

Article

Neighbor-joining analysis of morphological characters and DNA barcodes of COI sequences of rice black bugs from the Philippines

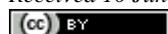
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Abstract

The rice black bugs (RBB) from the Philippines are argued to be comprised of many cryptic species. Still, they are also claimed to be only one species having many morphological variant forms. Arguably, RBBs are regarded as a ‘problem species’ with a ‘species problem’. To be able to understand the nature of the complexities observed in this group of insect pests, the Paleontological Statistics Software (PAST) was used to perform neighbor-joining analysis of thirty morphological characters, and DNA barcodes of COI sequences. The data matrix subjected to the Neighbor-Joining Method of analysis used the Kimura parameter bootstrapped 1000 times to determine the nature of morphological diversity. Genomic DNA (gDNA) from different RBBs extracted from the leg and head tissues, sequenced, and processed were also subjected to neighbor-joining analysis to determine how the different RBBs were phylogenetically related. Based on the analysis of morphological data, 11 out of 30 characters were consistent across all the RBB groups examined. Three morphologically differentiated groups were observed to differ from the standard taxonomic character traits of *S. coarctata*. The results of DNA barcoding for the RBB COI sequences showed only RBB Palawan was the only one to differ from the other RBB populations, thus indicating RBB Palawan could be a new species of *Scotinophara*. While morphometric variations were observed, the two Mindanao groups could be due to phenotypic plasticity, which may be attributed to adaptation in different environmental conditions.

Keywords congeneric; conspecific; phylogeny; taxonomy.

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1 Introduction

The predominantly invasive insect pest, *Scotinophara coarctata* (Fabricius) (Miyamoto et al., 1983) is argued to be composed of many cryptic species (Barrion et al., 2008). It is considered a species complex because RBB behaved differently in feeding habits, especially those collected from different rice hosts (Cruz et al., 2013;

Demayo et al., 2007; Torres et al., 2010, 2011, 2013a,b) and geographical locations (Sepe and Demayo, 2017; Sepe et al., 2019). The insect has shown to exhibit immense diversity regarding their general appearance, such as the shapes of the head, pronotum, scutellum, wings, and reproductive traits, thus referred to as a ‘problem species’ with a ‘species problem’. It was argued that rice black bugs are phenotypically plastic (Sepe et al., 2019; Sepe and Demayo, 2017; Torres et al., 2010, 2011, 2013a,b) and the many characters were just grouped based on symplesiomorphic resemblance (Wägele, 2005; Wägele and Mayer, 2007). Barrion et al. (2007) in their taxonomic matrix argued that RBBs in the Philippines is composed of many species. Still, these were only based on interpretation and explanation of the characters, which require several modifications and justifications, thus resulting in many disagreements on the taxonomic status of the insect (Krishnaiah et al., 2007). In our study, while it is argued that qualitative descriptions are very useful for the assessment of the taxonomic positions of the different morphological forms of *S. coarctata* (Cruz et al., 2013; Demayo et al., 2007; Torres et al., 2010, 2011, 2013a,b; Barrion et al., 2008), incorporating molecular methods such as DNA barcoding will further help resolve their species status (Moore, 1995; Hebert et al., 2003; Hebert et al., 2004; Lee et al., 2010; Witt et al., 2006; Yassin et al., 2008; Adamowicz, 2004; Coeur d’acier, 2008; Jousselin, 2009). We applied neighbor-joining analysis of both morphological characters and DNA barcoding of COI sequences to be able to determine whether those RBBs collected from different geographical locations are of the same species or not. The study will provide new information to understand the nature of complexities observed in the insect pest.

2 Methodology

RBB samples were collected in several rice-producing provinces in the Philippines (Fig. 1). The land areas of the study are covered with vast hectares of irrigated-lowland rice farms planted with different varieties.

