DIAGNOSTICS OF WILD STINGLESS BEES FROM NORTH EAST INDIA

M Bui*, H K. Singh, Aleminla A O, Avinash Chauhan and G T Behere**

Department of Entomology, School of Agricultural Sciences and Rural Development, Nagaland University, Medziphema 797106, Nagaland
**Division of Crop Protection, ICAR Research Complex for North East Hill Region, Umiam, Meghalaya
*Email: malar2uads@gmail.com (corresponding author)

ABSTRACT

This study is on the identification of wild stingless bee species by traditional and molecular taxonomy. Samples were collected from the previously tagged stingless bee hives at Medziphema, Nagaland and Upper Sabansari, Arunachal Pradesh. Laboratory analysis for taxonomic and molecular studies was carried out during 2016-2018 in the Insect Molecular Laboratory, Division of Crop Protection, ICAR Research Complex for North East Hill Region, Umiam, Meghalaya. Two genera were identified i.e., stingless bee colonies collected from Arunachal Pradesh identified as *Lepidotrigona arcifera* and three specimens from SARS RD, Nagaland were identified as *Tetragonula* sp. I (Black SARS RD and white SARS RD) and *Tetragonula* sp. II (underground SARS RD). The present study has generated a molecular data for the first time for *Lepidotrigona arcifera*.

Key words: *Lepidotrigona*, *Tetragonula*, morphology, *L. arcifera*, DNA barcoding, sequencing, diagnostics

Stingless bees of the family Apidae, subfamily: Meliponinae, tribe: Meliponini. Meliponini includes many genera. The total number of species within the Meliponini is estimated to be about 400 (Silveira et al., 2002). In India, very limited knowledge on stingless bee identification is available. North Eastern Region of India is a faunal hotspot. Many species of stingless bees are found in the northeast India and some of its species are not identified properly due to the overlapping morphological characters. The reliable identification of species is difficult even for expert taxonomist with morphological characters alone. Some genera can easily be separated using published keys (Sakagami et al., 1990; Michener, 2007). But there is a need for DNA based identification with mitochondrial gene cytochrome oxidase subunit 1 (COI) for establishing identity in some (Hebert et al., 2003). Molecular identification using COI gene has the advantage of not being limited by morphological polymorphism, sexual form and life stages of the target species (Asokan et al., 2011). The present study deliberates on the identification of wild stingless bees from the northeast India.

MATERIALS AND METHODS

Stingless bee samples for taxonomic and molecular studies were collected from previously tagged hives. The external features of the tagged hives such as shape and orientation of nest entrances were observed. Entrance funnels being one of the most diagnostic field traits for distinguishing closely related species were also observed along with internal nest structure. Stingless bee specimens were collected from all the hives. Ten worker bees from each hive were collected at the entrance tunnel of the hive by sweeping net. Specimens were preserved in 99% ethanol and laboratory analysis was carried out at ICAR, Umiam Meghalaya. Specimens stored in 99% ethanol were removed from vial and were kept at room temperature for one hour, on blotting paper to drain out and evaporate the excess ethanol. The were mounted, pinned and examined in microscope at the ICAR, Umiam Meghalaya. The pinned specimens were studied with taxonomic keys (Rasmussen, 2013), identified and labelled with all data. Head and thorax, abdomen hind and forelegs, wings were photographed. Rest specimens were preserved 100% ethanol in 10 ml screw cap vials for molecular analysis.

In order to extract the DNA, ethanol preserved specimens were air dried on sterilized blotting paper for an hour at room temperature, and the DNA was extracted from whole adult specimens by Phenol: Chloroform modified protocol of Behere et al. (2007). Specimens having similar morphological characters were grouped and analysed in the groups made. The testing for *Wolbachia* infection was done by *Wolbachia* coat protein genes specific primers. Two pairs of *Wolbachia* genes specific primers viz., wspF and wspR and Wol16SF and Wol16SR were used.
PCR reaction was carried out in a total volume of 10 μl by using 2 μl of template DNA (40-60ng), 5 μl PCR master mixes (2X), 0.5 μl each of forward and reverse primers. PCR profile had initial denaturation of one cycle at 94 ºC for 2 min followed by 35 cycles 94 ºC at 1 min, 50 ºC for 45 sec and 72ºC for 1 min with one cycle of final extension at 72 ºC for 5 min and samples were hold at 10ºC. Gel electrophoresis and gel documentation were performed following the recommended material and Equipment and Reagents. After the completion of PCR amplifications of all the samples, the success of PCR amplification was tested on 1.5% agarose gel. For staining the PCR fragments, a 2 μl of Ethidium bromide was pre mixed in 1.5% agarose gel just before the solidification and mixed thoroughly. The separation of PCR fragments were undertaken at 160 V for 20 min. In addition to the master mix sample, 100 bp molecular ladder/marker was also loaded to determine the PCR product size. After completion of electrophoresis, the images of gels were visualized under UV light illuminator and images were documented in Gel Documentation system (Carestream Gel Logic 212 Pro). Separate PCR reaction was carried out for LepF1/LepR1 and LCO/HCO primer pairs. Both primer pairs target the same region of COI gene. For testing the success of PCR amplification, PCR reaction was carried out in a total volume of 10 μl by using 2 μl of template DNA (40-60 ng), 5μl PCR master mixes (2X), 0.5 μl each of forward and reverse primers.

