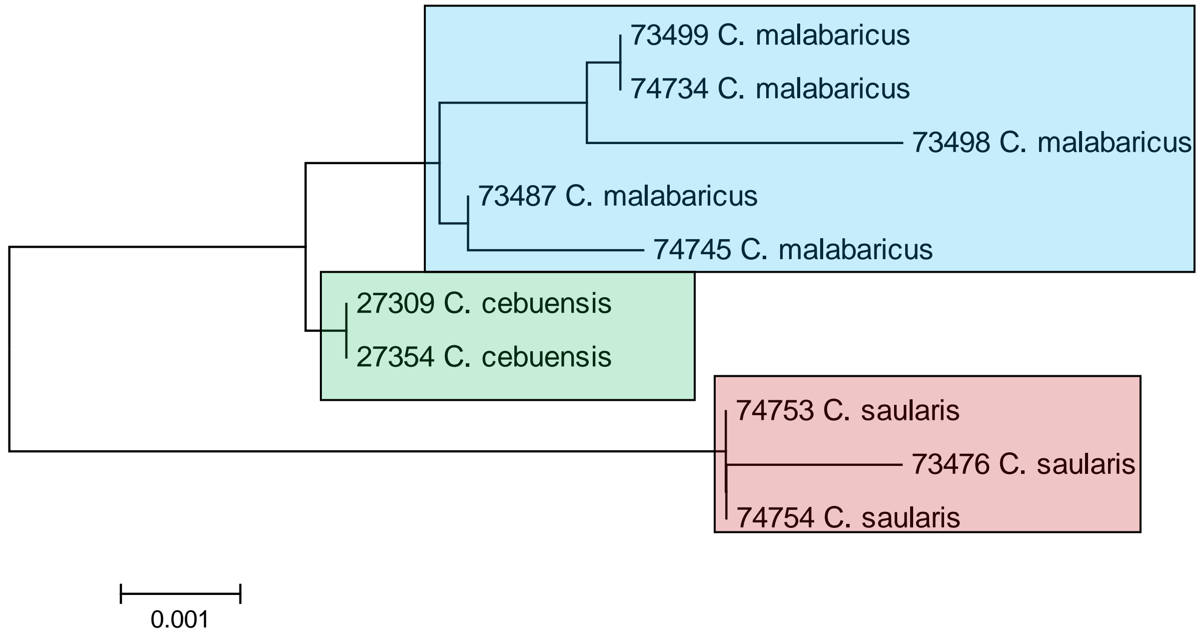

Figure 4. Maximum liklihood phylogenetic tree depicting a more recent common ancestor between *C. cebuensis* and *C. malabaricus* than either species has with *C. saularis*.