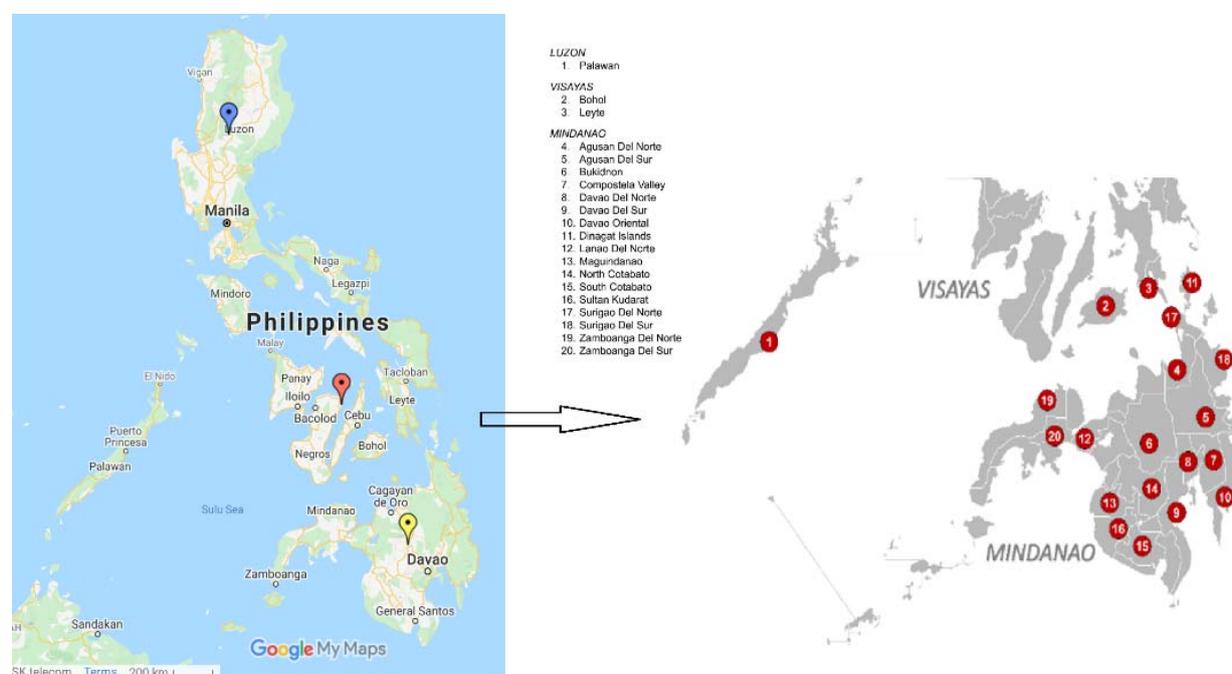


Fig. 1 Location map of the study areas.

Thirty rice black bugs were collected from rice fields through ‘light-trapping’ using a 1000-watt super bulb, and kept in a container soaked with 95% ethanol. Images of the head, pronotum, scutellum, genital plates, and forewings were taken using a SONY DSC-W830 digital camera under a stereomicroscope (Fig. 2).

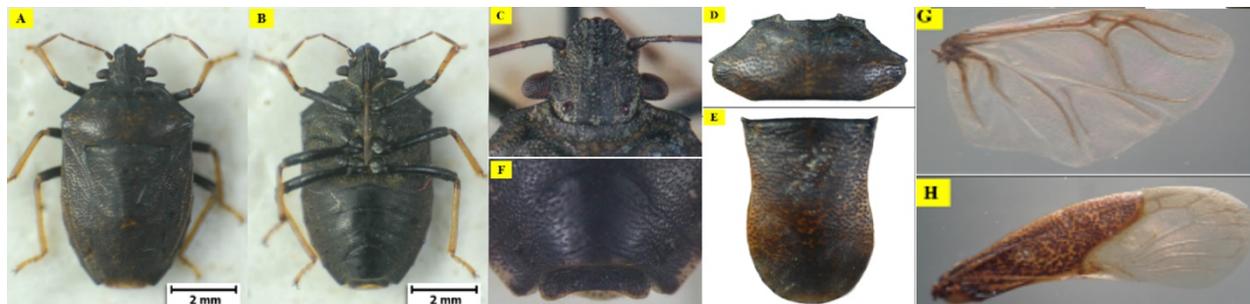


Fig. 2 Male RBB illustrating its (A)-dorsal view, (B)-ventral view, (C)-head, (D)-pronotum, (E)-scutellum, (F)-genital plate, (G)-hindwing and (H)-forewing.

Thirty adult morphological characters of the male RBB were selected for coding. The ‘presence’ and ‘absence’ of a particular character based on an understandable description of the external morphology of organisms (Nei et al., 2010; Sneath and Sokal, 1973) were used (Table 1). All of the observed characters were assigned values of 0, 1, 2, 3, or 4... and these values were used to conduct the phenetic analysis using PAST version 3.0 software (Hammer et al., 2001). The groups' characteristics were then equally weighted and treated unordered for quantification, objectification, and efficient classification. The matrix was then subjected to the Neighbor-joining method using the Kimura parameter with 1000 replicates bootstrapping to determine patterns of morphological diversity.

