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Neurigona susanrocesae, the first *Neurigona* species from the Philippines

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ABSTRACT

The first species of the genus *Neurigona* is described from the Philippines. The diagnosis, description and molecular data based on the partial DNA sequence of mitochondrial cytochrome oxidase subunit I gene were documented. The *Neurigona* species exhibits distinct characteristics, including a sub-apical dorsal stylus resembling an arista, a peduncle on the seventh abdominal segment, a flat dorsal postcranium, elongated legs devoid of prominent setae or bristles, and a distinctive rectangular bend in wing vein M. Based on the DNA sequence analysis, the unknown species is 90.06% similar to *Cyrtana* sp. and *Neurigona zhejiangensis*. However, 10% of its DNA sequences are significantly variable making it a totally different species. The specimen was verified by a dipterist as a species belonging to Genus *Neurigona* under Family Dolichopodidae. These flies are recognized for their lengthy legs and sizable yellow bodies. The species is named as *N. susanrocesae* as a tribute to the late Susan Roces, the "Queen of Philippine Movies".

Keywords: Bohol Island, DNA barcoding, new species, phylogenetic analysis, taxonomy

INTRODUCTION

Although taxonomists have estimated one to 1.7 million species in the order Diptera, they have only just scratched the surface in terms of documenting and describing its immense diversity, as the dipterans are believed to represent some 10-15% of the world's biodiversity (Stork 1996; Brown 2005). Dolichopodidae, or the long-legged flies are the largest dipteran family with about 7,500 species described globally (Bickel 2009). These flies are cosmopolitan and inhabit diverse ecosystems including freshwater and marine environments, forests, grasslands, and wetlands. Notably, they constitute the most prevalent true fly group, with adult members typically measuring between a mere 1.8 and 10

millimeters in size. (Grootaert and Meuffels 2004). Despite their small size, the adults and larvae are both known to be avid feeders. They mostly consume chironomid, ceratopogonid, and culicid larvae. Adults take out prey larvae from their burrows and secure them between their fleshy labellae throughout the feeding process. The prey is then punctured via the concealed hypopharynx, which mimics a dagger as bodily fluids are sucked up (Coulbaly 1993).

A high diversity of dolichopodids is normally thought to indicate an undisturbed ecological system, whereas a low diversity of these insects is linked with some kind of ecological disruption. Grichanov et al. (2021) noted that diversity of Dolichopodidae in East Asia is much higher compared to other parts of the world. In Malaysia, for instance, there was a lack of



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knowledge of dolichopodid pointing to the single study done even prior to World War II despite the country being recognized as a biodiversity hotspot (Parent 1935). The team of Grootaert and Meuffels (2004) surveyed for only three days in Endau-Rompin Park in Malaysia and revealed the presence of 83 species. Their paper highlights the need for more studies to understand the biogeography and evolutionary history of Dolichopodidae in biodiverse regions of the world.

The Philippines is in the “center of the center for biodiversity” and is likewise considered a hotspot for the discovery of new species of Dolichopodidae due to its unique geography (Ramos et al. 2018). Apart from colonization, the high species richness and endemism of insects in the country is believed to be explained by speciation and phylogenesis. Their limited dispersal abilities and small size prevent these insects from traveling long distances, leading to high levels of isolation and speciation in islands with diverse habitats and oceanic barriers (Heaney 2000). Several species of Dolichopodidae have been recently reported in various habitats in the Philippines, including mangroves, freshwater streams, and forests (Ramos et al. 2018; Ramos and Grootaert 2018; Jose et al. 2022; Wang et al. 2022; Grichanov 2023). The continued exploration and documentation of Dolichopodidae diversity in the Philippines are crucial in understanding the biogeography and evolutionary history of this diverse family.

In this paper, a new species belonging to the *Neurigona* genus of Dolichopodidae from Bohol Island, Philippines is reported. The recently discovered species bears a striking resemblance to *N. squamifera*, previously documented in Singapore (Parent 1935; Grootaert and Foo 2019). This finding marks the first record of its kind within the Philippines, enriching our comprehension of Dolichopodidae biodiversity in the country. The *Neurigona* genus is widely distributed globally, with only 25 known oriental species out of the 150 identified species worldwide (Yang et al. 2006), making this finding particularly significant.

