BIOSYSTEMATIC STUDIES ON STINGLESS BEES (APIDAE: MELIPONINI) OF KERALA

by

FASEEH P

(2016-11-097)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"BIOSYSTEMATIC STUDIES ON STINGLESS BEES (APIDAE: MELIPONINI) OF KERALA"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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20-06-2018

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CERTIFICATE

Certified that this thesis entitled **"BIOSYSTEMATIC STUDIES ON STINGLESS BEES (APIDAE: MELIPONINI) OF KERALA"** is a record of research work done independently by Mr. FASEEH P under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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Dedicated to my parents

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LIST OF ABBREVIATIONS

mm	-millimeter
cm	-centimeter
m	-meter
%	- per cent
HW	-Head width
HTL	-Hind Tibia Length
WL2	-Wing diagonal
IOD	-Inter Ocellar Distance
OOD	-Ocello Orbital Distance
F3	-Length of 3rd Flagellomere
OD	-Ocellar diameter
DNA	-Deoxyribonucleic acid
Fig.	-Figure
et al.	-And others
Viz.	-Namely
sp.	-Species (Singular)
sp. nov	-New species
aff.	-Affinis

Introduction

1. INTRODUCTION

Stingless bees are small to medium-sized eusocial bees belonging to the subfamily Apinae of the family Apidae. They are key pollinators of the tropical and subtropical flora. Stingless bees possess vestigial or under developed stinger and they use mandible as a defensive tool against intruders. They are commonly known as "Dammer bee" and are named "Cheru theneecha" in Malayalam, the local language in Kerala.

Stingless bees originated before the continental drift (Wille, 1983) and are distributed all over the tropics. Neo-tropical realm has the richest fauna of stingless bees and new species are being discovered every year from different regions (Freitas *et al.*, 2009). More than 600 species of stingless bees are known globally (Wille, 1983, Rasmussen and Cameron, 2010).

Stingless bees live in colonies comprising the queen, workers and drones where all the castes are morphologically different as in honey bee(Wille, 1979). The diverse nesting and feeding habits make stingless bees the most diverse eusocial bees in the world. There are nectar and pollen collectors, tear drinkers, sweat drinkers, necrophagous and parasitic types of stingless bees and their nesting habitats are varied. They nest inside crack and crevices, tree hollows, foundations of buildings, termite mounds, wasp nests, ant nests, bird nests, lime stones etc.

Stingless bees are naturally not abundant in temperate world (Amano *et al.*, 2000). Ability to withstand higher temperature, polylecty, year-round activity, easiness of transportation, restricted foraging range (Amano *et al.*, 2000), adaptability to human habitats (Pavithra *et al.*, 2013; Vieiria *et al.*, 2016) and safety to beekeepers make them more suitable to agriculture and greenhouse ecosystems than honey bees.

Stingless bees pollinate a wide variety of trees, agriculture and horticulture crops (Slaa *et al.*, 2006: Norowi *et al.*, 2010), besides being used as efficient pollinators in glass houses in temperate regions of the world.

Changing climate and increased pesticide use have a greater impact on major pollinators such as honey bees and bumble bees, which put pressure on us to find a new better alternative source of native pollinators. Stingless bees are the largest and the most diverse group among social bees. They are well known as the key pollinators of the tropical and subtropical flora (Roubik, 1989), that has gained increasing attention recently. They also resemble honey bees in most of the characters (Polylecty, shows floral constancy, lives in perennial colonies, keeping food reserves etc) (Heard, 1999). They are promising candidates for future commercial pollinators in conservative or protected cultivation (Slaa *et al.*, 2006).

The knowledge of insect biosystematics is very important to map the biodiversity pattern of a particular region or cropping system. The study of melittofauna in different areas is limited, so the taxonomic knowledge of stingless bees in various regions and groups are also limited (Rasmussen, 2013; Anguilet, 2015). Hence there is a chance of encountering novel species from different parts of the world (Rasmussen, 2013). Currently, three genera (*Tetragonula*, *Lisotrigona* and *Lepidotrigona*) comprising 11 species are recognized from Indian subcontinent. Among them, *Tetragonula iridipennis* (Smith) and *Tetragonula laeviceps* (Smith) are common throughout India (Rahman *et al.*, 2015). However, bulk of the diversity remains unknown to science. Stingless bee resource of India is less studied and poorly understood as most of the land area is poorly sampled (Rathor *et al.*, 2013). Three species of stingless bees *viz., Lisotrigona mohandasi* (Jobiraj and Narendran), *Lisotrigona chandrai* (Viraktamath and Jose) and *T. iridipennis* have been reported to occur in Kerala. However, presently only two species *viz., Lisotrigona cacciae* (Nurse) and *T. iridipennis* (Smith, 1854) are known to occur (Rasmussen *et al.*, 2017).

The genus *Tetragonula* still remains poorly characterized and taxonomically difficult (Sakagami, 1978). To recognize the species complex of *iridipennis* group, more research has to be conducted on this fauna (Rasmussen, 2013). There is an exponential growth of research papers published on the stingless bees from 1980 to 2010 with a figure close to 120 publications per year from different parts of the world (Rasmussen, 2013). Study on wing morphology showed the existence of patterns of variability in stingless bees of India and the need of thorough investigation of taxonomy and biology of these tiny bees (Francoy *et al.*, 2016). The morphometry and use of molecular analysis as complementary tools will facilitate the study of diversity, ecology, and biology of the stingless bees.

Nest entrance of stingless bee differs with genus (Kelly *et al.*, 2014) and attributes of nest structure and nesting habits can be used as a tool for ecological, phylogenetic and taxonomical studies (Lima *et al.*, 2013).

Detailed sampling and data generation after recording morphological parameters and conducting molecular analysis along with the knowledge on the nest characters can be used to exploit the diversity of stingless bees in Kerala. The work on "biosystematics" will strengthen the research efforts in mapping the beneficial stingless bees through systematic characterization. As there exists only very few detailed taxonomic studies on this group from India, the present study on this economically important group was carried out with the following objectives.

- To study the stingless bee diversity of Kerala
- To document the nest entrance architecture of stingless bees.

Review of literature

2. REVIEW OF LITERATURE

The literature pertaining to origin, distribution, diversity and nest architecture of stingless bees are reviewed here under.

2.1. ORIGIN AND DISTRIBUTION

Based on the differences in the nest architecture and anatomical characters such as the dorsal blood vessel, ventral nerve cord, and alimentary canal Wille (1979) divided the Meliponinae into two tribes Meliponini and Trigonini. Reduced wing venation, vestigial sting and presence of penicillium on the outer apical margin of the hind tibia, a unique character of Meliponini differentiate stingless bees from the other bees (Wille, 1983). Stingless bees are native to the new world and exhibit remarkable richness in Neotropics (Camargo and Pedro, 1992).

South America is regarded as the center of origin as it has more species diversity than Africa, Asia, and Australia (Kerr and Moule, 1964) but the evidences such as the wide acceptance of the plate tectonics and continental drift, finding of the fossils of Dammer bees in the Late Eocene in Europe and the presence of a primitive character of more developed sting in the Meliponinae of Africa, supports Africa as the centre of origin of stingless bees (Wille, 1979).

Africa has fewer of species (about 35) as compared to Neotropical region (200-300) which is due to the geographical changes that happened, providing more favorable environmental conditions to stingless bees in the South and Central America than Africa (Willie, 1979). The greatest diversity of stingless bees has been observed in Neotropics and then in the Indo-Malayan region (Camargo and Pedro, 1992).

Wille (1983) reviewed genera and subgenera in stingless bees and proposed a new classification for the subfamily Meliponinae, in which he recognized 8 genera and 15 subgenera.

Nineteen species of African stingless bees were classified into 6 genera *viz*., *Cleptotrigona* Cockerell, *Dactylurina* Cockerell, *Plebeina* Moure, *Hypotrigona* Cockerell and *Liotrigona* Moure recognized by Eardley (2004). Two more stingless bees were added to the Thailand fauna of stingless bees after the identification of *Trigona binghami* (Schwarz) and *Trigona minor* (Sakagami), which were 30 previously (Klakasikorn *et al.*, 2005).

Sakagami and Inoue (1985) described three bicolorous species of *Tetragonula*, *T. sarawakensis* (Schwarz), *T. dreshcheri* (Schwarz) and *T. minangkabau* (Sakagami and Inoue) which is the darker. Dollin *et al.* (1997), redescribed 6 species of Australian stingless bees, *T. clypearis* Friese, *T. sapiens* Cockerell, *T. carbonaria* Smith, *T. hockingsi* Cockerell, *T. mellipes* Friese and *T. fuscobalteata*. Boontop *et al.*, (2008), recorded 11 species from 3 genera of stingless bees in Thong PhamPhum district of Thailand, out of 11 species *Trigona ventralis, T. sirindhornae* (Michener and Boongird), *T. collina* (Smith) and *T. terminata* (Smith) were more frequent. A study conducted by Hamid *et al.* (2016) in urban and forest areas of Penang Island of Malaysia revealed that out of six stingless bees collected, (*Heterotrigona itama, Lepidotrigona terminata, Tetrigona apicalis, T. iridipennis, T. laeviceps, Tetragonula pagdeni*). *Heterotrigona itama* was most abundant and diverse in both urban and forest areas and in a general view, diversity was more observed in the forest ecosystem than in the urban areas. The author also mentioned that *T. iridipennis, T. pagdeni,*and *H. itama* were observed from both forest and urban areas.

2.2. STINGLESS BEES IN INDIAN SUBCONTINENT

Asiatic stingless bee *Trigona (Tetragonula) iridipennis* Smith is one of the most primitive honeybee found in India. The distribution of stingless bees in Indo-

Malayan/Australasian region extends from India to Solomon Islands and from China to Australia (Rasmussen and Camarago, 2008). Tropical climate with varying physiographic environment, higher altitudes, and luxuriant flora suits Indian subcontinent to have a rich and wide distribution of stingless bees (*T. iridipennis*) (Pavithra *et al.*, 2013).

The presence of stingless bees were reported from Haryana (Chaudary and Singh, 2007) Rajasthan (Gupta *et al.*, 2011) and Jammu Kashmir (Rahman *et al.*, 2015) of the North India. Stingless bees also recorded from south Indian states such asKerala, Tamil Nadu, Karnataka, Andhra Pradesh and northeast states Assam, Meghalaya, Arunachal Pradesh, Nagaland and also from Maharashtra which represent the central zone (Rahman *et al.*, 2015). Reports showed that Kani Tribes in the Karayar area of Western Ghats are involved in stingless bee (*Tetragonula iridipennis*) keeping (Kumar *et al.*, 2012).The research works conducted on the diversity of stingless bee in the Indian subcontinent is less known (Makkar *et al.*, 2016).

The most common species of stingless bee found in India was known by the name *Mellipona iridipennis* Smith. The species found in Kerala (Mohan and Devanesan, 1999) was identified by Roubik as *Trigona iridipennis* which is now known as *Tetragonula iridipennis* (Rasmussen, 2013).

Jobiraj and Narendran(2004) described and illustrated a new species of stingless bees *Lisotrigona mohandasi* from Kerala and gave a revised key to the species of *Lisotrigona*.

The *T. iridipennis* and *T. laeviceps* were found to be dispersed in all zones studied in the survey. *Lepidotrigona arcifera* (Cockerell), *Lisotrigona cacciae* (Nurse), *Lisotrigona mohandasi* (Jobiraj and Narendran), *Tetragonula* aff. *laeviceps* (Smith), *Tetragonula bengalensis* (Cameron), *Tetragonula gressitti* (Sakagami), *Tetragonula iridipennis* (Smith), *Tetragonula praeterita* (Walker), and *Tetragonula*

ruficornis (Smith) are reported from India (Rasmussen, 2013). He summarized the distribution of stingless bees throughout the Indian subcontinent and emphasized that additional collection and studies are urgently needed to clearly define the species limits of the complex "*iridipennis*" species complex. *Tetragonula gresetti* was newly reported from Arunachal Pradesh, confirming the need of more extensive studies on less sampled regions of the country in order to exploit new species (Rathor *et al.*, 2013). Rahman *et al.*, (2015) reported two genera of stingless bees comprising of 6 species from India and the study also revealed that out of six species, 5 were distributed well in north east India and four in south India.

Francoy *et al.* (2016) sampled bees from different regions of India and claimed theexistence of patterns of variability in stingless bees of the country. The study also indicated the need of thorough investigation of taxonomy and biology of these tiny beesand reported patterns of variability found in stingless bees in India using wing morphology and opined that, little is known about the stingless bee biology and taxonomy.

Study on genetic diversity in the *Trigona* population reveals the possibility of theexistence of more species in Coimbatore and Sittlinghi in Tamil Nadu, India. A new population which is having a nest entrance almost similar to *Trigona hockingsi* has been observed near Sittlinghi forest in Tamil Nadu (Sriram *et al.*, 2004).

