Acta Biol. Univ. Daugavp. 16 (1) 2016
ISSN 1407 - 8953

DNA BARCODING OF SELECTED *PACHYRHYNCHUS* SPECIES (COLEOPTERA:CURCULIONIDAE) FROM MT. APO NATURAL PARK, PHILIPPINES

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Anzano Cabras A., Dela Cruz R. 2016. DNA barcoding of selected *Pachyrhynchus* species (Coleoptera:Curculionidae) from Mt. Apo Natural Park, Philippines. *Acta Biol. Univ. Daugavp., 16 (1): 111 – 118.*

Pachyrhynchus Germar, 1824 is a genus with a center of diversity in the Philippines and has roughly 90% of endemism. Despite their narrow distribution often confined to a single mountain range or island, a wide range of interspecific variation is present which provides difficulty in its classification and identification. Currently, the subtle difference in genitalia structure is the only most conclusive basis in delineating species. To address this problem combining morphological traits and DNA barcoding in identifying species is believed to be helpful especially for problematic groups. For this study, three species of *Pachyrynchus sympatrically* found in Mt. Apo Natural Park, Mindanao which shows low morphological variation were selected for DNA barcoding. The first partial DNA sequence of the mitochondrial c cytochrome oxidase (cox1) gene of the genus *Pachyrhynchus* is presented in this paper. DNA extraction was conducted using modified CTAB-PVP method and Promega Wizard Genomic DNA Purification Kit. Gene amplification was done using LCO1490 and HCO 2198 primers producing 600-800 bp. Neighbour-Joining (NJ) and Maximum Likelihood were used to measure evolutionary distance. Three distinctive topologies with high bootstrap (100%) were generated. Thirty five nucleotide positions of COI were found to vary which shows the capacity of COI gene in distinguishing *Pachyrhynchus* to the species level. The data support the initial finding that *P. apoensis* Yoshitake, 2012 and *P.pseudamabalis* Yoshitake, 2012 are closely related because of the structure of their reproductive organ. DNA barcoding together with morphological characters should be used in delineating species especially in problematic groups for taxonomic and conservation purposes.

Key words: DNA barcode, cytochrome oxidase 1, Mindanao Island, endemic, beetles.

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INTRODUCTION

One of the problematic taxa in the order Coleoptera is the family Curculionidae which comprises the family of the "true" weevils or "snout beetles". It is the third-largest animal family, with over 50,000 species described worldwide (Oberprieler et al. 2007). They are recognized by their distinctive long snouts and geniculate antennae with small clubs. Curculionids have considerable diversity of form and size and some are polymorphic (Oberprieler et al. 2007). Hence, difficulty in its classification even in the higher classification is observed as there is a wide range of variation present (Wink et al. 1997).

Pachyrynchini is a tribe of the family Curculionidae or true weevils and subfamily Entiminae with its center of diversity in the Philippines. Majority of its genera are found in the country and has 90% of endemism for its species in the Philippines. One of the genera of the tribe which is highly endemic in the Philippines is the genus *Pachyrhynchus*. The species under this genus are known to have a narrow distribution often confined to a single mountain, mountain range or island due to their flightless characteristic. Interspecific variations in the elytral markings as well as color of integuments have been observed from various collections from the different mountains and islands. These variations provide a difficulty in identifying them to the species level. Accurate taxonomic identification is deemed necessary especially for their conservation since they are forest dweller species and our rapid habitat loss threatens their survival in the wild.

DNA barcoding of animals using mitochondrial cytochrome c oxidase subunit 1 was established by Herbert et al. (2003) as a new approach to taxon recognition and has been an important tool in delineating species, identifying new species and in establishing phylogenetic relationships (Herbert et al. 2003).Mitochondrial cytochrome c oxidase subunit 1 which is one of the most widely used genes in animal DNA barcoding has been proven to have the capacity to show high divergence among closely allied taxon in the animal kingdom(Hebert et al. 2003). Data on the sequence of cytochrome c oxidase subunit 1 (COl) gene will not only help solve problematic taxa but also help provide these species evolutionary relationship.

In this study, three closely related species of *Pachyrhynchus* which occur sympatrically in Mt. Apo Natural Park were subjected to DNA barcoding which will provide the first data of mitochondrial cytochrome c oxidase subunit 1 (COl) gene sequence of the genus *Pachyrhynchus*. A phylogenetic tree was also be inferred to show their evolutionary relationship with other species of Pachyrynchini.

MATERIAL AND METHODS

Specimen collection

Samples were obtained from shrubs and trees at an elevation between 1000 to 2000 masl which was the recorded distribution of the selected *Pachyrynchus* species in Mt. Apo Natural Park (Cabras et al. 2016). Specimens were collected through beating sheet and handpicking and killed in vials with ethyl acetate. Specimens were then soaked in 95% ethanol. Identification prior to DNA extraction was done using taxonomic descriptions provided by Yoshitake (2012).