METHODS

Sampling Area, Species Collection and Sorting

With permission to sample and later an issuance of a gratuitous permit from Department of Environment and Natural Resources – Region VII (Wildlife GP 2019-11, series of 2019), collections were made from June to August 2016 at Magsaysay Park, Rajah Sikatuna Protected Landscape (RSPL), Bilar, Bohol Island, Philippines. The vegetation of the habitat where the species was collected are predominantly planted natural regenerants of big-leaf mahogany and several species of *Ficus*. Malaise traps deployed in areas with minimum disturbance: Trap 1 (9.70431°N, 124.1239°E) and Trap 2 (9.70359°N,

124.1252°E) from June to August 2016. The traps were allowed to remain open during the entire sampling period and the bottles containing 70% unmethyated ethanol alcohol were changed every week. Collected specimens were sorted into various families and placed in separate vials with screw caps and labels/codes for molecular work. Sorting of specimens was based on “The Families of Diptera of the Malay Archipelago” (Oosterbroek 1998) and verified by taxon-specific experts from the National University of Singapore (NUS).

Digital Referencing and Morphological Characterization of Specimen

Images of the unidentified specimen were acquired at National University of Singapore using a Dun Inc. Passport II microphotography system, fitted with a Canon 65mm 5X MPE lens. The images were compiled using Zerene Stacker and digitally processed using Photoshop CS5. To confirm new species identification, taxonomic experts compared the undetermined specimens with specimens from a digital reference collection, a physical reference collection of specimens used for taxa identification that needs to be identified routinely by taxonomists from different backgrounds and updated identification tools for dipterans (Ang et al. 2012).

Molecular Identification using CO1 Gene Marker for DNA Barcoding

To further confirm the identification of the unknown dolichopod, DNA barcoding was conducted using mitochondrial CO1 as the gene marker. A partial sequence of 313 bp from the CO1 region was amplified and compared to other dipteran DNA sequences. Two DNA-sequence based approaches were conducted for species discrimination and identification namely, the “best match” approach where the DNA sequence is directly compared to all the barcodes in the database and the tree-based identification using phylogenetic analysis (Meier et al. 2016; Barrett and Hebert 2005; Blaxter et al. 2005)

DNA Extraction and Sequencing

DNA extraction from the leg portion of the sample were subscribed from the methods of Wong et al. (2014). Primer pairs (Macrogen) designed to target the mitochondrial cytochrome c oxidase I (COI) gene were used for polymerase chain reaction (PCR) amplification. Conditions for the PCR were set at the following: initial denaturation at 95°C (3 m); 1 cycle of 94°C (1 m), annealing 47°C (1 m), and 72°C (1 m), followed by 40 cycles with final extension set at 72°C (5 m). Amplicons were then purified using Sure Clean (Bioline), quantified in equimolar ratios using Nanodrop (Quiagen), and pooled prior to library preparation and Next-Generation Sequencing with Illumina MiSeq and HiSeq 2500 sequencing platforms. Sequencing libraries were prepared by AIT biotech

using the TruSeq Nano DNA Library Preparation Kit (Illumina), according to the manufacturer's protocol. Illumina MiSeq runs were provided by AITbiotech with the use of MiSeq Reagent Kit v3 (2 X 300 bp read lengths) while HiSeq runs were provided by Singapore Centre for Environmental Life Sciences Engineering (SCELSE) with HiSeq 2500 System and Rapid SBS Kit v2 (2 X 250 bp read lengths). The respective COI mitochondrial DNA sequence has been submitted to National Center for Biotechnology Information (NCBI) with accession number ON023659 for data referencing.