Engel (2000) reviewed the distribution of *L. cacciae* in southeastern countries such as northern Borneo, northern and central Thailand, Cambodia, Malasia, India, Laos, and Vietnam. *L. carpenteri* was reported from low elevations of Vietnam and *L. furva* was known from the central and northwestern regions of Thailand (Engel, 2000). *L. cacciae* was reported from Sri Lanka for the first time (Karunaratne *et al.*, 2017). Recently two new species of stingless bees which belong to genus *Lisotrigona* were reported from India, *L. revanai* and *L. chandrai* (Virakatamath and Jose, 2017) which were synonimised (Rasmussen *et al.*, 2017).

2.3. NEST ENTRANCE ARCHITECTURE

Tree hollows, human constructs, pillars of wooden structures and caves are reported as a common nesting sites for *T. laeviceps* (Sakagami *et al.*, 1983). Most frequent nesting sites of stingless bees are reported as cavities of trees, crack and crevices in mud and stone walls. Tree hollows and wooden pillars are also serving as a nesting site for *T. carabanaria* (Heard, 1988). *Trigona cilipes* was found to be associated with the nest of social wasp *Epipona tatua* (Hymenoptera; Vespidae), which are normally observed to share their nest with aerial nests of *Azteca* ants (Rasmussen, 2004). In Thailand, termite mounds are found to be the most common site for the nesting of *T. collinae* followed by subterranean cavities and tree cavities, which accounted for 42.6 per cent, 33.75 per cent and 15.63 per cent nesting respectively (Jongjitvimol and Wattachaiyingcharoen, 2007). Couvillon *et al.* (2007) mentioned that stingless bees construct species-specific nest entrances. Studies on attributes of nesting habitat and nest structure can be used for better understanding of taxonomy, phylogeny, and ecology of stingless bees (Lima *et al.*, 2013).

Eltz *et al.* (2003) reported that around 86 per cent of the total stingless bee community and 97 per cent of the *T.collinae* are associated with the living trees of dipterocarp forest in Sabah, Borneo. Rasmussen and Camarago (2008) reported four different kinds of nesting habitats in *Trigona* sp; exposed nest, association with termite colonies, subterranean colony, association with wasp and ant colony, semi-exposed in tree cavities. The nest of *T. iridipennis* is restricted to narrow closed space so that they can arrange clustered brood cells (Danaraddi *et al.*, 2012). Nesting attributes of *T. iridipennis* show that they prefer medium sized opening than large and small opening and oval-shaped mouth over the circular and irregular type. Even though they use grease, resin, wax, wooden piece, mud, tar, blue paint, pollen, stone, cow dung, animal feces etc, most common is resin, mud, and wax (Pavithra *et al.*,

2013), 83.4 per cent of nests were constructed in the interiors like human inhabited areas (quarters, offices, educational institutions etc.) than that constructed in the exterior area (facing towards the road) (Pavithra *et al.*, 2013).

Siqueira *et al.,* (2012) mentioned that hollow tree cavities act as a nesting site for 64 per cent of the nests distributed in Araguari river valley in Minas Gerais, Brazil. Most frequent nesting site was observed to be hollows of trees during an assessment of 6 different stingless bees *Tetragonisca fiebrigi, Scaptotrigona depilis, Partamona cupira, Oxytrigona tataira* and *Tetragona clavipes* (Lima *et al.*, 2013). Virkar *et al.* (2014) reported that, nesting habitat of *T. iridipennis* ranged from cracks, crevices of trees, walls, and even underground nests. Roopa *et al.* (2015) reported hollows of tree trunks old wall cavities, old pipes and termite mounds as nesting site of *T. iridipennis* and also observed that congregation of the nest was found more in old buildings than tree cavities. Most of the colonies of the *T. laeviceps* were found to be in the wall cavities (85.29%) followed by tree cavities (8.8%) followed by pole cavities (5.88%) (Patel and Pastajia, 2016).

Chinh *et al.* (2005) observed small nests of *L. carpentere* in manmade structures and nests of *T. laevicepes* and *T. ventralis* in living tree cavities. Nests of *L. chandrai* were observed in the foundation wall, tree cavities and laterite rocks of Kerala (Viraktamath and Jose, 2017).

Even though nest architecture is important parameter it cannot be considered as sufficient criteria for species differentiation in *Trigona* groups (Franck *et al,* 2004), but it can act as a supporting information for taxonomic and social evolution (Couvillon, 2007).

2.3.1. Nest Entrance

Roubik (2006) considered nest entrance as an important character as it indicates the presence of predators, parasites and symbionts and it also gives information on the age of the nest as well as the microclimate inside the colony.

Banziger *et al.*, (2011) reported *Pariotrigona klossi* from calcareous rocks of Thailand where the nests were having unique character of dozens of entrance tubes for a single colony and they differed with ornamentation (coral-like appearance) and lack of blackish entrances compared to other minute bees such as *L. cacciae, L. furva, L. carpenteri* and *T. fuscobalteata*. They also mentioned that no other stingless bees were reported constructing complex next entrance with dozens of entrance tubes.

The *Tetragonula* group showed differences in their nest entrances within the genus (Kelly *et al.*, 2014) as well as within the species (Suriwanto *et al.*, 2017).

2.3.2. Number of Guard Bees

Guard bees are recognized by their unique posture and behavior and they are found on the mouth of nest entrance, allowing only the inward movement of foraging bees by moving back to facilitate entry of arriving bees. Couvillon *et al.* (2007) reported that, the colony having more number of guard bees will attack intruders even for slight disturbance but at the same time colonies with one or two guard bees were observed to be timider and they retreat in to the nest while disturbed. They also noted that, with an increase in the number of guard bees, the bees have a tendency to be more aggressive.

Roubik (2006) and Nkoba (2012) reported a positive correlation between the number of guard bees and size of the entrance apex for bee trafficking.

Nunes *et al.* (2008) verified the ability of guard bees to take a decision on acceptance and rejection of nestmates and non-nest mates by recognizing the cuticular cues. The number of guard bees in a colony differed according to the time of

a day and seasons with more guard bees in honey flow season than dearth period (Jose, 2015).

A study conducted by Jayalekshmi (2015) showed that the mean number of guard bees ranged from 6 to 9 in *T. iridipennis*. Divya (2016) reported that the mean number of guard bees of *T. iridipennis* were more in Midland location (7.6) than in uplands (6.4).

The nest entrances of the minute stingless bee *Pariotrigona klossi* was found having several minute entrances for a single colony, where each entrance was guarded by a single bee (Banziger, 2011).

2.3.3. Design of Nest Entrance

The shapes having larger entrances avoided or limited overlapping during take-off and landing of forager bees, they also showed increased defensive behaviour (Biesmeijer *et al.*, 2007).

The shape of nest entrance varies as long tube with longer than wide, short tube wider than long and sometimes even without a nest entrance tube (Rasmussen and Cameron, 2007).There is no phylogenetic association for three characters, the shape of nest entrance, coprophily and necrophagy (Rasmussen and Camerago, 2008).

Couvillon *et al.* (2007) noted toad-mouth nest entrance as an extraordinary design which better manages bee's defensive nature as well as foraging traffic. Pavithra *et al.* (2013) reported that, *T. iridipennis* prefers entrance shape of oval, over irregular and round opening. The nest entrance was widest and mount shaped in case of *Trigona thoracica* of Malasia compared to 4 other species *viz.*, *Trigona itama*, *Trigona thoracica*, *Trigona terminata*, *Trigona laeviceps*, and *Hypotrigona scintillans* (Kelly *et al.*, 2014). The nest entrance of all colonies *of L. arcifera* observed by Vijayakumar (2014) was less round and trumpet at the apex.

Suriwanto *et al.* (2017) observed that, nest entrance of *T. biroi* and *T. sapiens* varied from mount-like, round-ringed and funnel shape with some color variation, whereas *T. fuscobalteata* nest was funnel-shaped with soft rigidity and funnel-shaped nests of *T. laeviceps* were characterized by hard rigidity.

2.3.4. Length and Width of Entrance Mouth

Biesmeijer *et al.* (2007) reported that, there was a positive relation between colony size and nest entrance size. He also mentioned that the size of nest entrance is a settlement or an agreement between colony size, defense, foraging traffic, and nest microclimate. They categorized stingless bees into three groups based on nest entrance size as a defensive colony, nest which shows any level of defense and as timid colony. As the size of the nest entrance increases it is easy for the predator and parasites to identify or recognize the stingless bee colony and thereby they increased the defensiveness in large entrance colonies.

Strong colonies with higher traffic show a positive correlation with the width of the entrance mouth, and those having a large mouth entrance have more number of guard bees for defensive purpose. Hence those nests having smaller entrance mouth can easily defend themselves (Couvillon *et al.*, 2007).

Sakagami *et al.*, (1983) observed downward directed, apically slightly tapering entrance having a length of 4-5cm in *T. laeviceps*, with the smooth inner surface whereas outer surface was rough and even. Less sticky circular entrance was found as commonest. The entrances of nest found in the hollow tree cavities have a small orifice (Rasmussen and Camarago, 2008).

Danaraddi and Viraktamath (2009) recorded width of entrance tube as 3.32 mm in tree cavities and 5.08 mm in the walls for *T. iridipennis* group. Pavithra *et al.* (2013) reported that *T. iridipennis* prefers entrance mouth of dimension 0.8 cm to 1.4 cm. The nest entrance of *T. iridipennis* ranged from 6-18 mm in length and 3-14 mm

in width (Roopa *et al.*, 2015). The reports of Jose (2015) showed the diameter of *T. iridipennis* nest entrance ranged from 1.03 to 3.13 cm.

Length and width of entrance mouth reported as 1.53±0.47 cm and 2.04±0.69 cm in *T. itama*, 3.97±1.29 cm and 4±0.92 cm in *T. throracica*, 1.84±0.38 cm and 1.96±0.1 cm in *T. terminata* and 1.85±0.35 cm and 2.75±0.75 cm in *T. laeviceps* (Kelly *et al.*, 2014).

Banziger *et al.*, (2011) reported minute root like nest entrances of *Pariotrigona klossi* having nest entrance size of 2.5 mm to 3 mm which makes them more groom defends against intruders, and they manage foraging traffic by the construction of multiple entrances for a single nest.

An average length of 5.8 mm having a trumpet appearance with the entrance wider than base has reported in *Lepidotrigona ventralis* nest (Vijayakumar, 2014).

The outer diameter of nest entrance was reported as 0.48-1.1 cm in *L. carpenteri* and 1-4.2 cm in *T. laeviceps* (Chinh *et al.*, 2005).

2.3.5. Length of Entrance Tube

Sagakami *et al.* (1983) reported entrance tube length of *T. laeviceps* as 2-5 cm with a maximum of 10 cm. Starr and Sakagami (1987) observed simple, dark brown, smooth entrance tubes in *T. sapiens* and *T. fuscobalteata*. The external entrance tube length varied from 1 cm to 5 cm in *L. carpenteri* and 2-15 cm in *T. laevicepes* (Chinh *et al.*, 2005). Danaraddi and Viraktamath (2009) recorded length of entrance tube to be 10.88 cm in tree cavities and 9.66 cm in the walls for *T. iridipennis* group. Roopa *et al.* (2015) reported that, the length of entrance tube of *T. iridipennis* ranged from 0.6 cm to 1.8 cm and width of the same ranged from 0.3-1.4 cm in old wall cavities. Length of entrance tube observed by Jose (2015) was in a range of 1.06 cm to 10.26 cm. The study conducted by Ramya *et al.*, (2015) indifferent agro climatic zones of Karnataka showed that, the length and width of nest

entrance tube of *T. iridipennis* vary from 0.5 cm to 9 cm and 0.3 cm to 1 cm respectively.

Kelly *et al.* (2014) reported the entrance tube length in different species as 7.84±7.39 cm in *T. itama*, 7.38±3.65 cm in *T. throracica*, 7±2.02 cm in *T. terminata* and 4.25±1.75 cm in *T. laeviceps*. Length of entrance tube of *T. laeviceps* in pole cavities was found to be very short as compared to the entrance tubes in the wall cavities and tree cavities (Patel and Pastajia, 2016). Suriwanto *et al.* (2017) noticed average nest entrance length of *T. biroi* as 2.23±2.52 cm, *T. sapiense* 1.88±0.99 cm and *T. fuscobalteta* as 3.70±3.88 cm.

2.3.6. Height from Ground Level

The nest entrance of *T. collina* was reported at a height of 1-90 cm from the ground level (Jongjitvimol and Wattachaiyingcharoen, 2007). Danaraddi and Viraktamath (2009) reported *T. iridipennis* group nest as having being found at a height of 22.26 cm in tree cavities and 19.26 cm height in wall cavities.

Lepidotrigona ventralis nest was observed 1-6 m above the ground level mostly in tree cavities with thin-walled dull white to yellow colored entrance tube (Vijayakumar, 2014).

An entrance tube of *L. carpenterei* was found at an average height of 0.9 m from the ground level and the highest observed were 2.5 m above the ground. A nest of *T. laeviceps* was found 2-4 m above ground level (Chinh *et al.*, 2005).