Table 1 List morphological character selection and coding of RBB males from different populations in the Philippines.

| NO. | CHARACTER | CODING | NO. | CHARACTER | CODING |
|-----|------------------|---|-----|---|---|
| 1 | Body size: | (0) small, 6.5 to 7.0 mm; (1) medium, 7.0 to 7.5 mm; (2) large 7.5 to 8.5 mm | 16 | Size of anterolateral spine: | (0) absent; (1) small; (2) moderate; (3) largely triangular |
| 2 | Body color: | (0) brown; (1) dark brown; (2) brownish yellow; (3) reddish brown; (4) grayish black; (5) black | 17 | Size of prehumeral spine: | (0) absent; (1) small; (2) moderate; (3) largely triangular |
| 3 | Head notch: | (0) distinct; (1) absent | 18 | Prehumeral spine vs. Anterolateral spine (ALS): | (0) PHS = ALS; (1) PHS < ALS; (2) PHS > ALS |
| 4 | Tylus vs. Jugum: | (0) Tylus = Jugum; (1) Tylus > Jugum; (2) Tylus < Jugum; (3) Tylus ≤ Jugum; (4) Tylus ≥ Jugum | 19 | Lateral sides of scutellum: | (0) well constricted; (1) not strongly constricted |
| 5 | Antennifers: | (0) not distinct; (1) slightly cleft to cleft; (2) oblique-pointed to pointed; (3) | 20 | Tip of scutellum: | (0) rounded; (1) slightly cleft; (2) truncate; (3) truncate to slightly |

| | | | | | |
|----|---|--|----|---|---|
| | | not cleft or blunt | | | emarginated; (4) emarginated; (5) pointed |
| 6 | Number of antennal segments: | (0) four; (1) five | 21 | Spines in tibia II: | (0) present; (1) absent |
| 7 | Length of antennal segment I vs. II | (0) I = II; (1) I > II; (2) I < II | 22 | Tibia color: | (0) brown; (1) dark brown; (2) brownish yellow; (3) reddish brown |
| 8 | Ocelli color: | (1) red; (2) dark red; (3) orange red; (4) orange yellow; (5) dark brown | 23 | Length of femur I vs. femur II | (0) I = II; (1) I > II; (2) I < II |
| 9 | Cicatrices humps of pronotum: | (0) absent; (1) present | 24 | R+M - CuA triangle of hindwings: | (0) absent; (1) moderate; (2) large |
| 10 | Serration on lateral margins of pronotum: | (0) serrated, minute; (1) serrated, large; (2) not serrated | 25 | Junction of vein R+M of hindwings: | (0) heavily sclerotized; (1) lightly sclerotized |
| 11 | Direction of anterolateral margins of pronotum: | (0) absent; (1) oblique-upward; (2) latero-upward; (3) oblique-posterad | 26 | Number of closed marginal cells of forewings: | (0) 0; (1) 1-2; (2) 2-3; (3) 3-5; (4) 4-5 |
| 12 | Length of anterolateral margins of pronotum: | (0) absent; (1) very short, slightly beyond eye; (2) distinctly long, approximately 2x eye length | 27 | Number of longitudinal veins of forewings: | (0) 3; (1) 4; (2) 5; (3) 6; (4) 7 |
| 13 | Shape of anterolateral margins of pronotum: | (0) absent; (1) slightly concave; (2) concave; (3) straight | 28 | Proboscis reach: | (0) abdominal segment I; (1) coxae III; (2) coxae II; (3) coxae I |
| 14 | Concavity of lateral margin of pronotum: | (0) slightly sinuate; (1) concave; (2) slightly concave; (3) straight; (4) doubly convex and oblique | 29 | Shape of Midanterior margin of sternite VII in the abdominal tip: | (0) V-shaped; (1) widely V-shaped |
| 15 | Direction of anterolateral spine: | (0) absent; (1) obliquely upward; (2) laterad; (3) posterad; (4) posterolaterally | 30 | Shape of tergite X of genital plate: | (0) narrowly concaved; (1) broadly concaved |