Phylogenetic Analysis

The DNA analysis pipeline was based on the method of Meier et al. (2016). Paired-end reads were merged using PEAR 0.9.6 (Zhang et al. 2014). Reads from each PCR product were assigned to their corresponding specimen using uniquely labeled primer pair, and the dominant read was identified as the specimen barcode using a Python script (Srivathsan and Meier 2012). To sort the data, the reads in each sample were counted, grouped identical reads into sets, identified the most common set, and merged it with similar-length variants. These were also compared to the number of reads in the biggest set and the second-largest set (Meier et al 2016).

For purposes of quality control, barcoding of a particular sample was only considered successful if (i) the total read count was > 50x, (ii) the total barcode count was > 10x, and (iii) and the most dominant read was at least five times that of the second most dominant read (Meier et al. 2016). These sequences were then used for NCBI-BLAST (Basic Local

Alignment Search Tool) to search for sequences that matches (> 97% identity) the taxa. The top twenty specimens with the highest percentage match (86-88%) were retrieved for phylogenetic analyses.

All retrieved sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) v7. Aligned sequences in fasta format were further analyzed for phylogenetic inference using Molecular Evolutionary Genetics Analysis (MEGA X) (Kumar et al. 2018). Evolutionary relationships of each taxon were inferred using the Neighbor-Joining method (Saitou and Nei 1987) with 1000 bootstrap support. Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). In addition, a time tree was generated using the strict-clock method and the Tamura-Nei model (Tamura and Nei 1993) with an estimated log likelihood value of -5435.14 at a global rate of 0.001. The tree is drawn to scale with branch lengths measured in the relative number of substitutions per site.

RESULTS

Neurigona susanrocesae sp. nov.

Type material. Philippines, island of Bohol, province of Bilar, Magsaysay Park, 08.30.2006, ♀ holotype, 1 (Figure 1), leg. RP Jose, Entomology Laboratory, National University of Singapore (after the photo was taken, the specimen was desiccated and securely archived within a collection).

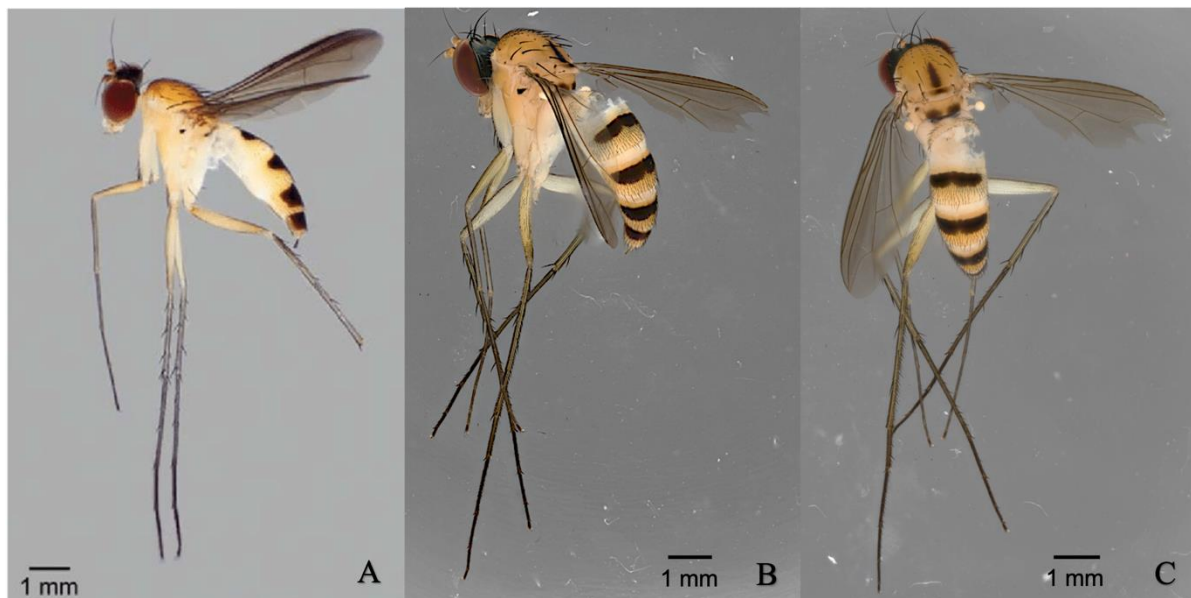


Figure 1. Gross morphological comparison between a parent *Neurigona squamifera* (A) from ZRCBDP0007646 (Photograph: K.Q. Chin) and the unidentified dolichopod discovered from Bohol Island. Holotype, female (P1F32R20) showing the (B) dorsal and (C) lateral habitus of the specimen. Scale bar =1 mm.