2.4. MICROMETRY

The genus *Tetragonula* is recognized by posteriourly projected mesoscutellum over the propodeum and presence of sericeous area on the metabasitarsus (Sakagami, 1978, Engel *et al.*, 2017., Rasmussen, 2017). Engel *et al.*, (2017) mentioned that the

stingless bees of Asia are closer and very difficult to identify and they differ in minute aspects of size, coloration and setation.

Most of the morphometric works are carried out through the morphological study of worker bees (Wille, 1979; Rasmussen, 2013; Anguilet *et al.*, 2015). The morphometry and use of molecular analysis as a complementary tool will facilitate the study of diversity, ecology, and biology of the stingless bees (Anguilet *et al.*, 2015).

The study conducted in Kerala by Devanesan *et al.* (2003) considered 18 morphometric characters of the queen and worker bees of *T. iridipennis*. The body length of *T. iridipennis* in Tamil Nadu varies from 5.8 mm in Sittlinghi and 4.8 mm in Coimbatore (Sriram *et al.*, 2004). The mean body length of worker was mentioned as 4.07 mm and that of the queen was 10.07 mm. The body size of stingless bees collected from hilly zone was bigger than that collected from plains of Karnataka (Kuberappa *et al.*, 2005).The body length of *T. iridipennis* varies from 3.93 mm to 4.12 mm in parts of Karnataka (Danaraddi and Viraktamath, 2009; Danaraddi *et al.*, 2012). Research conducted in Punjab showed that the body length of *T. iridipennis* differd from 3.59 to 3.69 mm (Makkar *et al.*, 2016). The study conducted by Tej *et al.* (2017) revealed that the length and width of bees collected from the Coimbatore district were comparatively larger than that from Erode and Tiruppur districts of Tamil Nadu.

Rasmussen (2013) observed that the length of the body varies from 4.70 mm in *L. arcifera*, 3.55 mm in both *T. iridipennis* and *T. bengalensis*, 3.45 mm in *T. ruficornis*, 3.33 mm in *T. praetereta* and 2.90 mm in *L cacciae*. Rahman *et al.* (2015) reported body length of *T. iridipennis* as 3.57 mm, *T. bengalensis* as 3.75 mm, *T. praeterita* as 3.27 mm, *T. laeviceps* as 4.04 mm, *T. ruficornis* as 3.43 mm and *L. arcifera* as 3.48 mm from India. Suriwanto *et al.* (2017) reported that body length of four different stingless bee species in Indonesia ranges from 3.47-3.54 mm in *T.*

fuscobalteata, 3.40-3.43 mm in *T. laeviceps*, 4-4.17 mm in *T. biroi* and 3.69-3.80 mm in *T. sapiense*.

Vijayakumar (2014) noted that the body size of *L. ventralis* ranged from 4.2-4.5 mm. The body length of *L. chandrai* was recorded as 2.78 mm, and *L. revani* measured 2.58 mm whereas the body length of males of *L. chandrai* was found to be 3.01 mm and that of queen was reported as 4.24 mm (Viraktamath and Jose, 2017).

A strong positive correlation of body size and flight distance was observed in 6 species of stingless bees *viz.*, *Cephalotrigona capitata* Smith, *Melipona marginata* Lepepeetier, *Melipona quadrifasciata* Lepeletier, *Trigona spinipes* Fabricius, *Melipona compressipes* Fabricius and *Plebeia droryana* Friese, and it was also observed that the body size has a direct influence on the dispersion capacity of a population (Araujo *et al.*, 2004).

A study conducted on 17 sympatric stingless bees revealed that, 4 species, *T. nitidiventris, T. itama, T. eryithrogastra* and *T. thoracica* having long tongue have hairy and tubular glossae (Nagamitsu and Inoue, 1998).

2.4.1. Head

A study conducted in two populations of stingless bees of Sittlingi and Coimbatore of Tamil Nadu revealed that there is variation in head length in both populations (Sriram *et al.*, 2004). The head width of *T. iridipennis* workers varied from 1.52 mm to 1.61 mm (Danaraddi and Viraktamath, 2009; Danaraddi *et al.*, 2012), 1.5 mm to 1.68 mm (Vijayakumar and Jayaraaj, 2014), 1.59 mm to 1.61 mm (Makkar *et al.*, 2016) 1.60 mm (Rasmussen, 2013).The head is 1.1 times wider than long and compound eyes were 2.7 times longer than wide in *T. gresitti* (Rathor *et al.*, 2013).

The length and width of the head of *T. iridipennis* collected from the Coimbatore district were 1.53 mm and 1.76 mm which was greater than that of Erode district and Tirupur districts of Tamil Nadu. Length and width of the head of bees

from Erode were 1.23 mm and 1.62 mm respectively and were comparatively smaller than the others. Antennal length ranged from 1.67 mm to 1.87 mm from Erode to Coimbatore (Tej *et al.*, 2017).

Rasmussen (2013) reported the head width of *L. arcifera* as 1.89 mm and Vijayakumar (2014) reported that, head width ranges from 1.65 mm to 1.82 mm in *L. arcifera*. Jobiraj and Narendran (2004) recorded the head width and length of *L. mohandasi* as 1.28 mm and 1.08 mm respectively and the study also reported the measurements of scape length (0.389 mm), flagellar length (0.91 mm), eye length (0.86 mm), eye width (0.35 mm), post ocellar length (0.3 mm) and ocellarocular length (0.2 mm). The head width of *L. cacciae* and *L. furvae* ranged from1.08-1.81 mm and 1.25-1.35 mm respectively (Michener, 2007). Rasmussen (2013) reported the head width of *L. cacciae* as 1.19 mm. Viraktamath and Jose (2017) recorded the mean head width of *L. chandrai* as 1.19 mm and of *L. revani* as 1.14 mm and the mean head width of *L. chandrai* males and queen was measured as 1.18 mm. The mean value of other head parameters measured were 0.47 mm (scape length) 1.38 mm (length of antenna), 0.07 mm (length of third flagellomere), 0.10 mm (width of third flagellomere) in *L. revanai* and 0.45 mm (scape length), 1.41 mm (antennal length), 0.07 mm (fourth flagellomere), 0.11 mm (width of fourth flagellomere) in *L. chandrai* (Viraktamath and Jose, 2017).

2.4.2. Thorax

Schwartz (1948) reported that, stingless bees having small size are having fewer hamuli than that of the robust ones and also the bees having more number of hamuli are more stable in their flight and have increased air range. The number of hamuli in the tribe Trigonini normally varies from 5 to 8 (except in *Meliponula*, *T. capitata* and *T. thoracica* where it is 9) and in Meliponini it varies from 9 to 16 in each wing (Wille, 1979). Presence of five hamuli in *T. iridipennis* was reported by Devanesan *et al.*(2003), Kuberappa *et al.* (2005), Danaraddi *et al.* (2009) and Makkar

et al. (2016). The number of hamuli noted by Rasmussen (2013) in *T. iridipennis, T. laeviceps, T. bengalensis* and *T. ruficornis* was five and it was six in *L. arcifea* and *L.cacciae*. A study conducted by Tej *et al.* (2017) in 3 districts of Tamil Nadu (Coimbatore, Tiruppur, and Erode) also reported the number of hamuli in *T. iridipennis* as five. The number of hamuli reported in the workers of *L. cacciae* (Rasmussen, 2013), *L. chandrai* and *L. revanai* were five, but in the queen of *L. chandrai* has four.

Thorax width of *T. iridipennis* ranged from 1.38 mm to 1.61mm in a study conducted throughout Karnataka (Danaraddi and Viraktamath,2009).

Wing length and width of *T. iridipennis* differed from 3.54 mm to 3.78 mm and 1.17 mm to 1.41mm respectively (Danaraddi and Viraktamath, 2009). The wing length including the tegula has found to be ranging from 3.2 mm to 3.9 mm in workers of *T. iridipennis* and wing length of males ranges from 3.1 mm to 3.8 mm (Vijayakumar and Jayaraaj, 2014). The length of forewing including tegula, ranged from 3.73 mm to 3.75 mm in *T. iridipennis* found in Punjab (Makkar *et al.*, 2016). Mean value of length and width of forewing in three different locations of Tamil Nadu were observed as 4±0.07 mm and 1.53±0.02 mm in Coimbatore, 3.38±0.07 mm, and 1.52±0.03 mm in Trippur, 3.32±0.07 mm and 1.17±0.03 mm in Erode (Tej *et al.*, 2017).

Vijayakumar (2014) stated that the length of wing including tegula was in a range of 4.3-4.7 mm in *L. arcifera*. The length of forewing excluding tegula and width of forewing measured was 2.49 mm and 1.04 mm respectively in *L. revanai*. The length of forewing ranged from 2.38-2.60 mm and the width of forewing ranged from 0.92-1.04 mm in *L. chandrai* (Viraktamath and Jose, 2017).

Hind tibial length of males range from 0.96 mm to 1.23mm and that of worker ranged from 1.29 mm to 1.57 mm in *T. iridipennis* (Vijayakumar and Jayaraaj, 2014). The tibial length of hind leg varied from 1.29 mm to 1.57 mm in workers of *T. iridipennis* (Makkar *et al.*, 2016). Tej *et al.* (2017) reported that the length and width of hind tibia varied in Coimbatore (1.62±0.03 mm and 0.49 mm),

Trippur (1.34±0.03 mm and 0.43±0.01 mm) and Erode (1.31±0.04 mm and 0.49±0.01 mm)

Tibial length of stingless bee reported from North-East India (*L. arcifera*) was 1.43 mm (Rasmussen, 2013) and Vijayakumar (2014) recorded it in the range of 1.36 mm to 1.41 mm. The length and width of hind tibia was observed as 0.86 mm and 0.31 mm in *L. cacciae* (Rasmussen, 2013), and length and width ranged from 0.83 to 0.89 mm and 0.28 to 0.30 mm in *L. revanai* and 0.81 mm to 0.92 mm and 0.30 mm to 0.37 mm in *L. chandrai* (Viraktamath and Jose, 2017).

Meta basitarsus was observed to be 1.4 times longer than wide in *T. gresitti* (Rathore *et al.*, 2013). Length and width of basitarsus of *T. iridipennis* from three different locations of Tamil Nadu was recorded as 0.57 mm, 0.42 mm, 0.44 mm and 0.26 mm, 0.21 mm, 0.23 mm respectively (Tej *et al.*, 2017). The mean value of length and width of basitarsus was recorded as 0.35 mm and 0.18 mm in *L. cacciae* (Rasmussen, 2013), 0.38 mm and 0.31 mm in *L. chandrai* and 0.42 mm and 0.23 mm in *L. revanai* (Viraktamath and Jose, 2017).

2.4.3. Abdomen

The width of the abdomen in *T. iridipennis* differed from 1.27 mm to 1.51 mm (Danaraddi and Viraktamath, 2009).

2.4.4. Genitalia

The shape of gonostylus and penis valve is the most reliable character of male genitalia, which is considered as the best diagnostic character for *iridipennis* species complex (Schwarz, 1939; Vijayakumar and Jayaraj, 2017).Vijayakumar and Jayaraaj (2017) reported males of *T. iridipennis* with long and slender gonostyle having apical hairs and a robust penis valve which had almost same length of the gonostyle and tapered only at the apex. Gonostylus having a width of 0.63 mm was observed in case

of *L. chandrai*. Length and width of valves range from 0.50 mm to 0.54 mm and 0.20 mm to 0.27 mm respectively (Virakthamath and Jose, 2017).

Materials and Methods

3. MATERIAL AND METHODS

The present study on 'Biosystematic studies on stingless bees (Apidae: Meliponini) of Kerala' was carried out in the Department of Agricultural Entomology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala, with an objective of studying the stingless bee diversity of Kerala and to document their nest entrance architecture. The materials used, techniques adopted and the observations made for the present study are presented in this chapter.

3.1. STUDY OF STINGLESS BEE DIVERSITY

3.1.1. Collection of Specimen

3.1.1.1. Study Area

A total of 225 colonies were sampled from different parts of Kerala state including every district (Fig 1). Sampling was done in such a way that all the geographic area of the state was covered. Most of the samples were collected from natural colonies in the planes, coastal areas, and hill regions.

3.1.1.2. Habitats

Specimens were collected from foundations of buildings, mud walls, concrete walls, meter boards, raincoat, tree hollows and also from various beekeepers in the state.

3.1.1.3. Time of Collection

Specimens were collected in the months of August, September, October, November and December during the year 2017 and in January and February during the year 2018.