For DNA barcoding, approximately 10mg were isolated from the legs and head of one male to obtain genomic DNA (gDNA) samples. The extraction and sequencing were processed at the Philippine Genome Center (PGC) at the University of the Philippines - Diliman, Quezon City. The extraction was done using the CTAB extraction method. The extracted samples were then checked on a 1% agarose gel electrophoresis (AGE) to detect the quality and quantity of gDNA using a UV gel documentation system. The partial DNA gene sequence of the COI gene for RBB was done using the universal primers of the LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') for forward and the HCO2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3') for reverse. The PCR amplification was carried with an

annealing temperature of 50°C. The PCR products were fractionated on a 1.0% agarose gel electrophoresis and are visualized under UV light and photographed using gel documentation and then sequenced. Samples with PCR bands, the products obtained should be near the expected size of 750bp sequence.

The forward and reverse sequences were aligned for high percentage yield to locate the consensus sequence using Geneious version 11 1.4 software. The forward and reverse sequences were trimmed and edited using BioEdit version 7 2.5 software for low percentage yield. All of the consensus sequences for each RBB group were then exported as a fasta file (sequences/alignment) before phylogenetic analysis. The search for identification similarity for each consensus sequence was performed using NCBI Nucleotide BLAST and compare related species found in NCBI Genbank provided with an accession number.

The fasta file data of all species/outgroups were aligned and analyzed using the MEGA7 software for phylogenetic analysis (Kumar et al., 2016). In the sequence data obtained, the constant sites and variable sites within sequences were done to determine the nucleotides in all sequences for comparison of outgroups. A constant site is a site containing the same nucleotide in all sequences. A variable site contains at least two types of nucleotides, but some can be singleton or parsimony-informative. In order of the increasing computation complexity, the phylogeny tree-building was implemented by a Neighbor-Joining method (NJ) using Kimura-2 parameter with 1000 replicates bootstrapping. The Pairwise Distances using Kimura-2 parameter with 1000 replicates bootstrapping was computed to estimate evolutionary distances between sequences by calculating the proportion of nucleotide differences between each pair of sequences (Kimura, 1980; Felsenstein, 1985; Saitou and Nei, 1987). The method of NJ was provided for comparative purposes since it is the most common and fast-performing algorithm widely used with the DNA barcoding data. The species used for comparison were the following (based on accession Number): *Scotinophara scotti* (GQ292233), *Podisus serieventris* (HQ106266), *Podisus maculiventris* (KR039482), *Neottiglossa leporina* (KM022331), *Neottiglossa tumidifrons* (KR038107), *Oechalia schellenbergii* (EF641159) and *Tolumnia basalis* (KY492344). *Scotinophara bispinosa* did not match any of the BLAST searches but was still added in the analysis since it has GenBank sequence (JX469139) and belongs to the same genus of rice black bugs.

3 Results

All RBB groups were observed to be polymorphic for 19 traits that include the direction of anterolateral margins of pronotum, shape of anterolateral margins of pronotum, concavity of lateral margin of pronotum, size of anterolateral spine, size of prehumeral spine, lateral sides of scutellum, tibia color, number of longitudinal veins of forewings, proboscis reach, shape of mid anterior margin of sternite VII in the abdominal tip, and shape of tergite X of genital plate. A closer look at the NJ tree (Fig. 3) and assessing the characters of the different populations that made them different from others show RBB from Maguindanao, Surigao Del Sur, and Lanao Del Norte show distinctness for their relative lengths of tylus and jugum. The RBB Davao Oriental on the number of closed marginal cells of forewings, RBB Surigao Del Norte and Davao Del Norte on the shapes of the tip of the scutellum either rounded or pointed tips, RBB Davao Del Sur, North Cotabato, and South Cotabato have either orange-red or dark brown ocelli, RBB Zamboanga Del Sur, Agusan Del Sur, Agusan Del Norte, and Dinagat Islands have dark brown, brownish-yellow and grayish-black color, RBB Compostela Valley and Bohol on the lengths of antennal segment I and II, RBB Leyte, Zamboanga Del Norte, Bukidnon and Sultan Kudarat on the body size that are either small, medium or large sizes. All of the RBB individuals from all the populations were found to be consistent in 11 out of 30 characters (Table 2). Results show the NJ tree has three RBB distinct groups from the morphologically known *S. coarctata*. RBB Palawan, another group that includes RBB from Maguindanao, Surigao Del Sur, Lanao Del Norte, and the rest is forming another group.