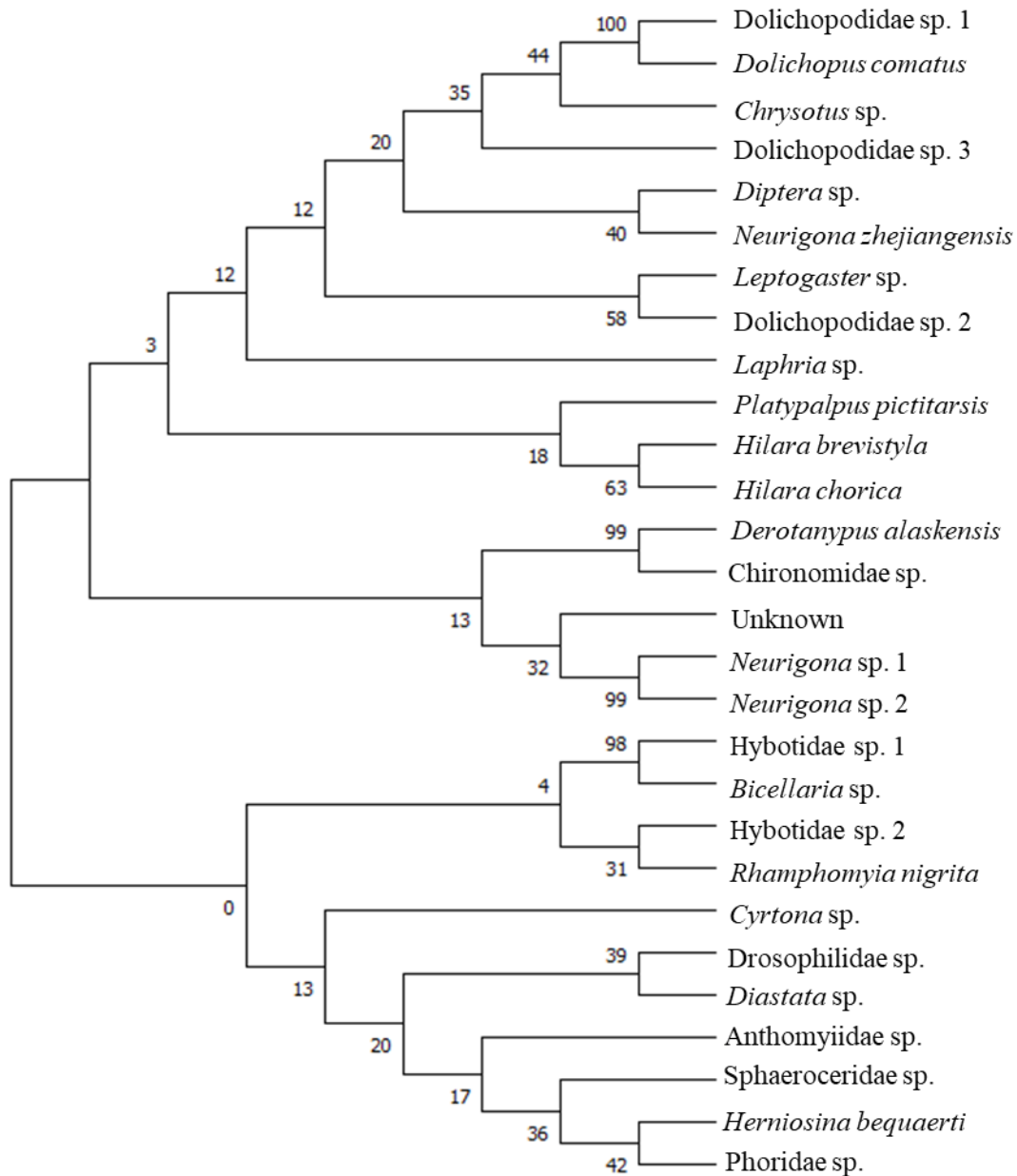


Figure 2. Phylogenetic analysis using MEGA X. The top thirty species sharing the highest similarity to the unknown DNA sequence were inferred for phylogenetic analysis using the Neighbor-Joining method with 1000 bootstrap support. The unknown sample clustered together with two conspecific *Neurigona* species with 32% bootstrap support. Numbers next to branches indicate percentage of replicate trees where associated taxa clustered together in the bootstrap test.

Dimensions. L: 6 mm, H: 3 mm, W: 5 mm.

Description. Female body length: 6 mm, wings: 5 mm, antenna: yellowish. Arista with basal segment yellowish, base of apical arista segment pale becoming black towards the tip. Palpus light brown elongate with bristling. Thorax: light brown with a brown prescutellar patch. Acrostichals biseriata. Scutellum brown, border yellow. Ventral border of postnotum is brown. Legs: yellow, tarsomeres entirely black, hind femur ventrally yellow. Fore coxa with

minute yellowish white anterior bristles on basal half. Tibia with black bristles-like structures. Wings transparent brownish tinged; anterior third darker brown than the posterior part. Haltere with knob anteriorly dusky yellow, otherwise almost entirely white. Abdomen: first tergite yellow with long black marginal bristles. Base, tip, and sides of tergite two and three yellow, the rest is brown to black. Tergite four with only a narrow yellow band at the base and tip, side brown. Tergite five dorsally yellowish white, a small brown patch at the side, cercus yellowish white.

The DNA sequence analysis of the unknown species showed a 90.06% similarity to *Cyrtona* sp. and *Neurigona zhejiangensis*, with 10% of its DNA sequences being significantly variable, indicating it is a distinct species. Figure 2 illustrates the evolutionary relationships between 30 closely related species with 88-90% similarities, using the Neighbor-Joining (N-J) method with 1000 bootstrap support. The unidentified species was found to be closely related to two different species of *Neurigona*, with 32% bootstrap support, and clustered as one group with them, suggesting it is highly likely to belong to the *Neurigona* genus. The percentage of replicate trees where associated taxa clustered together in the bootstrap test is indicated next to the branches.