DNA extraction protocol

Two DNA extraction protocols were used in the study. For species with numerous specimens, a CTAB- PVP protocol which followed that of Mega and Revers (2011) with some modification was used. For species with limited number of specimens Promega Wizard Genomic DNA Purification Kit was used following the protocol from its manual. The tissues used for DNA extraction of *Pachyrhynchus* were the legs since it has lots of muscle which is rich in mitochondrial DNA (Cox et al. 2013). Legs were obtained from each *Pachyrhynchus* specimen and weighed. For the CTAB-PVP method, legs were crushed partially and added with liquid nitrogen and immediately crushed further into a finer powder

and placed in a 1.5ml sterile microcentrifuge tubes. Crushed tissues were mixed with PVPP (Sigma, P6755) in a proportion of 100mg of PVPP per 1g of grinded tissue, 600µL of extraction buffer (50mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.1M NaCl, 0.4M LiCl, 1% CTAB, 2% PVP40 (Sigma), 0.5% Tween 20, 0.2% β- mercaptoethanol) and poured in a 1.5ml micro centrifuge tubes and mixed thoroughly. The tubes were then placed in water bath at 60°C for 25 minutes. Then the tubes were inverted to obtain a good solution of the crushed tissues with the buffer. The tubes were cooled at room temperature and 600µL of chloroform: isoamyl alcohol mixture (24:1) was added. The tubes were mixed gently by inversion to form an emulsion during 4 minutes. After that the tubes were centrifuged (Eppendorf Centrifuge 5415R) at 10.600g for 5 minutes at room temperature. The aqueous phase present in each tube were transferred to a new 1.5ml micro centrifuge tube gently. Each tube was added with 250µL of 5M NaCl and 750µL of cold (-20°C) isopropanol. NaCl degrades polysaccharides. The solutions were kept in the freezer at -20ºC for 20 minutes to improve precipitation nucleic acids. Samples were then centrifuged at 10,600g (Eppendorf Centrifuge 5415R) for 10 minutes at room temperature and the supernatant poured off. The DNA pellets were washed with 1000µL of cold 76% ethanol. Next, the samples were spun quickly and the excess ethanol was removed with a micropipette. Washed DNA pellets were dried by leaving the tubes uncovered at 37°C for 20 minutes. DNA samples were dissolved in 50µL TE (10mM Tris HCl pH 8.0, 0.1mM EDTA pH 8.0) and treated with 1µL of RNAase A (10mg mL-1) at 50°C for 20 minutes.

Gel Electrophoresis

Extracted DNA were run on a 1% agarose gel in 0.25x TBE buffer. Electrophoresis was done at 100V for 30 min and visualized using Gel Doc EZ Imager (BIO-RAD).

DNA amplification and sequencing reactions

Polymerase chain reaction (PCR) was carried out using the universal barcoding primers LCO 1490 (sequence: 5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (sequence: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') to yield an approximately 600-900 bp fragment of the cytochrome oxidase subunit I (COI) region of the mitochondrial DNA (Folmer *et al*., 1994; Cox et al. 2013). The PCR reaction contained 1x PCR buffer, 0.8 mM $MgCl_2$, 1.2 mM dNTP mix, 0.2 mM forward and reverse primer, 1 unit Taq DNA polymerase and 100 ng genomic DNA. Cycling condition was: 2 min at 95°C; 43 cycles of 30 s at 94°C; 30 s at 45°C; 5 min at 65°C, and a final elongation step of 5 min at 72°C. A PCR blank control was incorporated. PCR product was again run on a 1% agarose gel in a 35ml 0.25x TBE buffer. Electrophoresis was done at 100V for 30 min and visualized using Gel Doc EZ Imager (BIO-RAD).

DNA Analysis

Mitochondrial cytochrome oxidase subunits 1 genomic sequences ranging from 600 to 800 bp of the 7 individuals of *Pachyrynchus* belonging to three species were aligned with Clustal W. Sequences from National Center for Biotechnology Information genbank were was used as well in constructing a phylogenetic relationship. Maximum Composite Likelihood method (Tamura et al. 2004) was used to compute the evolutionary distance of the species and Neighbour-Joining (NJ) method (Saitou and Nei 1987) was selected for the construction of phylogenetic trees. Mega 7.0.14 was used in creating a phylogenetic tree. Pairwise distance was also computed using Mega 7.0.14. For haplotypes, variation in sequences was identified by Mega 7.0 Sequence Data Explorer and was selected, identified in a table and compared to produce haplotypes.