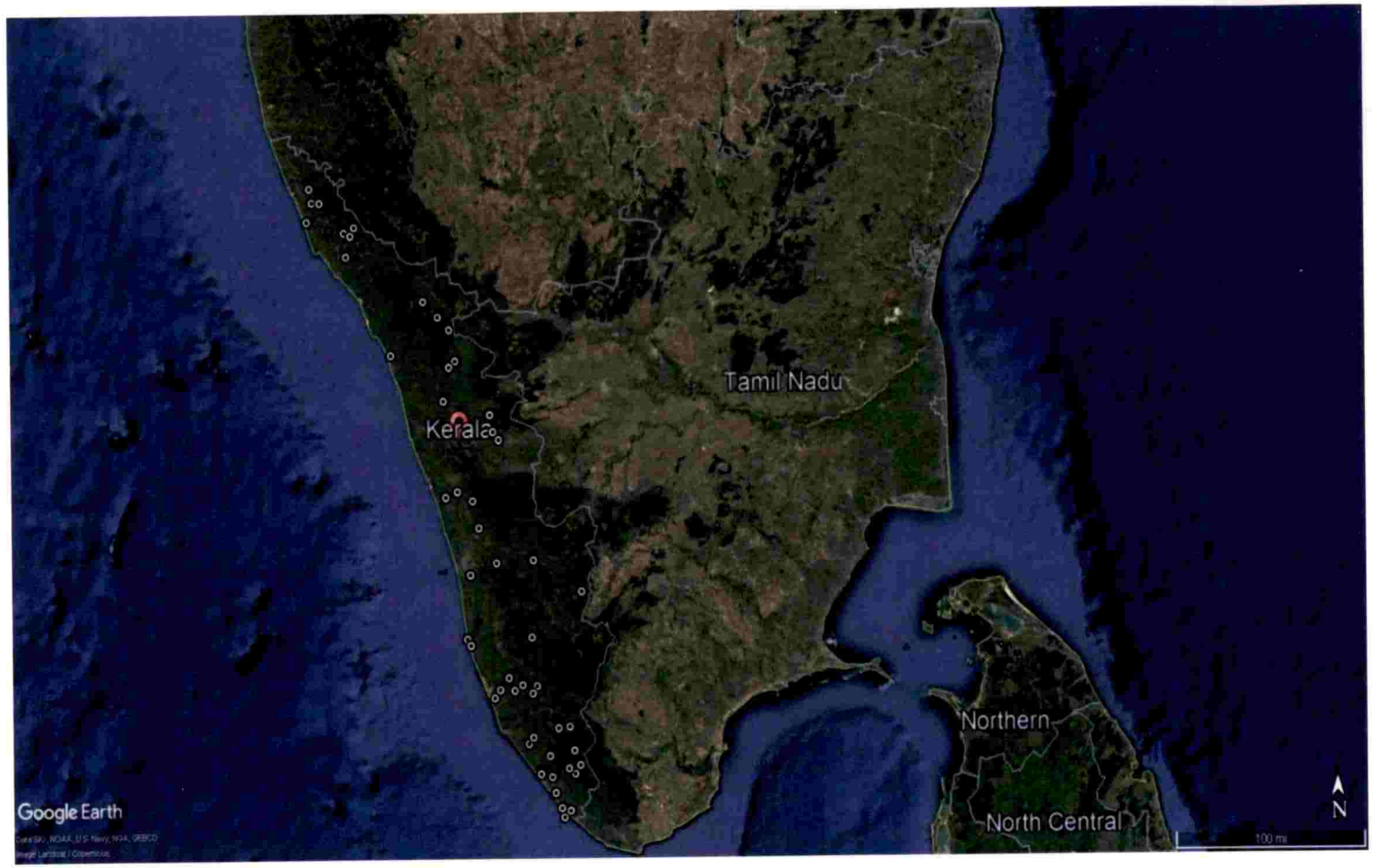


Fig 1. Location of samples collected from Kerala

3.1.1.4. Identification of Feral Colonies.

Natural colonies were located in different areas by observing the movement of bees in bright sunlight, presence of bees in the nearby flora, and by following the stingless bee swarms. Stingless bee farmers are also contacted to identify the nest location.

3.1.1.5. Collection of Specimen

Identified colonies were kept undisturbed and nest parameters *viz* number of guard bees, design of nest entrance, width and length of entrance mouth, length of entrance tube and height from ground level were recorded. Each nest entrance was photographed with a Nikon D80 camera mounted with 55 mm macro lens.

A transparent bottle was kept in front of the nest entrance (Plate 1A) and the bees were collected by gently tapping nearby the nest entrance. The disturbed bees got trapped in the bottle while moving out of the nest entrance. The bees which did not come out even after tapping were collected using aspirator. These trapped bees were killed using cotton dipped in ethyl acetate. Immediately after killing, the bees were transferred into 1.5 ml vials containing 70% ethyl alcohol. The date of collection and location of the collected specimens were labeled using a pencil in white paper strips.

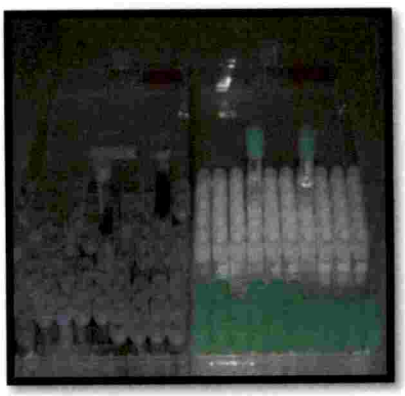
3.1.2. Preservation of Specimens

All specimens were preserved either as dry or wet, and were neatly labeled and stored in insect boxes.

3.1.2.1. Wet Specimen Preservation

The wet specimens were preserved in 70% ethyl alcohol (Plate 1B).

A. Bee trapping method



B. Storage methodology

C. Dry preservation

D. Labelling

Plate 1. Bee trapping and storage of specimen

3.1.2.2. Dry Specimen Preservation

Few of the specimens from each sample were pinned (Plate 1C) using entomological pins of size 000 and also by carding. Specimens were spread in such a way that all possible diagnostic characters were properly visible. The pinned specimens were kept in a hot air oven at 45° C for drying.

3.1.2.3. Labelling

Acid-free paper was used for labeling of the dry specimens. Each specimen was labelled (Font:Times New Roman, Font Size: 5) with country, state, geographical coordinates, sample number, date of collection, and name of the collector (Plate 1D).

3.1.2.4. Storage

Both dry and wet specimens were stored at normal room temperature. Vials containing wet samples were kept in cryo cube boxes (Plate 1B), which could accommodate 100 vials each. Pinned dry specimens were stored in airtight insect boxes.

3.2. IDENTIFICATION OF SPECIMENS

3.2.1. Morphological Characterization

The collected specimens were processed for the study of external morphological characters of mouth parts, antennae, sclerites and sutures of head capsule, characters of leg and genitalia (only for *Tetrgonula iridipennis*). Leica stereoscopic microscope (Model M-165) mounted with DFC90 digital camera was used for taking photographs.

Male genitalia from specimens were dissected out from the abdomen and boiled in 0.5% KOH solution.

3.2.1.1. Preparation of Permanent Slides

Permanent slides of antennae, mouthparts, legs, wings and abdominal segmentswere prepared.

3.2.1.1.1. Clearing of muscles

The specimens were heated in 10% KOH solution on a heat block at low mode for 30 to 40 minutes.

3.2.1.1.2. Passing through alcohol series

After KOH treatment, the specimens were rinsed with distilled water and passed through alcohol series of 70%, 90%, and 100%.

3.2.1.1.3. Dissection of body parts

Various body parts such as head, thorax, legs, wings, tergites and sternites were dissected out in a cavity block containing ethyl alcohol. Fine forceps and pointed needles were used for dissection. Smooth brushes were used for transferring dissected parts to the slides. Proboscis and sternites were stretched with a drop of alcohol on a clean slide and fixed with coverslips to obtain better measurements.

Males were examined by detaching male genitalia and the shape of male gonostyle and penis valve were recorded (Sakagami., 1978).

3.2.1.1.4. Mounting

Dissected body parts were carefully aligned and DPX was used as mounting agent, immediately after which, coverslips were placed over it. The coverslip was gently pressed to remove excess DPX.

3.2.2. Micrometry

A total of 50 different body measurements and 17 body ratios were taken for analysis of specimens. Measurements of various body parts were done with an ocular micrometer attached to the stereoscopic microscope.

3.2.2.1. Head

3.2.2.1.1. Measurements of compound eyes and ocelli

Length of compound eye, width of compound eye, upper interorbital distance, lower interorbital distance, diameter of median ocellus, interocellar distance, ocellorbital distance, length (clypeal apex to vertex) and width of the head capsule, ratio between length and width of head, alveorbital distance, alveocellar distance, alveolar diameter, length of clypeus, maximum width of clypeus, intertentorial distance, clypeocellar distance, length of malar space, length of hairs on clypeus, length of hairs on frons, length of hairs on vertex. (Plate 2)

3.2.2.1.2. Measurements of antennae

Length of antennae, length of scape, the diameter of scape, theratio between length and diameter of scape, diameter of the third flagellomere, length of pedicel+flegellomere, length of the first flagellomere, length of the second flagellomere, length of the third flagellomere. (Plate 3A)

3.2.2.1.3. Measurements of mandible

Length of the mandible, width of the mandible, the ratio between length and width of mandible. (Plate 3B)

3.2.2.2. Thorax

Length of mesoscutum, width of mesoscutum, width of scutellum, length of scutellum, length of hairs on scutellum. (Plate 4C)

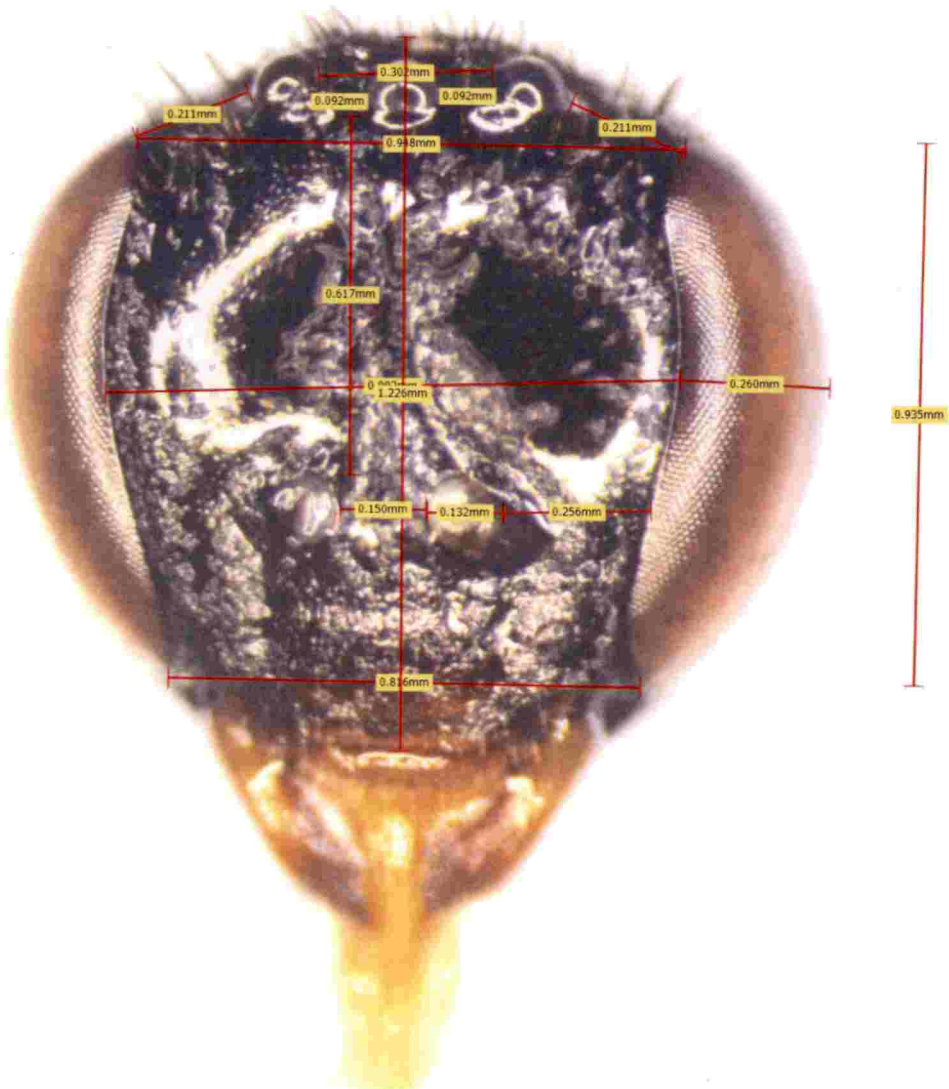
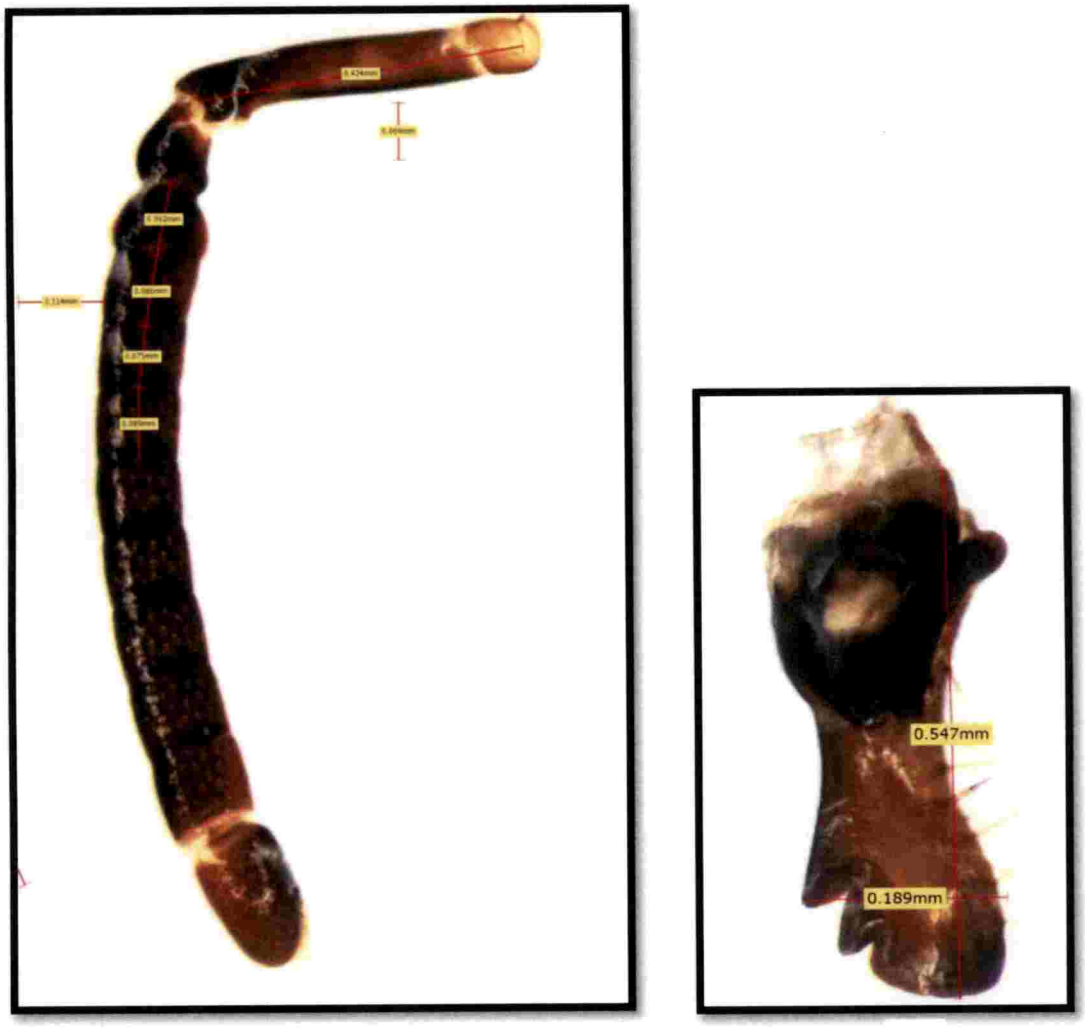
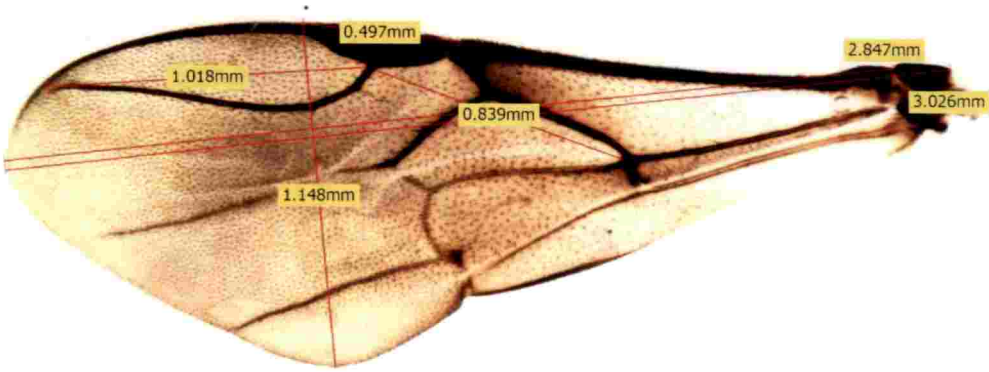