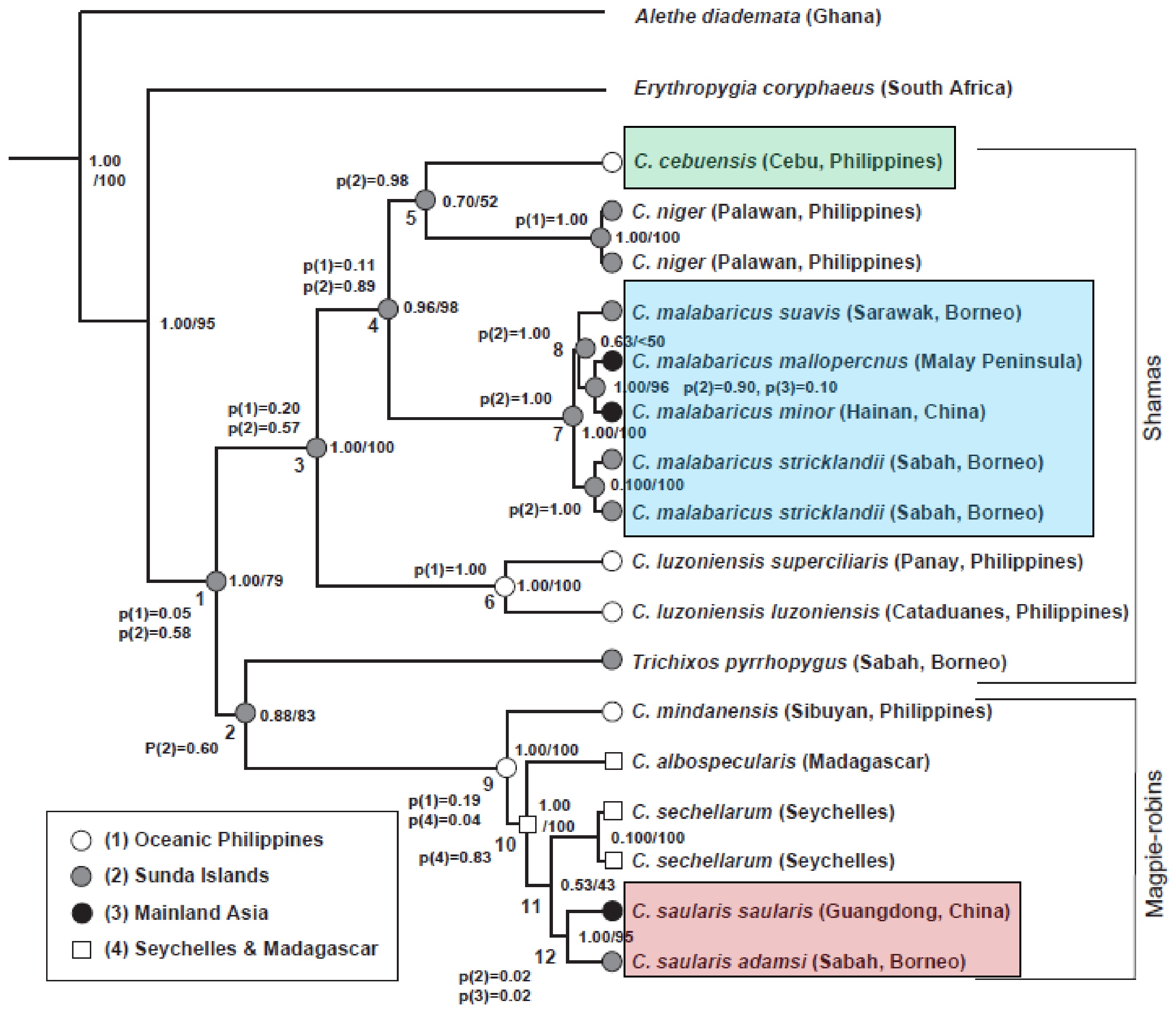

Figure 5. Phylogeny of Copsychus and Trichixos estimated by Bayesian analysis and maximum likelihood (ML) bootstrapping (Lim *et al.* 2010). *C. cebuensis* and *C. malabaricus* have a more recent common ancestor than either has with *C. saularis*.

1. Specimen Collection Information

LSUMZ B-73487	*Copsychus malabaricus*	Gawin, Dency F. 65	Ethanol	Malaysia: Sabah: : Kudat; Baggi; Kamping Kalangkaman N 0 17' 50.7" E117 04' 44.4" 27M
LSUMZ B-73498	*Copsychus malabaricus*	Gawin, Dency F. 76	Ethanol	Malaysia: Sabah: : Tawaii; Tawaii Hills Park HQ N 29' 8.73" E 117 55' 22.57"
LSUMZ B-73499	*Copsychus malabaricus*	Gawin, Dency F. 77	Ethanol	Malaysia: Sabah: : Tawaii; Tawaii Hills Park HQ N 29' 8.73" E 117 55' 22.57"
LSUMZ B-74734	*Copsychus malabaricus*	Sheldon, Frederick H. 759	Ethanol	Malaysia: Sarawak: : MT. mulu Nationel park, Deer Cave; 04 Degrees 01' 24" N, 114 Degrees 49' 14"E; limestone forest.
LSUMZ B-74745	*Copsychus malabaricus*	Sheldon, Frederick H. 769	Ethanol	Malaysia: Sarawak: : MT. mulu Nationel park, Deer Cave; 04 Degrees 01' 24" N, 114 Degrees 49' 14"E; limestone forest.
LSUMZ B-73438	*Copsychus saularis*	Gawin, Dency F. 20	Ethanol	Malaysia: Sabah: : Jalan, Tnlawk; Selinsing,Klias.
LSUMZ B-73457	*Copsychus saularis*	Gawin, Dency F. 39	Ethanol	Malaysia: Sabah: : Kampung Nundulong, Mendulong, hipitag
LSUMZ B-73476	*Copsychus saularis*	Gawin, Dency F. 54	Ethanol	Malaysia: Sabah: : Kudot, Kota Mamdi, Lompkon N 6 34'18.54 E116 42' 19.5", 36 m
LSUMZ B-74753	*Copsychus saularis*	Sheldon, Frederick H. 776	Ethanol	Malaysia: Sarawak: : Mulu Nationel park Head Quaters ; 04 Degrees 02' 33" N, 114 Degrees 48' 49"E.
LSUMZ B-74754	*Copsychus saularis*	Sheldon, Frederick H. 777	Ethanol	Malaysia: Sarawak: : Mulu Nationel park Head Quaters ; 04 Degrees 02' 33" N, 114 Degrees 48' 49"E.