RESULTS AND DISCUSSION

DNA Barcoding Result

For *Pachyrhynchus apoensis* Yoshitake, 2012, 606-bp amplicons were generated

from mitochondrial cytochrome oxidase gene while *Pachyrhynchus hirokii* Yoshitake, 2012 generated 708 bp and 797 bp for *Pachyrhynchus pseudamabilis* Yoshitake, 2012.

NJ Analysis and percent divergence

DNA sequences of *Pachyrhynchus* from Mt. Apo Natural Park fell into three major topologies (Fig.1) each with 100% bootstrap support. *P. apoensis* and *P. pseudamabilis* formed two closely related clades by bootstrap value of 92% while *P. hirokii* is separate from the other two species of *Pachyrhynchus* in Mt. Apo. *Metapocyrtus hederaephilus* Yoshitake, H., S. Miyahara, M. Nishino, and K. Suzuki, 2012 is an outgroup and belongs to another genus in this tribe. It is the only *Metapocyrtus* species with a DNA barcode in NCBI.

A high topology for the phylogenetic tree using partial COI gene shows the high discrimination power of COI gene in delineating Pachyrynchini fauna to the species level (Herbert *et al*. 2003). The phylogenetic result shows a closer evolutionary relationship between *P. apoensis* and *P. pseudamabilis*. This is also supported by the morphology of *P. apoensis* and *P. pseudamabilis* as described by Yoshitake (2012). The major differences between the two species are the scaly patterns of their elytra. The tree shows that *P. hirokii* evolved independently among the three species of *Pachyrynchus*. *P. hirokii*'s morphology is quite distinct from the other two species by its more slender and stouter rostrum and body compared to *P.apoensis* and *P.pseudamabilis* and heavily punctured elytra. Despite the closer elytral patterns of *P. pseudamabilis and P. hirokii* other morphological traits including the shape of prothorax, rostrum and elytra as well as the extensive punctures of *P. hirokii*'s elytra, clearly distinguishes the two species. In case of the structure of the aedeagus which is now considered as the most conclusive distinguishing morphological character of Pachyrynchini, *P.apoensis* and *P.pseudamabilis* have more similar structure which means that these two are more closely related as compared to *P.hirokii*. It can be inferred that scaly markings of the elytra is not the main morphological distinguishing character for *Pachyrhynchus* but more characters that varies such as the shape of rostrum, pronotum and elytra and especially the aedeagus which is a reproductive part. Conducting more DNA barcoding of other *Pachyrhynchus* species will validate this inference.

DNA barcoding and Haplotype construction

Haplotype which refers to the variation of nucleotides selected in a region is used to easily identify the nucleotide variations of the selected species to facilitate sequence analysis. For the three species of *Pachyrhynchus* in Mt. Apo Natural Park, 35 haplotypes were recognized. This is a high number of haplotypes and reveals the high discrimination power of COI gene in distinguishing species. In other taxon such as plants, a combination of one or two genes are used to generate a high number of haplotypes but in the case

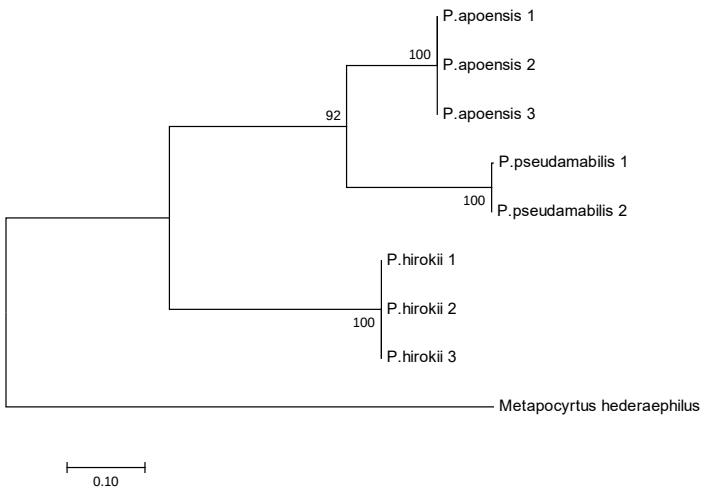

Fig. 1. Molecular Phylogenetic analysis by Maximum Likelihood method.