Plate 2. Measurements of head

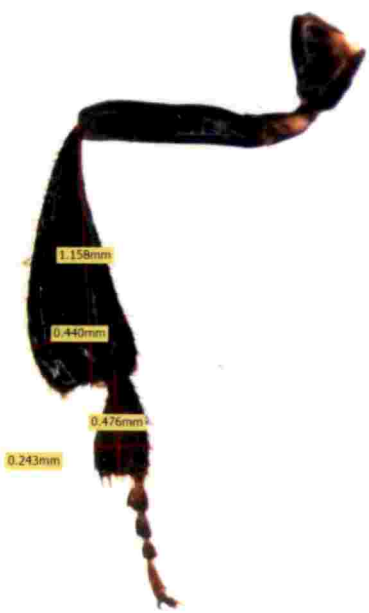


A B

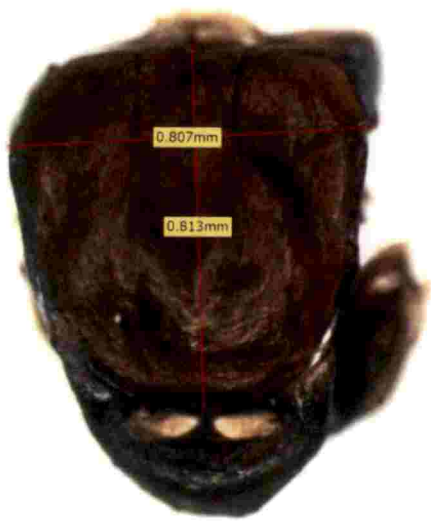
Plate 3. Measurements of Antennae and Mandible



A



B



C

Plate 4. Measurements of wing, leg and antennae

3.2.2.2.1. Wing measurements

Length of forewing excluding tegula, length of forewing including tegula, width of forewing, length of pterostigma, width of pterostigma, ratio between length and width of pterostigma, length of marginal cells, width of marginal cells, length of abscissa of M, length of abscissa of Cu, length of wing diagonal, number of hamuli. (Plate 4A)

3.2.2.2.2. Measurements of legs

Length of tibia III, width of tibia III, ratio between length and width of tibia III, length of basitarsus III, width of basitarsus III (Plate 4B), ratio between length and width of basitarsus, ratio of length of basitarsus III and head width

3.2.2.3. Abdomen

The maximum width of tergum III was measured.

3.2.2.4. Body Ratios

A total of 17 ratios from measurements obtained through morphological characterization are taken to analyse the specimens. They are, ratio between length and width of head (HW), length and width of mandible, length and diameter of scape, length and diameter of pterostigma, length and diameter of tibia 3, length and width of basitarsus 3, basitarsus 3 and head width, wing diagonal (WL2) and HW, length of tibia 3 (HTL) and HW, HTL and WL2, Interocellar distance (IOD) and Ocello orbital distance (OOD), malar space and diameter of 3rdflagellomere (F3), inter alveolar distance and alveolar diameter, IOD and ocellar distance (OD), length of mesoscutum and width of mesoscutum, length of eye and the length of scape were calculated.

3.2.3. Statistical Analysis

The morphometric data were subjected to principle component analysis using SPSS software to know the variation within the stingless bees.

3.2.4. Molecular Characterization

Selected specimens identified through morphometric characterization were subjected to DNA sequencing.

3.2.4.1. Genomic DNA Isolation

Genomic DNA was isolated from the tissues using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

Tissues were placed in a 1.5 ml micro centrifuge tube. 180 µl of T1 buffer and 25 µl of proteinase K was added and incubated at 56°C in a water bath until the tissue were completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. 200 µl of B3 buffer was added and incubated at 70°C for 10 minutes. 210 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

3.2.4.2. Agarose Gel Electrophoresis for DNA Quality check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml

ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad) (Figure 1).

3.2.4.3. PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers.

Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
COX1	LCO	Forward	GGTCAACAAATCATAAAGATATTGG
	HCO	Reverse	TAAACTTCAGGGTGACCAAAAAATCA

(Folmer *et al.*, 1994)

PCR amplification profile

COX1

98 °C - 30 sec

98 °C - 5 sec
45 °C - 10 sec
72 °C - 15 sec
} 10 cycles

98 °C - 5 sec
50 °C - 10 sec
72 °C - 15 sec
} 30 cycles

72 °C - 60 sec
4 °C - ∞

3.2.4.4. Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.2.4.5. ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 30 minutes followed by enzyme inactivation at 80°C for 15 minutes.

3.2.4.6. Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	-	10-20 ng
Primer	-	3.2 pM
Sequencing Mix	-	0.28 µl
DMSO	-	0.30 µl

5x Reaction buffer - 1.86 µl

Sterile distilled water - make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes.

3.2.4.7. Post Sequencing PCR Clean up

1. Master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol were prepared.
2. 12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed.
3. 52 µl of master mix II was added to each reaction.
4. Contents were mixed by inverting and incubated at room temperature for 30 minutes
5. Spun at 14,000 rpm for 30 minutes
6. Decanted the supernatant and added 100 µl of 70% ethanol
7. Spun at 14,000 rpm for 20 minutes.
8. Decanted the supernatant and repeated 70% ethanol wash
9. Decanted the supernatant and air dried the pellet.

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer.

3.2.4.8. Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1. Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1

DNA sequences were analyzed by using CLC Sequence viewer software. Specimens were sequenced to know the intra specific variation in the sequences.

Phylo-genetic tree was constructed using UPGMA (Un-weighted Pair Group Method with Arithmetic averaging).

3.3. NEST ARCHITECTURE

Nest entrance characters of observed colonies were recorded. Characters such as number of guard bees, length of entrance tube, horizontal length (width) and the vertical length of the opening of entrance and height from ground level of natural colonies were recorded.

3.3.1. Number of Guard Bees

A number of individual sitting on the nest entrance (Plate 5C) were counted as guard bees. Nest was kept undisturbed while taking observation and in case of aggressive colonies, a waiting time of 5 minutes was given for the guard bees to settle after which they were counted.

3.3.2. Length of Entrance Tube

A measuring scale of 30cm was used for measuring the length of entrance tube. Measurements were taken from the base of the protruding part of entrance to the tip of the entrance (Plate 5B).

3.3.3. Width of the Entrance

The horizontal length of entrance is considered as the width of the mouth of entrance tube. The maximum horizontal distance was measured using centimeter scale and recorded (Plate 5A).

3.3.4. Length of the Entrance

The vertical length of the entrance (Plate 5A) is taken as the length of the entrance mouth, measured using a centimeter scale.

A. Entrance opening

B. Length of entrance tube

C. Number of guard bees

Plate 5. Observations recorded on nest entrance of stingless bees

3.3.5. Design of Entrance

The shape of the entrance was categorized into heart, oval, slit, square, round, triangular, semi-circular and multiple entrances.

3.3.6. Height from the Ground Level

Height from the ground level was recorded for different colonies.

Results

4. RESULTS

A total of 225 stingless bee (Apidae: Meliponini) colonies were sampled from different locations in Kerala. Species belonging to two genera under the tribe Meliponini *viz.*, *Tetragonula* and *Lisotrigona* were identified during the study. Two species of stingless bees that occur along with the most common stingless bee species *Tetragonula iridipennis* are identified as new to science. This chapter also provides information on new records for the genus *Lisotrigona* in Kerala. Nest entrance characters of the stingless bees encountered during the study are also discussed.

4.1. STUDY OF STINGLESS BEE DIVERSITY

A total of 225 different colonies of stingless bees were sampled from all districts of the state. The sampling altitude ranged from 8 m (Kakkanad, Alappuzha district) to 1064 m (Pampadumpara, Idukki district) above mean sea level.

Two hundred and seven bees collected were identified as *T. iridipennis*, the most common stingless bee of the Indian subcontinent. This species is well distributed through and various geographical areas of the state and were recorded from all districts of Kerala (Fig 2).

A new species *Tetragonula* sp. nov. 1 has been recorded from Chullikkara area of Kasargod district as well as Perunna in Changanaserry of Kottayam district. Another species, *Tetragonula* sp. nov. 2 was collected from 9 different colonies from various locations in Kerala. They were obtained from Thiruvananthapuram, Kollam (Ambanad), Pathanamthitta (Kumbaya), Kannur (Poovam, Manakkadavu) and Kasargodu (Karadukka) districts.These bees were identified as new based on morphometric characters in the keys provided by Rasmussen (2013, 2017) and was verified with DNA sequencing. The distribution patterns of these bees are provided in Fig 3 and Fig 4.