All the samples were analyzed in sequence analysis software Staden Package (Staden et al., 2000). All the sequences were also checked manually within the software for accuracy. The messy 5' and 3' ends of sequences were trimmed for all the sequences. The Single Nucleotide Polymorphism (SNP) if any within different individuals of same species were detected and followed by further steps of BLASTN. All the analyzed sequences were subjected to BLASTN search in online portal of National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The nucleotide blast (nr) option was used for BLASTN search. The species 99-100% homology was considered as similar species.

Multiple sequence alignment of all the nucleotide sequences was performed in the Clustal W software (Thompson et al., 1997). All the sequences were also translated into protein under invertebrate genetic code. Protein sequences were also aligned using Clustal W software. The synonymous and non-synonymous nucleotide substitutions in between sequences were also determined in Clustal W software. The representative sequence of partial COI gene of each species identified in this study was deposited to NCBI and accession numbers of all the submitted sequences were obtained.

RESULTS AND DISCUSSION

The collected stingless bee species were identified based on the taxonomical keys developed by Rasmussen (2013). Thermorphological characters viz., colour of head, presence of thoracic vittae, color and orientation of antennae, overall colour pattern of scutum and abdomen were observed in conformity with those given in Rasmussen (2013). These revealed that the head and thorax are with dense tessellation. Dense plumose hair present at the mesoscutum, hind wing with six hamuli (Fig. 1). It was also confirmed that all the stingless bee colonies collected from the Arunachal Pradesh was Lepidotrigona arcifera (Cockrell 1929) and three species/colonies collected from SASRD Nagaland were identified as Tetragonula sp. I (Black SASRD and white SASRD) and Tetragonula sp. II (underground SASRD). Colonies collected from Arunachal Pradesh are identified as Lepidotrigona arcifera (Cockrell 1929). It is holotype and confirmed by taxonomist Dr. C A Viraktamath, Emeritus Scientist, Department of Entomology, University of Agricultural Sciences, GKVK, Bengaluru.

In the specimens of all the colonies from Arunachal Pradesh, the nucleotide length of final good quality DNA sequence of partial COI gene was 575bp which encoded 191 amino acids. The barcode developed and deposited to the international GenBank NCBI (National Centre for Biotechnology Information) are vide Accession Numbers: MH347232 to MH347236. Among five colonies, the DNA sequences of three colonies (NH2, NH5 and LAH6) were 100% identical. However, colony NH4 has three mutation (Nucleotide polymorphism) and colony LAH5 has two mutations similar except Colony NH4 has three mutation. Even with these three and two mutations within in sequences of colonies collected from Arunachal Pradesh, the overall identity at taxonomic and molecular level did not changed and all these colonies were belonged to L. arcifera because all these mutations were silent mutations. It is worth to note that, the present study has generated a molecular data for the first time for L. arcifera. The molecular data/DNA barcodes generated from this study would be certainly useful to the other researchers working on L. arcifera across the globe. This result is conformity with the findings reported by Rasmussen (2013) who
reported that *L. arcifera* is identified with diagnostic dense tessellation on head and thorax and densely plumose hairs on the margin of mesoscutum. Similar finding was reported by Vijayakumar (2014) that worker bee of *L. arcifera* had usually the mesonotum usually enclosed by a border of short thick scale like or tomentose yellowish to whitish hairs.

Similarly, specimens from three stingless bee colonies were collected from Nagaland, viz., Black SASRD, white SASRD and underground SASRD. Distinct variations in number of hamuli present in hind wings in different specimens were detected. Abdominal variation also observed in white SARSD, black SASRD, and underground SASRD. Difference in structure and color of the entrance funnel was observed in these colonies prominently. White SASRD and black SASRD stingless bee colonies were identified as *Tetragonula* sp. I (Table 1) based on the morphological characters. In present investigations it was revealed that all the specimens collected from Nagaland were having head and thorax with dark coloration. Antennae are ventrally