DISCUSSION

The unidentified species shares key characteristics with other *Neurigona* species within the Dolichopodidae family found in Asia. These distinctive features include a flat dorsal postcranium, a sub-apical dorsal stylus resembling an arista, a face covered in dense pruinosity, proepisternum adorned with setae, a mesonotum with a flat posterior slope, noticeably elongated legs without major setae, ventral modifications on abdominal segments four or five, a well-defined peduncle on abdominal segment seven, and a spherical hypopygium (Bickel and Lian-Meng 1996; Bickel 2009).

This recently discovered species closely resembles *N. squamifera* in several aspects. Its antenna is distinctly yellow, with a notably wide third antennal segment. The palpus exhibits a vibrant yellow coloration adorned with yellow bristles. The thorax displays a uniform yellow hue, while the prescutellar depression, although subject to slight variability in intensity, generally appears as a faint brown shade, never dark brown. The scutellum features brown coloring, bordered by a striking yellow outline. The ventral border of the postnotum is also brown in appearance.

The arrangement of acrostichals is consistently biseriate throughout, adding to its distinctive characteristics. In the basal half, the dorsocentrals are short and multiseriate, followed by a row of six uniseriate dorsocentrals, which gradually lengthen toward the scutellum.

The legs of this species are primarily yellow, with a notable exception: the fore tarsus possesses entirely black apical three tarsomeres, while the hind femur exhibits a darkened ventral surface. The fore coxa bears pale hairs on its basal half and transitions to black hairs on the apical half. Additionally, there are three prominent black bristles on the side and six at the tip of the fore coxa. Tarsomeres four and five display a dorsal comb of black squamiform bristles, with the

longest ones situated at the base of tarsomere four and decreasing in length toward the tip of tarsomere five.