Fig 2. Distribution of *Tetragonula iridipennis* in Kerala

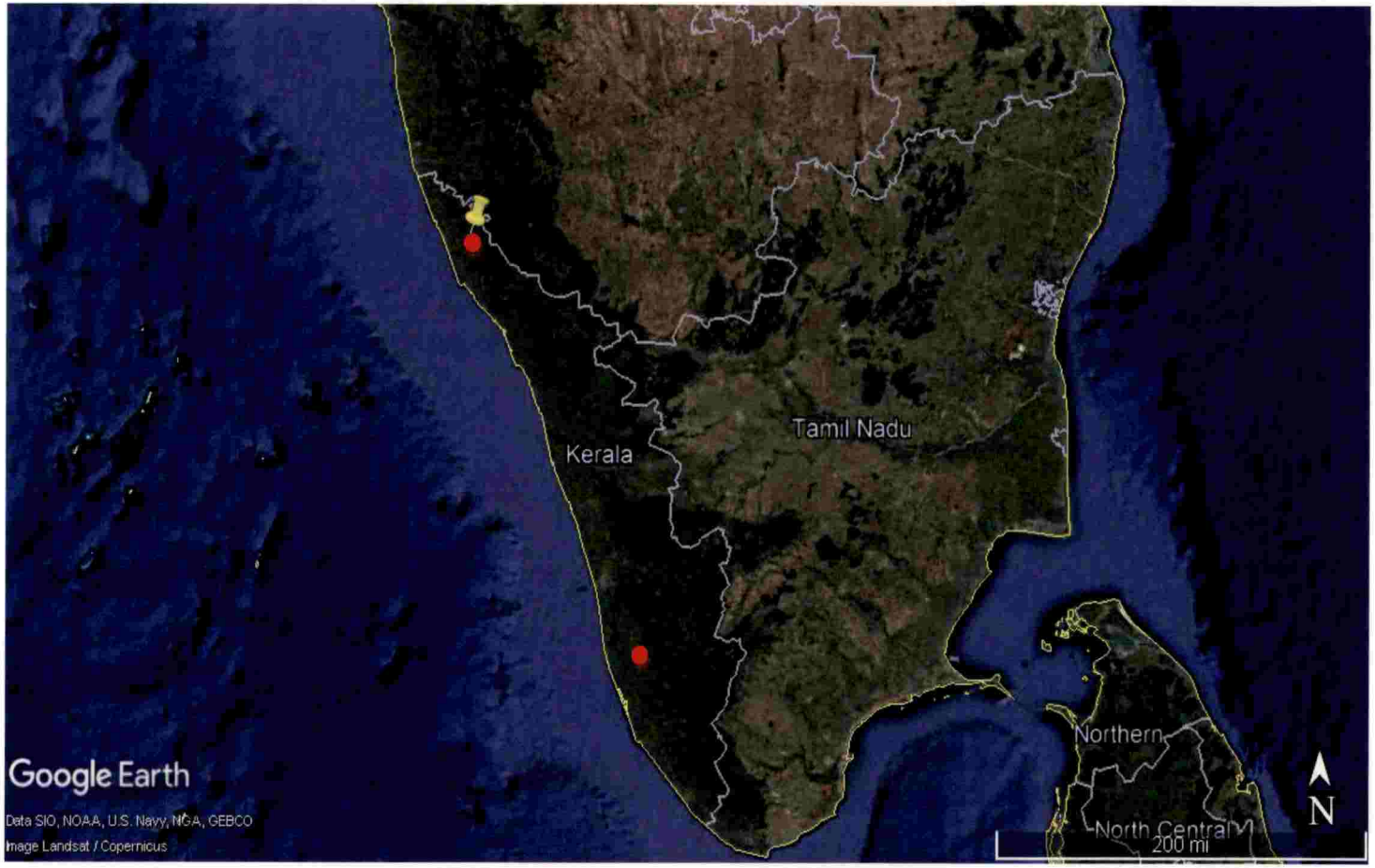


Fig 3. Distribution of *Tetragonula* sp. nov. 1 in Kerala

Fig 4. Distribution of *Tetragonula* sp. nov. 2 in Kerala

It was observed that, the species *L. cacciae* was also well distributed in the northern and central districts of the Kerala (Kasaragod, Kannur, Malappuram, Thrissur), but they did not occur in the southern districts of the state (Fig 5).

All specimen studied are specimens collected were deposited in the Travancore insect collection.

4.2. IDENTIFICATION OF STINGLESS BEES

Stingless bees can be easily distinguished from other bees by noting reduced wing venation, presence of Penicillium on hind tibia and the absence of a functional stinging apparatus. They also lack an auricle in the hind tibia and wax glands of these bees are found on the dorsal side.

It was very difficult to differentiate the specimens at field level as they all look similar in general appearance. However, two genera *viz.*, *Tetragonula* and *Lisotrigona* can be easily differentiated by their body size and nest entrance. There were wide variations in the nest entrances of *Tetragonula* genus but critical examination was required to identify the specimens at the species level.

4.2.1. Morphological Characterization

All the specimens were observed under Leica stereoscopic microscope (Model M-165) to differentiate the bees collected from different locations. The most widely distributed bee *T. iridipennis* could be differentiated based on distinct hair bands with glabrous interspaces.

In general appearance, all the collected specimens look similar to *T. iridipennis*. A critical examination of each specimen was made and the specimens were segregated into three groups *viz.*, *T. iridipennis, Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2 (Plate 3). The body size of the *T. iridipennis* and *Tetragonula*. sp. nov. 1 were similar whereas *Tetragonula* sp. nov. 2 was larger and robust in

Fig 5. Distribution of *Lisotrigona cacciae* in Kerala

appearance. The integument was black in *T. iridipennis* and *Tetragonula* sp. nov. 2 whereas *Tetragonula* sp. nov. 1 was light brown colored. The compound eyes were brown in *T. iridipennis* and *Tetragonula* sp. nov. 1 whereas orange brown in the *Tetragonula* sp. nov. 2.

The hair pattern differed among the three species. In *T. iridipennis*, frons as well as clypeus was densely covered with plumose hairs (Plate 6B), In *Tetragonula.* sp nov. 1 plumose hair on clypeus was denser and they did not obscure the integument (Plate 6D) whereas in *Tetragonula* sp. nov. 2 plumose hairs on the lateral sides were larger and denser than that on the clypeus (Plate 6F).

The colouration of clypeus was different in the three species of bees. It was black in *T. iridipennis* (Plate 6B); reddish brown in *Tetragonul.* sp. nov. 1, (Plate 6D) and with distinct yellow sub apical border in *Tetragonul.* sp. nov. 2 (Plate 6F).

The inner margin of mandibles in *T. iridipennis* and *Tetragonula* sp. nov. 2 are angulated at the basal portion of second tooth and the base of mandible (Plate 7A, Plate 7C), whereas it is more curved in the *Tetragonula* sp. nov. 1 (Plate 7B).

The dissected out mouthparts of the three species were similar in appearance (Plate 7D, Plate 7E, Plate 7F). The scape of *Tetragonula* sp. nov. 2 appeared robust as compared to the other two species.

The mesoscutum of *T. iridipennis* had distinct six hair bands with glabrous inter spaces (Plate 8A). It was observed that, *Tetragonula* sp. nov. 1 was more similar to *T. iridipennis* in hair band formation, but the stout hairs along with plumose hairs were light brown in color (Plate 8B). The hair pattern of *Tetragonula* sp. nov. 2 are distinguishable from the former two bees. In *Tetragonula* sp. nov. 2, plumose hairs were comparatively very few in the hair bands but they were more concentrated on the anterior mesoscutm (Plate 8C). The mesonotum of the *Tetragonula.* sp. nov. 2 is

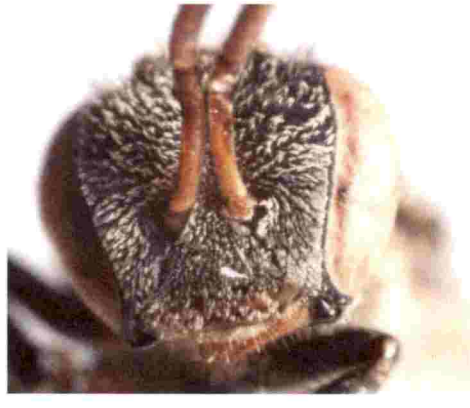
A. *Tetragonula iridipennis*

B. *Tetragonula iridipennis*

C. *Tetragonula* sp. nov. 1

D. *Tetragonula* sp. nov. 1

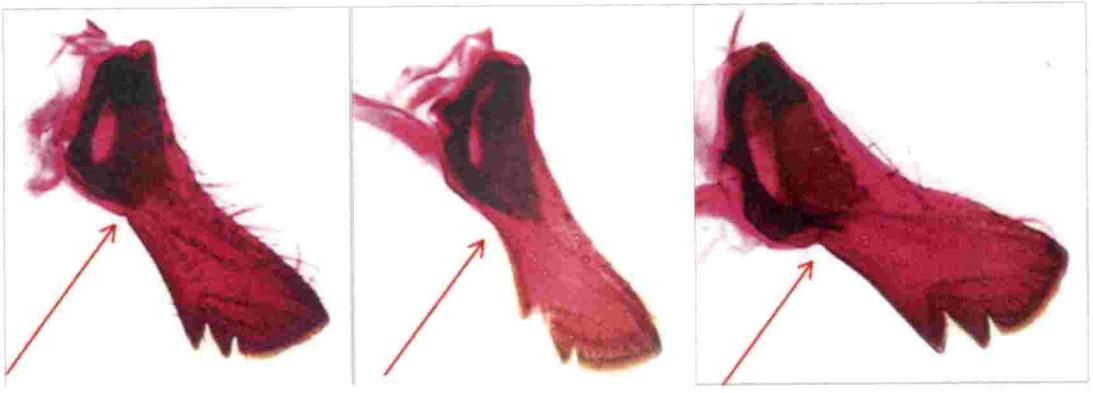
E. *Tetragonula* sp. nov. 1



F. Tetragonula sp. nov. 2

Plate 6. Lateral and anterior view of *Tetragonula iridipennis,*

Tetragonula sp. nov. 1 *and Tetragonula* sp. nov. 2

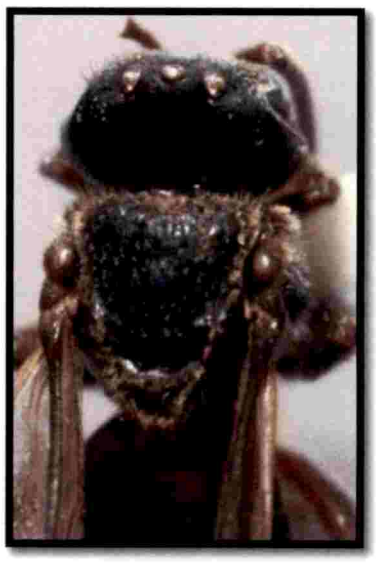


A. *Tetragonula iridipennis* B. *Tetragonula* sp. nov. 1 C. *Tetragonula* sp. nov. 2

D. *Tetragonula iridipennis* E.T*etragonula* sp. nov. 1 F. T*etragonula* sp. nov. 2

Plate 7. Mandibles and mouth parts of *Tetragonula iridipennis, Tetragonula* sp. nov. 1 and Tetragonula sp. nov. 2

A. *Tetragonula iridipennis*



B. *Tetragonula* sp. nov. 1

C. *Tetragonula* sp. nov. 2

Plate 8. Thorax of *Tetragonula iridipennis, Tetragonula* sp. nov. 1

and *Tetragonula* sp. nov. 2

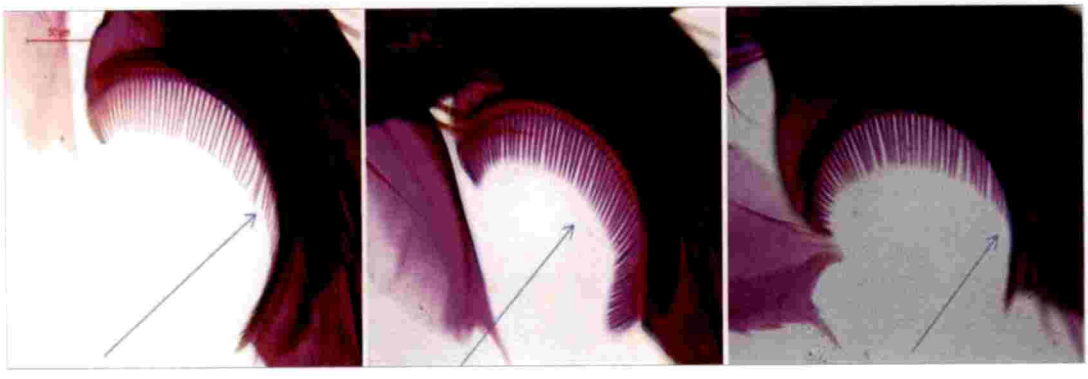
characterized by the presence of more stout black hairs which are distributed widely. These stout hairs were also present in the inter spaces of hair bands.

The lateral view of the antennal comb of the three bees differed. The entire antennal comb of *Tetragonula* sp. nov. 1 was clearly visible in a lateral view (Plate 9B), whereas in *T. iridipennis* and *Tetragonula* sp. nov. 2, the comb was partially visible (Plate 9A, Plate 9C). The difference in the curvature of the antennal comb is clearly observed when slide mounted.