Table 1. Sample of haplotypes sequence variation in the three *Pachyrhynchus* species. The number indicates the nucleotide position with variation

Gene	Mitochondrial Cytochrome Oxidase 1																		
Species	66	108	125	145	180	191	199	203	223	249	263	280	292	295	318	320	345	377	399
P.a.1	G	C	G	A	G	C	G	A	G	G	A	A	A	A	A	A	A	A	T
P.a.2	G	C	G	A	G	C	G	A	G	G	A	A	A	A	A	A	A	A	T
P.a.3	G	C	G	A	G	C	G	A	G	G	A	A	A	A	A	A	A	A	T
P.h.1	A	G	A	A	T	G	T	T	T	A	T	T	T	C	C	C	C	A	T
P.h.2	A	G	A	A	T	G	T	T	T	A	T	T	T	C	C	C	C	A	T
P.h.3	A	G	A	A	T	G	T	T	T	A	T	T	T	C	C	C	C	A	T
P.p.1	T	T	T	C	A	A	C	G	A	T	G	G	G	T	T	T	T	G	G
P.p.2	T	T	T	C	A	A	C	G	A	T	G	G	G	T	T	T	T	G	G

Legend: P.a (*P. apoensis*), P.h (*P.hirokii*), P.p (*P. pseudamabilis*)

Table 2. Pairwise differences (% range) in COX I sequences among species of *Pachyrhynchus*

	1	2	3	4	5	6	7	8	9
[1]									
[2]	0.00								
[3]	0.00	0.00							
[4]	0.25	0.25	0.25						
[5]	0.25	0.25	0.25	0.00					
[6]	0.41	0.41	0.41	0.42	0.42				
[7]	0.41	0.41	0.41	0.42	0.42	0.00			
[8]	0.41	0.41	0.41	0.42	0.42	0.00	0.00		
[9]	0.57	0.57	0.57	0.61	0.61	0.57	0.57	0.57	

Legend: [1]*P.apoensis*1, [2]*P.apoensis*2, [3] *P.apoensis*3 [4]*P.pseudamabilis*1, [5]*P.pseudamabilis*2, [6] *P.hirokii*1, [7]*P. P.hirokii* 2, [8] *P. P.hirokii* 3 [9] *Metapocyrtus_hederaephilus*

of COI one gene alone can give such a high number of haplotypes (Pang et al. 2011, Herbert et al. 2003). The high discrimination power of COI gene was also well studied by Herbert *et al*. (2003) and was found to be very effective in identifying taxa up to the species level except for some cases of Lepidoptera and Cnidaria which has a short divergence time. As for other taxon like Odonata, one gene is not sufficient in distinguishing species in some families and

they use a combination of two genes (Chen et al. 2013). In the case of *Pachyrhynchus*, COI was observed to be effective in delineating species and clearly distinguishes one species from another. The analysis of the haplotypes gives clear and definite nucleotides that may serve as potential barcode (Maranan & Diaz, 2013). Table 1 shows the regions of the nucleotides which show variation reveals that no between species variation was present in *P. apoensis* as well as *P. hirokii* and *P. pseudamabilis*.

The numbers of base differences per site from between sequences are shown. The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 527 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Pairwise alignment (Table 3) shows that among the three *Pachyrynchus* species sequenced from Mt. Apo Natural Park, *P.apoensis* is closer to *P.pseudamabilis* (i.e. d=0.25) while *P.apoensis* is more distant compared to *P.hirokii* (i.e. d=0.41). Both, *P.apoensis* and *P.pseudamabilis* are distinguished morphologically from the pattern of their elytra while *P.apoensis* and *P.hirokii* have wider range of difference in the elytral pattern as well as shape of rostrum, pronotum and elytra. For cryptic species or species group with high level of variation, DNA barcoding should be further used together with morphological data in identifying species. A precise and accurate identification especially *Pachyrhynchus* should be established since these are highly endemic and is highly threatened due to the loss of their habitats. For *Pachyrynchus* species complex such as *P. venustus* group and *P.speciousus* group which shows remarkably high variation in every mountain and island, DNA barcoding should supplement morphological data in delineating species. Similar recommendation was given by Bondoc (2013) in supplementing phenotypic traits with DNA barcodes in authenticating buffalo species. For highly endemic species such as *Pachyrhynchus* which also faces high extinction rates due to deforestation, accurate identification is highly important in assessing abundance, habitat threats and actual distribution and endemism. DNA barcoding of *Pachyrhynchus* using mitochondrial COI gene will be very helpful in accurate identification together with morphological data.

CONCLUSION

A high sequence variation using mitochondrial cytochrome c oxidase gene was observed among the three species of P*achyrynchus* found in Mt. Apo Natural Park, Mindanao Island. No within species sequence variation was observed for *P.apoensis, P.pseudamabilis* and *P.hirokii*. Distinctive clades with higher bootstrap of 100% for the three *Pachyrhynchus* species showing high discrimination power of COI gene in delineating species of *Pachyrhynchus* was observed. Results reveal the high usability of COI gene as aid in identifying *Pachyrhynchus* to the species level. This is highly helpful especially in problematic group of species under the genus which shows remarkable variation in every locality. DNA barcoding supports the idea of using reproductive character such as the aedeagus in delineating species. DNA barcoding using mitochondrial COI gene should support morphological characters and aedeagus in identification of species especially for problematic taxon and for conservation purposes.

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Received: 04.05.2016.
Accepted: 12.05.2016.