Table 2 The character data matrix used for the phenetic analysis of RBB in the Philippines.

| R B B | CHARACTERS (See Table 1 for descriptions) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---|
| | 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 | 28 | 29 | 30 | |
| A | 1 | 2 | 0 | 1 | 2 | 1 | 0 | 2 | 1 | 2 | 3 | 2 | 1 | 1 | 3 | 2 | 3 | 2 | 0 | 5 | 0 | 2 | 2 | 2 | 2 | 0 | 2 | 2 | 1 | 1 | 0 |
| B | 1 | 1 | 0 | 2 | 2 | 1 | 2 | 5 | 1 | 2 | 1 | 2 | 1 | 2 | 3 | 3 | 3 | 0 | 1 | 0 | 0 | 2 | 2 | 2 | 2 | 0 | 3 | 1 | 2 | 0 | 1 |
| C | 2 | 2 | 0 | 0 | 2 | 1 | 2 | 3 | 1 | 2 | 2 | 2 | 2 | 1 | 3 | 3 | 3 | 0 | 0 | 3 | 0 | 2 | 2 | 2 | 0 | 1 | 1 | 2 | 0 | 1 | |
| D | 2 | 2 | 0 | 1 | 2 | 1 | 2 | 2 | 1 | 2 | 3 | 2 | 2 | 1 | 3 | 3 | 2 | 1 | 1 | 0 | 0 | 0 | 2 | 2 | 0 | 2 | 1 | 2 | 0 | 1 | |
| E | 2 | 2 | 0 | 1 | 2 | 1 | 1 | 5 | 1 | 2 | 2 | 2 | 1 | 2 | 3 | 2 | 3 | 2 | 1 | 2 | 0 | 2 | 2 | 2 | 0 | 4 | 1 | 1 | 0 | 1 | |
| F | 1 | 2 | 0 | 0 | 2 | 1 | 2 | 5 | 1 | 2 | 3 | 2 | 1 | 1 | 3 | 3 | 3 | 0 | 0 | 5 | 0 | 2 | 2 | 2 | 0 | 2 | 2 | 1 | 0 | 1 | |
| G | 1 | 0 | 0 | 1 | 2 | 1 | 0 | 5 | 1 | 2 | 1 | 2 | 2 | 1 | 3 | 2 | 3 | 2 | 1 | 2 | 0 | 0 | 2 | 2 | 0 | 3 | 2 | 2 | 1 | 0 | |
| H | 1 | 2 | 0 | 0 | 2 | 1 | 0 | 5 | 1 | 2 | 3 | 2 | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 5 | 0 | 1 | 2 | 2 | 0 | 3 | 1 | 1 | 0 | 1 | |
| I | 0 | 1 | 0 | 0 | 2 | 1 | 2 | 3 | 1 | 2 | 2 | 2 | 1 | 1 | 3 | 3 | 2 | 1 | 1 | 2 | 0 | 0 | 2 | 2 | 0 | 2 | 2 | 1 | 1 | 0 | |
| J | 1 | 2 | 0 | 2 | 2 | 1 | 1 | 5 | 1 | 2 | 3 | 2 | 1 | 1 | 3 | 3 | 2 | 1 | 1 | 0 | 0 | 0 | 2 | 2 | 0 | 3 | 2 | 1 | 1 | 0 | |
| K | 1 | 1 | 0 | 1 | 2 | 1 | 2 | 2 | 1 | 2 | 2 | 2 | 2 | 1 | 3 | 3 | 2 | 1 | 1 | 3 | 0 | 0 | 2 | 2 | 0 | 2 | 1 | 0 | 0 | 1 | |
| L | 1 | 2 | 0 | 2 | 2 | 1 | 2 | 5 | 1 | 2 | 3 | 2 | 3 | 1 | 3 | 2 | 2 | 0 | 1 | 4 | 0 | 0 | 2 | 2 | 0 | 2 | 2 | 0 | 1 | 0 | |
| M | 1 | 1 | 0 | 1 | 2 | 1 | 1 | 5 | 1 | 2 | 3 | 2 | 1 | 1 | 3 | 1 | 1 | 0 | 1 | 2 | 0 | 1 | 2 | 2 | 0 | 4 | 2 | 1 | 1 | 0 | |
| N | 1 | 1 | 0 | 1 | 2 | 1 | 2 | 3 | 1 | 2 | 2 | 2 | 1 | 2 | 3 | 3 | 3 | 0 | 1 | 0 | 0 | 1 | 2 | 2 | 0 | 2 | 1 | 1 | 1 | 0 | |
| O | 1 | 1 | 0 | 1 | 2 | 1 | 0 | 5 | 1 | 2 | 1 | 2 | 3 | 2 | 3 | 3 | 2 | 1 | 0 | 5 | 0 | 1 | 2 | 2 | 0 | 2 | 2 | 1 | 0 | 1 | |
| P | 2 | 2 | 0 | 0 | 2 | 1 | 2 | 5 | 1 | 2 | 3 | 2 | 1 | 1 | 3 | 3 | 3 | 0 | 1 | 0 | 0 | 0 | 2 | 2 | 0 | 3 | 1 | 1 | 0 | 1 | |
| Q | 0 | 2 | 0 | 1 | 2 | 1 | 2 | 5 | 1 | 2 | 3 | 2 | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 0 | 1 | 2 | 2 | 0 | 3 | 2 | 1 | 0 | 1 | |
| R | 1 | 2 | 0 | 0 | 2 | 1 | 2 | 5 | 1 | 2 | 3 | 2 | 2 | 1 | 3 | 1 | 2 | 2 | 1 | 4 | 0 | 1 | 2 | 2 | 0 | 2 | 2 | 1 | 1 | 0 | |
| S | 0 | 2 | 0 | 0 | 2 | 1 | 0 | 3 | 1 | 2 | 2 | 2 | 3 | 1 | 3 | 1 | 3 | 2 | 0 | 3 | 0 | 0 | 2 | 2 | 0 | 3 | 1 | 1 | 0 | 1 | |
| T | 2 | 4 | 0 | 1 | 2 | 1 | 2 | 5 | 1 | 2 | 3 | 2 | 1 | 2 | 3 | 3 | 3 | 0 | 1 | 2 | 0 | 1 | 2 | 2 | 0 | 2 | 1 | 0 | 0 | 1 | |
| U | 2 | 2 | 0 | 2 | 2 | 1 | 0 | 2 | 1 | 2 | 3 | 2 | 1 | 1 | 3 | 2 | 3 | 2 | 0 | 0 | 0 | 2 | 2 | 2 | 0 | 2 | 2 | 1 | 1 | 0 | |