As we couldn't collect the males of the all the three distinct species, we are unable to compare the male genitalial characters. The genitalia of the *T. iridipennis* studied displayed a robust penis valve tapering at its distal end; gonostylus with minute hairs in the apex which were raised from apex of gonocoxite.

Lisotrigona cacciae was recorded from Poovam (Kannur), Kokkadavu (Kasaragod), Erinjeri (Kasaragod), Nilambur (Malappuram), Peechi (Thrissur) and KFRI, Thrissur. Two feral colonies were recorded from Kokkadavu and the feral colony from Poovam was on a Jack fruit tree. Two nests, one live and another abandoned were recorded from Peechi. Few of the bees collected from KFRI Thrissur were getting attracted towards the eyes as well as sweat on hands. The record of four feral colonies from Nilambur (three were on a stone wall and one was on a Teak plant (*Tectona grandis*)) shows the spread of these tiny bees from Kannur, Kasragod and Thrissur district (Previously recorded) to Malappuram district of Kerala.

All the *L. cacciae* specimens collected were very small compared to *Tetragonula* bees; they were black; without hair bands on the mesoscutem and scutellum was not projected posteriourly as in *Tetragonula*. The characters of *L. cacciae* obtained from four different feral colonies are given in Table 1 and the photographs in Plate 10.



A. *Tetragonula iridipennis* B. *Tetragonula* sp. nov. 1 C. *Tetragonula* sp. nov. 2

Plate 9. Antennal comb of *Tetragonula iridipennis, Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2

Table 1. Characters of *Lisotrigona cacciae* collected from four different feral colonies of Kerala

Character	Peechi	KFRI Thrissur	KFRI Nilambur	Kokkadavu
Clypeus	Brown Apically yellow, subapically black. Bordered by plumose hairs	Brown Without yellow. subapically black. Bordered by plumose hairs	Brown Bordered by plumose hairs	Brown Bordered by only yellow margin
Frons	Black	Brown with very less hairs	Black	Black
Compound eye	Orange	Red brown with black coating	Orange	Red brown
Ocelli	Orange with black arch spot on it	Brown	Orange with black arch spot on it	White
Mandible	Orange yellow	Amber color	Light yellow	Amber
Scape	Ochraceous with apical $1/3^{rd}$ black	Brownish with lower $1/3^{rd}$ yellow	Ochraceous with apical $1/3^{rd}$ brown	Ochraceous with apical $1/3^{rd}$ brown
Gena	Imbricate with minute punctuation	Imbricate with minute punctuation	Imbricate with minute punctuation	Minute punctures.
Vertex	With punctuation	With punctuation	With punctuation	With minute punctuation.
Pronotum	Yellow spots on black sclerites laterally	Brown with plumose hairs	Yellow spots on black sclerites laterally	Light brown spot on dark black sclerite.
Mesoscutem	Punctuate	Punctuate	Punctuate	Minut punctuation numerous.
Metanotum	Imbricate	Imbricate	Imbricate and brown	Imbricate

Propodium	Imbricate	Imbricate	Imbricate	Imbricate with minute punctures.
Tegula	Brown and white	Brown and white	Brown and white	White yellow
Coxa	Brown	Brown	Brown	Dark brown
Trochanter	Ochraceous	Ochraceous	Ochraceoys	Ochraceous
Fore tibia	Brown	Brown	Brown	Brown
Hind tibia	Yellow band on keriotrichea region	No yellow band, light brown	No yellow band	No yellow band
Basitarsus	Yellow in inner side	Yellow in inner side	Yellow in inner side	Black with yellow border

A. Anterior view

B. Dorsal view

C. Dorso-lateral view

D. Lateral view

E. Hind leg

Plate 10. *Lisotrigona cacciae*

4.2.2. Micrometry

The mean values of morphological characterization of the three stingless bee species (*T. iridipennis, Tetragonula* sp. nov. 1, *Tetragonula* sp. nov. 2) are given in Table 2. Out of 50 different measurements taken, the mean values of three bees were distinct in head width, intertentorial distance, clypeocellar distance, length of pedicel+flagellomere, length of forewing excluding tegula, length of forewing including tegula, wing diagonal (WL2), width of fore wing, length of marginal cell, length of abscissa of cu, length of abscissa of m. the following ratios are also distinct in three bees, ratio between length and diameter of scape, length and width of mandible, length and width of pterostigma, alveolar orbital distance and alveolar diameter.

The measurement of all body parts were larger in *Tetragonula* sp. nov. 2, whereas body measurements of *T. iridipennis* and *Tetragonula* sp. nov. 1 were almost similar. The ratio between length of tibia 3 and head width length of hind tibia 3 and WL2, IOD and OOD, malar space and f3, inter alveolar distance and alveolar diameter, alveolorbiatal distance and alveolar diameter, inter ocellar distance and ocellar diameter, length of mesoscutum and width of mesoscutum, length of eye and length of scape gave distinct values which can clearly differentiate *Tetragonula* sp. nov. 1 from *T. iridipennis* and *Tetragonula* sp. nov. 2.

4.2.2. 1. Variation of Wing Hamuli

Out of various morphological characters observed, the number of hamuli was found varying within the *T. iridipennis* colonies. A total of 3302 wings from 69 different colonies have been observed. The variations observed in the number of hamuli of *T. iridipennis* are given in Table 3.

Table 2. Body measurements of *Tetragonula. iridipennis, Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2

Body parts	*Tetragonula iridipennis* (mm)	*Tetragonula* sp. nov. 1 (mm)	*Tetragonula* sp. nov. 2 (mm)
Width of head (HW)	1.6	1.53	1.7
Length of head	1.33	1.33	1.54
Length of compound eye	1.06	1.11	1.19
Width of compound eye	0.37	0.38	0.46
Upper inter orbital distance	1.05	1.00	1.14
Lower inter orbital distance	0.89	0.88	1.02
Diameter of median ocellus	0.15	0.14	0.16
Inter ocellar distance (IOD)	0.36	0.35	0.46
Ocellorbital distance (OOD)	0.22	0.21	0.28
Inter alveolar distance	0.16	0.14	0.19
Alveolloribital distance	0.32	0.27	0.27
Alveolocellar distance	0.68	0.69	0.75
Alveolar diameter	0.15	0.16	0.18
Length of clypeus	0.31	0.32	0.35
Maximum width of clypeus	0.71	0.71	0.78
Inter tentorial distance	0.45	0.47	0.51
Clypeocellar distance	0.89	0.93	0.97
Length of malar space	0.05	0.04	0.05
Length of scape	0.55	0.57	0.63
Diameter of scape	0.09	0.08	0.11
Diameter of 3 flagellomere (F3)	0.12	0.14	0.13

Length of pedicel+flagellomere	0.97	1.06	1.17
Length of first flagellomere	0.06	0.07	0.08
Length of 2nd flagellomere	0.11	0.12	0.11
Length of 3rd flagellomere	0.11	0.12	0.12
Length of mandible	0.62	0.6	0.65
Width of mandible	0.16	0.21	0.21
Length of fore wing excluding tegula	3.33	3.28	3.7
Length of fore wing including tegula	3.49	3.55	4.1
WL2	0.97	0.92	1.07
Width of fore wing	1.06	1.19	1.33
Length of pterostigma	0.52	0.54	0.59
Width of pterostigma	0.13	0.13	0.13
Length of marginal cells	1.17	1.22	1.35
Width of marginal cells	0.31	0.31	0.36
Length of abscissa of Cu	0.7	0.74	0.81
Length of abscissa of M	0.56	0.59	0.64
Hamuli	5	5	5
Length of mesoscutum	0.86	0.86	0.93
Width of mesoscutum	1.02	1.05	1.15
Width of scutellum	0.77	0.75	0.8
Length of scutellum	0.32	0.3	0.33
Length of tibia 3 (HTL)	1.42	1.41	1.51
Width of tibia 3	0.5	0.49	0.54
Length of basitarsus 3	0.46	0.49	0.55

Width of basitarsus 3	0.25	0.26	0.27
Width of tergum 3	1.15	1.26	1.35
Length of hairs o clypeus	0.05	0.05	0.05
Length of hairs on vertex	0.1	0.09	0.13
Length of hairs on frons	0.05	0.06	0.05
Length of hairs on scutellum	0.1	0.06	0.48
Length of body	3.6	3.23	3.37
Ratio between length and width of head	1.2	1.16	1.1
Ratio between length and diameterof scape	5.75	7.07	6.25
Ratio between length and width of mandible	3.95	2.88	3.19
Ratio between length and width of pterostigma	4.01	4.22	4.65
Ratio between length and width of tibia 3	2.85	2.9	2.82
Ratio between length and width of basitarsus 3	1.88	1.87	2.02
Ratio of length of basitarsus 3 and head width	0.29	0.319	0.33
WL2/HW	0.61	0.6	0.63
HTL/HW	0.89	0.92	0.89
HTL/WL2	1.46	1.53	1.42
IOD/OOD	1.64	1.68	1.64
Malar space/ F3	0.41	0.27	0.43

Inter alveolar/alveolar diametr	1.07	0.92	1.09
Alveolorbital/alveolar diameter	2.13	1.74	1.25
Inter ocellar distance/ocellar diameter	2.35	2.46	2.38
Length of mesoscutem/width of mesoscutum	0.84	0.82	0.84
Length of eye/scape length	1.93	1.96	1.88

Table 3. Variations observed in wing hamuli of *Tetragonula iridipennis* sampled from various locations of Kerala

Sample No/Location	Altitude (m)	Number of bees examined	Hamuli Pattern							Normal hamuli (%) (5*5)	Variation (%)
			5*5	L5*R6	L6*R5	6*6	L5*L4	L4*L5	4*4		
S69	8	7	6					1		85.71	14.29
S64	14	30	29				1			96.67	3.33
S66	14	36	35					1		97.22	2.78
S67	14	12	12							100	0
S70	14	32	27	2	3					84.38	15.63
S21	15	11	11							100	0
S22	15	24	24							100	0
S44	16	18	17		1					94.44	5.56
S63	19	50	46		2	2				92	8
S62	19	21	21							100	0
Vellayani	30	12	12							100	0

V3	30	30	27	1				2		90	10
V2	30	37	33				4			89.19	10.81
V1	30	24	23	1						95.83	4.17
V7	30	34	34							100	0
Trivandrum	31	12	11					1		91.67	8.33
V4	31	25	24					1		96	4
S46	38	20	19					1		95	5
S25	39	25	22		3					88	12
S1	42	25	23				2			92	8
S2	42	22	22							100	0
S10	42	18	16	2						88.89	11.11
S7	44	25	25							100	0
S4	44	12	12							100	0
S43	45	24	23					1		95.83	4.17
S41	49	18	18							100	0
S39	55	17	17							100	0
S40	55	22	20				1	1		90.91	9.09

Kallumoodu	56	32	32							100	0
S30	64	24	24							100	0
Vembayam	68	18	17				1			94.44	5.56
Bala 7	79	30	30							100	0
Bal 6	79	70	48	9	8	5				68.57	31.43
S28	82	34	31	3		1				91.18	8.82
S50	87	21	20	1						95.24	4.76
S31	91	31	30	1						96.77	3.23
S24	96	28	28							100	0
S51	111	18	17	1						94.44	5.56
S52	111	8	8							100	0
S37	115	29	28	1						96.55	3.45
S19-2	125	35	29				5		1	82.86	17.14
S20	125	17	17							100	0
S29	132	49	36	5	2	6				73.47	26.53
S58	150	18	14	2	2					77.78	22.22
S55	150	8	4	3	1					50	50

S56	150	4	4							100	0
S58	150	15	12	3						80	20
S47	158	21	18	2	1					85.71	14.29
S53	165	41	37		4					90.24	9.76
S54	165	16	16							100	0
S48	171	10	7	2	1					70	30
S61	184	13	13							100	0
Thenmala	189	10	8		2					80	20
Vithurai	226	45	43	2						95.56	4.44
S14	257	20	15	3	2					75	25
S13	257	27	24	2	1					88.89	11.11
S15	257	26	23	3						88.46	11.54
Amb 1	580	12	10		2					83.33	16.67
Amb 2	580	22	20		1		1			90.91	9.091
Amb 3	580	22	19	2		1				86.36	13.64
Amb 6	580	63	23	15	7	18				36.51	63.49
Ambalawayal	694	29	28				1		1	96.55	3.45

Mananthawadi	756	35	33		2					94.29	5.71
Koilandi	880	32	32							100	0
Kannur	972	9	7		2					77.78	22.22
Kasargod	1002	16	15		1					93.75	6.25
Cheriyan		18	16		1	1				88.89	11.11
Belloprar		7	7							100	0
S11		25	25							100	0
Total		**1651**	**1477**	**66**	**49**	**34**	**16**	**9**	**2**	**89.46**	**10.54**

Number of hamuli in *T. iridipennis* varied from 4 to 6. Number of hamuli varied within the colony as well as in the left and right wing of bees. Seven different combinations of hamuli are found in left and right wings of *T. iridipennis* bees, 5 hamuli in left wing and 5 hamuli in right wing (5*5), 5 in left wing and 6 in right wing (5*6), 6 in left wing and 5 in right wing (6*5), 6 in left wing and 6 in right wing (6*6), 5 in left wing and 4 in right wing (5*4), 4 in left wing and 5 in right wing (4*5), 4 in left wing and 4 in right wing (4*4). Out of seven combinations 3 were symmetric pattern, others were asymmetric pattern.