2. DNA Extraction and Elution Protocol

I cut a 10 mg piece from each tissue sample using a new razor blade and microscope slide for each sample. Each piece was placed in a 0.5 mL microcentrifuge tube. I added 180 µL of Buffer ATL and 20 µL of Proteinase K to each tube. The tissue samples were incubated at 56˚C for 2 hours, and vortexed every 20 to 30 minutes while incubating. After incubation, I added 200 µL of Buffer AL, vortexed, added 200 µL of 95% ethanol, and vortexed. The samples were transferred into a DNeasy Mini spin column, placed inside a 2 mL collection tube. The spin columns were centrifuged at 8000 rpm for one minute. The flow-through and collection tube were discarded, and the spin column placed in a new collection tube. I added 500 µL of Buffer AW1, and centrifuged at 8000 rpm for one minute. I placed the spin columns in new collection tubes and added 500 µL of Buffer AW2. The samples were centrifuged at 14,000 rpm for 3 minutes, and placed in a

new 2 mL-microcentrifuge tube. The DNA was eluted with 200 µL Buffer AE added to the center of the column membrane. The sample was incubated at room temperature (15 - 20˚C) for 5 minutes, then centrifuged for one minute at 8000 rpm. From the flow-through, 30 µL aliquots were placed in refrigerator stock tubes, and the remaining 170 µL was stored in the freezer.

3. **Gel Electrophoresis**

A 1.2% Agarose gel was poured and allowed to set for roughly 30 minutes using a 12-tooth comb. Mix 2 µL PCR product with 2 µL EZ Vision Dye. Inject the 4 µL solution into each well, leaving one well for the ladder and one well for the control (dH_2O). Use at least a 100 basepair ladder. Cover the gel with 1x SB Buffer, and run at 155 V for approximately 30 minutes, or until the dye reaches the edge of the gel.

4. **QIAquick PCR Purification Protocol**

Use the remaining 23 µL of each sample. Add 115 µL Buffer PB to PCR product. Transfer 138 µL solution to QIAquick spin column and centrifuge at 13,000 rpm for 1 minute. Discard the flow-through and return the column to the same collection tube. Wash each sample with 750 µL Buffer PE (ethanol added), and centrifuge at 13,000 rpm for 1 minute. Discard the flow-through, replace in the column, and centrifuge 1 additional minute. Discard the flow-through and the collection tube, and place spin column in a new 1.5 mL microcentrifuge tube. Elute the DNA with 50 µL Buffer EB, added to the center of the column membrane, and centrifuge at 13,000 rpm for 1 minute. Keep the flow-through in the microcentrifuge tube and discard the spin column. Yields approximately 48 µL of purified PCR product.

5. Sephadex Protocol

Add 390 μL of Sephadex to the wells of a filter plate placed on an ABI plate, and set for 10 minutes. Add 13 μL nanopure H_2O to the cycle sequencing product (~20 μL). Counterbalance the Sephadex plate and spin at 2300 rpm for 3 minutes. Add the cycle sequenced product to the Sephadex column, counterbalance, and spin at 2300 rpm for 4 minutes. Collect the cleaned product in the ABI plate. Top each sample with a mineral oil cap, wrap in foil, and freeze before sequencing.