A-Palawan, B-Bohol, C- Leyte, D-Agusan del Norte, E-Agusan del Sur, F- Bukidnon, G- Compostela Valley, H- Davao del Norte, I- Davao del Sur, J- Davao Oriental, K-Dinagat Islands, L- Lanao del Norte, M- Maguindanao, N-North Cotabato, O- South Cotabato, P- Sultan Kudarat, Q- Surigao del Norte, R- Surigao del Sur, S- Zamboanga del Norte, T- Zamboanga del Sur, U- *S. coarctata*

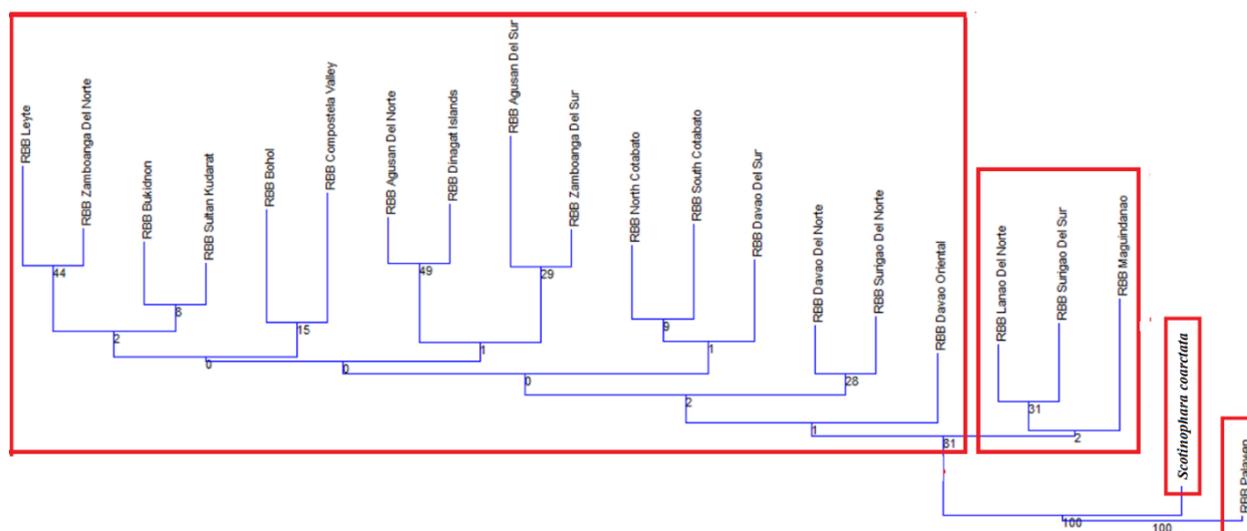


Fig. 3 Neighbor-Joining Tree analysis of selected RBBs collected in the Philippines.