<table>
<thead>
<tr>
<th>Species/Characters</th>
<th><em>Lepidotrigona arcifera</em> (Arunachal Pradesh)</th>
<th><em>Terragonula</em> sp I (white) Nagaland</th>
<th><em>Terragonula</em> sp I (black) Nagaland</th>
<th><em>Tetragonula</em> sp II (Nagaland)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head and thorax</td>
<td>Dense tessellation present. Densely plumose hair at the margin of mesoscutum</td>
<td>Dark head and thorax. Mesoscutum with gaurous interspace. No setose around and ocelli and mesoscutum.</td>
<td>Dark head and thorax. Mesoscutum with gaurous interspace. No setose around and ocelli and mesoscutum.</td>
<td>Dark head and mesoscutum</td>
</tr>
<tr>
<td>Antennae</td>
<td>Black ventrally</td>
<td>Reddish brown ventrally</td>
<td>Reddish brown ventrally</td>
<td>Dark brown ventrally</td>
</tr>
<tr>
<td>Abdomen</td>
<td>Dark band present. Yellow coloration present at propodeum.</td>
<td>Light brown, three number of band present</td>
<td>Uniform light brown</td>
<td>Dark brown, three lines with illuminated small dots present.</td>
</tr>
<tr>
<td>No. of hamuli</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Colour of entrance funnel</td>
<td>White/ light brown</td>
<td>White/ light brown</td>
<td>Black</td>
<td>Black</td>
</tr>
</tbody>
</table>

Table 1: Morphology of stingless bees of Arunachal Pradesh and Nagaland
reddish brown in all the specimens. Their mesoscutum were having broad glabrous interspaces. However, underground SASRD stingless bee specimen is identified as *Tetragonula* sp. II (Table 1) due to distinct variations in number of hamuli present in hind wings in different specimens, abdominal variation in infuscation, body colour and difference in structure and colour of the entrance funnel. This distinctness was further supported by molecular analysed result. Sequences of white SASRD and black SASRD are 100% identical and hence same species (*Tetragonula* sp. I).

The good quality DNA sequences were successfully obtained for the representative specimens of these three colonies. The nucleotide sequence length for colony; White SASRD and Black SASRD were 666 bp (221 amino acids) and 677 bp (225 amino acids), respectively. In absence of matching molecular data in international GenBank, the molecular identity for White SASRD and Black SASRD could not be established. The identity of these two colonies was established as *Tetragonula* sp. I. The DNA sequences of both the colonies were 100% identical. In case of colony referred as Underground SASRD collected from Nagaland, the nucleotide length of this colony was 666 bp which encoded into 225 amino acids. The multiple sequences alignment between White SASRD, Black SASRD and Underground SARSD revealed that, there was 10% variation at molecular level in case of Underground SASRD. That amount of genetic variation always exists in between species. Taxonomically, the specimens of Underground SASRD colony were unambiguously

Fig. 2. White SASRD, (*Tetragonula* sp. I) Stingless bee of SASRD, Nagaland
Fig. 3a: Antennae  
Fig. 3b: Head and thorax  
Fig. 3c: Abdomen  
Fig. 3d: Hind wing  
Fig. 3e: Fore wing  
Fig. 3f: Hind leg  
Fig. 3g: Overview

Fig. 3. Black SASRD, (Tetragonula sp. I) stingless bee of SASRD, Nagaland
Fig. 4. Underground bee of SASRD, *(Tetragonula* sp. II*) SASRD, Nagaland

Fig. 4a: Over view  
Fig. 4b: Thorax  
Fig. 4c: Abdomen  
Fig. 4d: Head  
Fig. 4e: Hind wing  
Fig. 4f: Fore wing

**L. arcifera**  
Black SASRD T-1  
Underground SASRD T-II

Fig. 5. Nest entrances of different shapes in *L.arcifera*, *Tetragonula* Sp-1 and *Tetragonula* Sp-2
identified as *Tetragonula* sp. II. At morphological and molecular level, Underground SASRD was significantly different from the White and Black SASRD colonies. The DNA barcodes of White SASRD, Black SASRD and Underground SASRD have been successfully submitted to the GenBank (NCBI) vide accession Numbers; MH347237, MH347239 and MH347238, respectively.

All the specimens collected from Nagaland were having head and thorax with dark coloration but underground SASRD specimen has body black in colour while other two are comparatively lighter in body colour (Figs. 2-4). It has also been noticed that white SASRD and underground SASRD have 6 hamuli in their hind wings. Whereas black SASRD has 5 hamuli. Abdomen of white SASRD brownish with three distinct bands and black SASRD specimens having pale brown uniformly. Whereas underground SASRD has dark brown with three distinct bands. In black SASRD, entrance gate bounded by projecting short and stout funnel in dark brown to black. White SASRD colony with white to pale brown funnel entrance gate of 3-4 cm in length. Whereas underground SASRD had blackish entrance gate without long funnel entrance tube (Fig. 5).

The present findings corroborate those of Rasmussen (2013), who reported that antennae of *Tetragonula* spp. orient ventrally mostly testaceous to ferruginous and *Tetragonula* species of the “iridipennis” species group are characterized by having a dark mesoscutum with four distinct hair bands separated by broad glabrous interspaces. Similarly, Rahman et al. (2015) reported that *Tetragonula* spp. have black head and mesosoma and brownish antennae and their head is devoid of pubescence.

Thus it can be concluded that the northeast India is having diversity of stingless bees, Arunachal Pradesh dominating population of *L. arcifera* Cockrell whereas Nagaland is having dominant *Tetragonula* spp. The molecular data/DNA barcodes generated from this study would be certainly useful to the other researchers working on stingless bees across the globe.

REFERENCES