The wings of this species are tinged with a brownish hue, with the anterior third appearing darker brown than the posterior portion. A distinctive feature is the strong rectangular bend in vein M. Finally, the squama is yellow with a brown border, adorned with long yellow setae, completing the unique characteristics of this species.

The haltere exhibits an anteriorly positioned dusky yellow knob, while the remainder of the structure appears almost entirely white. Moving to the abdomen, the first segment has a complete yellow coloring. On tergite two and three, the base, tip, and lateral aspects are characterized by a yellow hue, contrasting with the brown to black shading along the remaining portions. Tergite four presents a slender yellow band solely at its base and tip, while the sides adopt a brown shade. The fifth tergite displays a dorsal coloring that appears yellowish white, featuring a minor brown patch along its lateral region. Notably, the cercus displays a distinct white hue (Figure 1A). However, this newfound species exhibits notable morphological distinctions, particularly evident in the palpus. The palpus showcases an elongated structure with a light brown coloration, accompanied by bristling. Furthermore, the thorax displays a light brown hue, punctuated by a distinctive brown prescutellar patch. The ventral surface of the hind femur is adorned with a yellow coloring, while the wings uniformly exhibit a transparent brownish tinge.

The molecular analysis of the DNA sequence from the unknown species provided valuable insights into its genetic relationships. The species demonstrated a 90.06% similarity to *Cyrtona* sp. and *Neurigona zhejiangensis*, with a notable 10% variability in its DNA sequences, confirming its distinctiveness. Through the Neighbor-Joining (N-J) method and phylogenetic analysis, the species was shown to be closely affiliated with two distinct *Neurigona* species, clustering as a unified group. This clustering further strengthens the hypothesis that the unknown species falls within the *Neurigona* genus.

To establish the identity of the unknown dolichopod, DNA barcoding techniques using mitochondrial CO1 as the gene marker were applied. Both the "best match" approach and tree-based identification methods were employed. Although no exact matches were found using the "best match" approach, the species exhibited a striking 90.06% similarity to *Cyrtona* sp. and *Neurigona zhejiangensis*. The tree-based identification, using the N-J method, yielded a cluster of closely related species sharing 88-90% similarities, among which the unknown species aligned itself most closely with two *Neurigona* species. These combined results conclusively support the placement of the unknown species within the *Neurigona* genus. Thus, amalgamating the tenets of traditional taxonomy with insights gleaned from

molecular investigations, has unveiled this species as a distinct and hitherto unrecorded member of the *Neurigona* genus.

Through a meticulous scrutiny of its morphological attributes and a robust molecular analysis, the recently uncovered species from Bohol Island as *Neurigona susanrocesae* sp. nov is confidently designated. This chosen appellation serves as a tribute to the illustrious legacy of Jesusa Sonora Poe, affectionately known as Susan Roces, the revered "Queen of Philippine Movies". Roces, who adorned the silver screen with her presence in more than 130 films during her seven-decade career, garnered numerous accolades, including the prestigious "Lifetime Achievement Award" from the Film Academy of the Philippines. Beyond her status as a celebrity, Roces exemplified modest living and philanthropy through her support of various charitable endeavors, including the Rivers of Living Water Catholic community, Carmelite Sisters, and the Movie Workers Welfare Foundation (Mowelfund). As the widow of presidential candidate Fernando Poe Jr. and the mother of Senator Grace Poe (19th Congress), Roces left an indelible mark on Philippine society. Her passing on 20 May 2022 at the age of 80 adds a poignant context to the naming of this new species in her honor.

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ETHICAL CONSIDERATIONS

Field collection was approved and later granted permit through the issuance of a gratuitous permit from DENR – Region VII (Wildlife GP 2019-11, series of 2019).

DECLARATION OF COMPETING INTEREST

The authors declare that there are no competing interests to any authors.

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