Table 3 The constant (C) and variable (V) sites between aligned COI sequences of 433 bp length.

| RBB populations | <i>S. scotti</i> | | <i>S. bispinosa</i> | | <i>S. serieventris</i> | | <i>P. maculiventris</i> | | <i>N. leporina</i> | | <i>N. tumidifrons</i> | | <i>O. schellenbergii</i> | | <i>T. basalis</i> | |
|---------------------|------------------|-----|---------------------|-----|------------------------|-----|-------------------------|-----|--------------------|-----|-----------------------|-----|--------------------------|-----|-------------------|-----|
| | C | V | C | V | C | V | C | V | C | V | C | V | C | V | C | V |
| | Palawan | 373 | 60 | 372 | 61 | 369 | 64 | 371 | 62 | 377 | 56 | 374 | 59 | 369 | 64 | 378 |
| Bohol | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Leyte | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Agusan del Norte | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Agusan del Sur | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Bukidnon | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Compostela Valley | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Davao del Norte | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Davao del Sur | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Davao Oriental | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Dinagat Islands | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Lanao del Norte | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Maguindanao | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| North Cotabato | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| South Cotabato | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Sultan Kudarat | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Surigao del Norte | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Surigao del Sur | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Zamboanga del Norte | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |
| Zamboanga del Sur | 375 | 58 | 374 | 59 | 371 | 62 | 373 | 60 | 379 | 54 | 374 | 59 | 371 | 62 | 380 | 53 |

COI sequences of more than 500 bp were obtained from all the RBB's collected for DNA barcoding. The final aligned data that incorporated the NCBI GenBank sequences reached a nucleotide length of 433 bp representing the seven related species belonging to 5 genera (Table 3). Results showed similar constant and variable regions except for only the Palawan RBB (Table 3). This group differed for two nucleotides in either the constant or variable regions compared to the other groups of the insect. The identity of the different RBBs ranged between 87% to 89% similarity matches when compared to the other Pentatomid bugs (Table 4). The position of the different RBBs in the phylogenetic tree is shown in Fig. 4. RBBs collected were found to belong in only two groups- RBB Palawan and all others congregating to belong to just one group.

Table 4 Cross-reference of Closest Genbank identification with morphological identification.

| RBB Populations | BLASTn Hit (COI) | | | | | | | |
|---------------------|------------------|---------------------|------------------------|-------------------------|--------------------|-----------------------|--------------------------|-------------------|
| | <i>S. scotti</i> | <i>S. bispinosa</i> | <i>S. serieventris</i> | <i>P. maculiventris</i> | <i>N. leporina</i> | <i>N. tumidifrons</i> | <i>O. schellenbergii</i> | <i>T. basalis</i> |
| Palawan | 87% | – | – | – | 88% | – | 87% | 88% |
| Bohol | – | – | – | – | 88% | 87% | 87% | 89% |
| Leyte | – | – | – | – | 88% | – | 87% | 89% |
| Agusan del Norte | – | – | – | – | 88% | – | 88% | 88% |
| Agusan del Sur | – | – | 87% | 87% | 88% | 87% | 87% | 89% |
| Bukidnon | – | – | – | – | 88% | – | 88% | 89% |
| Compostela Valley | 87% | – | – | – | 88% | – | 87% | 89% |
| Davao del Norte | 88% | – | – | – | 88% | – | 88% | 89% |
| Davao del Sur | 87% | – | – | – | 88% | 87% | – | 88% |
| Davao Oriental | 88% | – | – | – | 88% | – | 88% | 89% |
| Dinagat Islands | 87% | – | – | – | 88% | 87% | 87% | 89% |
| Lanao del Norte | 87% | – | – | – | 88% | – | 87% | 89% |
| Maguindanao | – | – | – | – | 88% | 87% | 87% | 89% |
| North Cotabato | – | – | 87% | 87% | 88% | – | 88% | 89% |
| South Cotabato | 87% | – | – | – | 88% | – | 87% | 88% |
| Sultan Kudarat | – | – | – | – | 88% | – | 88% | 89% |
| Surigao del Norte | 87% | – | – | – | 88% | – | 87% | 89% |
| Surigao del Sur | 87% | – | – | – | 88% | – | 87% | 89% |
| Zamboanga del Norte | 87% | – | – | – | 88% | – | 87% | 89% |
| Zamboanga del Sur | – | – | – | – | 89% | – | 88% | 88% |

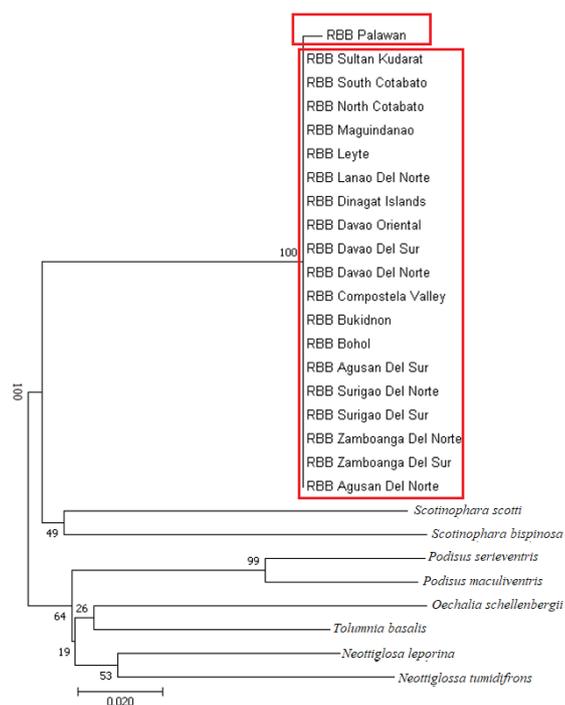


Fig. 4 Phylogenetic tree based on COI (K2P model) using Neighbor-joining method.

4 Discussion

The obtained results on phenetic analysis revealed that all RBBs were consistent in 11 out of 30 characters. The other nineteen traits were polymorphic, resulting in observed variations in combined characters within groups. It is, however, very interesting that three groups were found to be morphologically different from *S. coarctata* previously described (Sepe et al., 2019; Sepe and Demayo, 2017; Torres et al., 2010, 2011, 2013a,b). The variations between groups can be attributed to the observed differences in polymorphic characters within populations indicating RBBs in the Philippines are phenotypically plastic. This plasticity can be due to the ability to change its phenotype in response to changes in its habitat, or to its movement to a different habitat (Whitman and Agrawal, 2009), or changes in densities during the early stages of the insect's development resulting in remodeling of their phenotypes (Jannot et al., 2009). The delimitation of the four phenotypically differentiated groups using DNA barcodes showed that all groups except RBB Palawan belong to the same species group. There was a very high conspecific divergence observed, with 99.5% having identical nucleotide sequences. However, the divergence of RBB Palawan is consistent with the phenotypic analysis, where it was shown to differ for all the other groups of RBB (Fig. 4). Both DNA barcoding and morphological analysis results complement that RBB Palawan could be genetically distinct from all other RBB groups examined and could be a new species. The fragment size of COI has been shown to provide high resolution to identify cryptic species thus, the results for RBB Palawan suggest evolutionary shifts in the evolution of the RBB in the Philippines, leading to speciation (Hebert et al., 2004; Lee et al., 2010; Moore, 1995; West-Eberhard (2003); Losos and Thorpe (2004); Dumbacher and Fleischer (2001).

5 Conclusion

Results of the morphological and DNA barcoding analysis of the different RBB groups show while all the groups were morphologically consistent in 11 out of 30 characters, nineteen characters were polymorphic, resulting in the observations of three morphologically differentiated groups from the taxonomic character traits

of *S. coarctata*. However, the results of DNA barcoding for the RBB COI sequences showed identical nucleotide sequences in all groups except for RBB Palawan, which showed two nucleotide differences from the other groups. It can be concluded that RBB in the Philippines are phenotypically plastic except for RBB Palawan. The plasticity could result from the differences in adaptation of the species in response to variations in environmental conditions where they inhabit and may be undergoing evolutionary shifts, which could lead to speciation.

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