Normal hamuli pattern found in the *T. iridipennis* is, five each in both right and left hind wings. A variation of 10.54 % in the number of hamuli among the 1651 bees was observed. While excluding the symmetric patterns (4*4, 5*5, and 6*6) 8.49 per cent variation in the population observed. Among the six hamuli pattern (other than normal 5*5) 5*6 showed (4%) maximum variation followed by 6*5 (2.97%), 6*6 (2.06%), 5*4 (0.97%), 4*5 (0.55%) and 4*4 (0.121%). The rarest recorded hamuli pattern was 4*4.

Among the 69 colonies observed, the highest variation was recorded from Ambanad (Kollam) 63.49 per cent followed by Balaramapuram (Thiruvanathapuram) 31.42%, Kuranghan chola (Malapuuram) 26.54 per cent. More than one type of hamuli pattern was recorded from a single colony, whereas none of the colonies was recorded with all the type of hamuli.

As there is 10.54 percentage of variation in the number of hamuli among bees of *T. iridipennis*, this character cannot be a reliable indicator for defining the species.

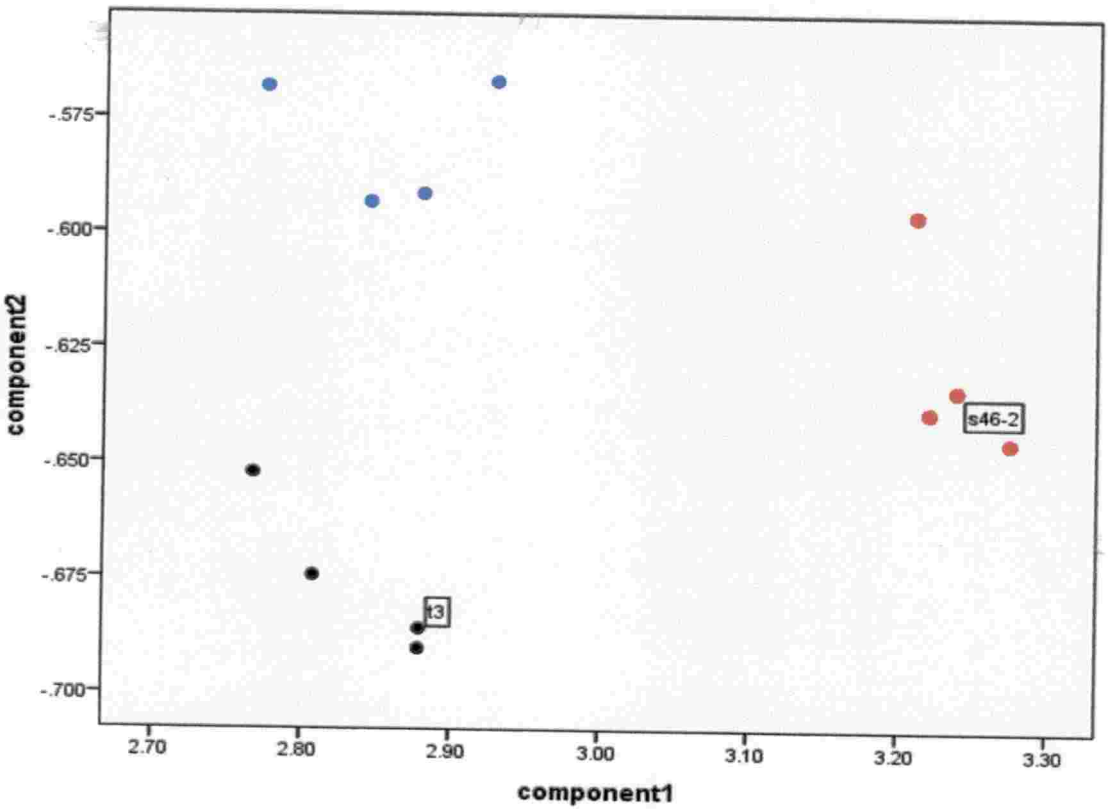
4.2.3. Statistical Analysis

The principle component analysis of the 50 different body measurements of the three *Tetragonula* sp. gave distinct clusters (Fig 6).

4.2.4. Molecular analysis

The observation on the morphological characterization, micrometric analysis and principle component analysis clearly indicated the presence of two more distinct species other than *T. iridipennis* and *L. cacciae* in Kerala. In order to confirm the identity of the bees discovered, the specimens were subjected to molecular analysis. It was confirmed that, the results of DNA sequencing also supports the previous observations.

The partial sequencing of mitochondrial COI gene showed more than 8% variation between the three *Tetragonula* bees. The phylogenetic tree constructed from the DNA sequences of 2 bees from different colonies of *Tetragonula* sp. nov. 2, three bees from different colonies of the *T. iridipennis* and two bees from different colonies of *Tetragonula* sp. nov. 1 is given in Fig 7. The tree reveals that, *Tetragonula* sp. nov. 1 is closely related to the common species *T. iridipennis* whereas *Tetragonula* sp. nov. 2 are distantly related. As boot strap value is more than 90, the data is more reliable. The result of genetic variation at the level of nucleotide base pair also indicates considerable variation in the three bees. The inter-specific genetic variation observed between *T. iridipennis* was in a range of 9.02-9.93 per cent and 19.49 to 20.76 per cent for *Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2, respectively for 554 continuous DNA base pairs. Whereas intraspecific variation was very narrow in each species, *T. iridipennis* (0.54-1.62%) *Tetragonula* sp. nov. 1 (0.38%) and *Tetragonula* sp. nov. 2 (2.71%).



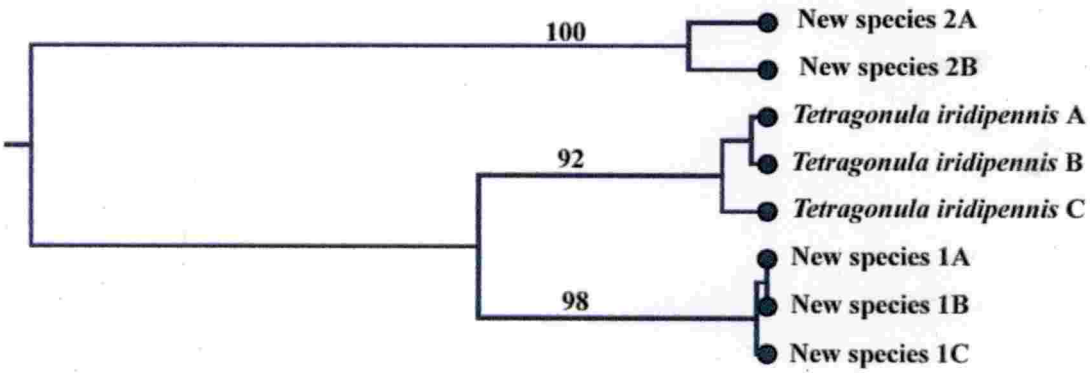
● *T. iridipennis*

● *Tetragonula* sp. nov. 1

● *Tetragonula* sp. nov. 2

Fig 6. Scree plot of PCA of morphometric data of *Tetragonula iridipennis*, *Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2

Fig 7. Phylogenetic tree (UPGMA)



Name	Node type	Branch length	Bootstrap value	Size
	Root	0.00		0
	Internal node	0.11	100	0
New Species2	Leaf	0.01		591
New Species2	Leaf	0.01		691
	Internal node	0.07	100	0
	Internal node	0.04	100	0
	Internal node	4.83E-3	98	0
Tetragonula iridipennis	Leaf	2.62E-3		575
Tetragonula iridipennis	Leaf	2.62E-3		579
Tetragonula iridipennis	Leaf	7.45E-3		580
	Internal node	0.05	100	0
	Internal node	1.71E-3	92	0
New Species1	Leaf	0.00		580
New Species1	Leaf	0.00		617
New Species1	Leaf	1.71E-3		602

4.2.5. Taxon description

4.2.5.1. *Tetragonula* sp. nov. 1

Holotype : Female (worker): Body length 3.25 mm (3.2–3.25 mm); forewing length, including tegula, 3.6 mm (3.4–3.65 mm); head length from anterior margin of clypeus to summit of vertex, in facial view 1.55 mm (1.287–1.386 mm); head width 1.485 mm (1.485–1.617 mm); length of scape 0.576 mm (0.56-0.576mm); length of 3rd flagellomere 0.103 (0.103-0.128 mm); meta tibia length 1.54 mm (1.384–1.435 mm); forewing diagonal from base of vein M to base of cross vein r-rs at margin of pterostigma 1.024 mm (0.923–1.024 mm).

Length of compound eye twice (1.96-2 times) the length of scape, the ratio of inter ocellar distance to the ocellar diameter more than 2.5 (2.43-2.57). Ocello orbital distance over half the inter ocellar distance. Inter alveolar distance slightly less or equal to alveolar diameter. Metasoma slightly flattened, narrower than head, and tapering towards the apex after first two segments.

Ratio between length and width of head 1.16 mm (1.1-1.26 mm), length and diameter of scape 7.07 mm (6.24-7.49 mm), length and width of mandible 2.88 mm (2.76-3 mm), length and width of pterostigma 4.22 mm (3.67-4.57), length and width of 3rd tibia 2.9 mm (2.75-3.11), length and width of basi tarsus 1.87 mm (1.78-2 mm), length of basi tarsus and head width 0.32 mm (0.31-0.33 mm), WL2/HW 0.57-0.69 mm, HTL/HW 0.87-0.95 mm, HTL/WL2 1.38-1.53 mm, IOD/OOD 1.55-1.89 mm, malar space/F3 0.25-0.3 mm, Inter alveolar distance/alveolar diameter 0.9-1 mm, alveolorbital distance/alveolar diameter 1.6-1.89 mm, IOD/OD 2.43-2.57 mm, length of mesoscutum/width of mesoscutum 0.78-0.85 mm, length of eye/scape length 1.94-2.01 mm.

Punctuations on vertex and posterior side of hind legs minute. Shape of clypeus differed. Clypeus concave at distal margin. Bees appear robust and big. Inner sides of mandible curved without an angle between second teeth and point of attachment.

Color: Integument light brown. Head black except at the clypeus which is red brown. Compound eyes are brown. Ocelli are yellow brown. Scape dark brown, pedicel dorsally dark brown ventrally ferruginous, First flagellomere yellow, remaining segments brown dorsally and ferruginous ventrally and with minute hairs and pits. Labrum brown. Mandibles amber colored and with apical dark border. Malar space light brown. Gena black. Neck light brown.

Mesoscutum black, scutellum light brown, hairs on the mesothorax are yellow brown. Tegula and wing sclerites brown. Metanotum yellow brown. Pleuron red brown with minute punctures. Costal vein and pterostigma are dark brown. Wing hyaline iridescent. legs brown anteriourly and yellow brown posteriourly. Basitarsus brown and lightening towards apex in tarsomeres. Penicillium yellow brown. Stout hairs on hind tibia are brown and plumose hairs on it are dull white. Posterior region of tibia with minute punctures. Arolium black and apically white.

Propodium red brown. First abdominal segment is light brown, remaining segments are dark brown. Apical terga brown. Each tergal segments with light border. Last abdominal terga yellow brown with white hairs.

Pilosity: Labrum with long yellow brown hairs and white short hairs. Plumose hairs are densely covered over clypeus and sparsely on frons, they won't obscure the integument. Frontal suture move through alveolar border. Hairs on

vertex are long and light brown. Gena with simple silver hairs. Neck with white simple hairs.

Pronotal knobs with dense white plumose hairs. Mesoscutum with plumose hair bands intermixed with few light brown stout hairs. Hairs on scutellum are brown in upper margin and white in lower margin which are mixed with dull white plumose hairs. Metanotum with white long hairs. Pleural area with silver white hairs. Metapleuron with dense long silver hairs. Propodium glabrous and without anyhairs. Anterior portion of mesopleuron with dense plumose hairs and posterior part with less branched plumose hairs. Trochanter having white spurs. Keriotrichea white brown. Fore tibia with white hairs on anterior side, Lamellate hairs on the posterior side. All hairs on antenna comb of the fore tibia is clearly visible. Middle tibia with mixture of both white and brown stout hairs along with white plumose hairs. Hind tibia with long stout brown hairs, outer margin with white plumose hairs. Keriotrichea on hind femur on a projected platform. White hairs anteriourly on the median line of basitarsus. Basal seraceous area more than half length of basitarsus. Abdominal terga with hairs except at the first two segments.

Male: Unknown

Material examined: Holotype: F: INDIA, KERALA, Ayarote, P. Faseeh, 31-X-2017; **Paratypes**: 3 F: Same data as that for Holotype.

Distribution: INDIA (Kerala).

4.2.5.2. *Tetragonula* sp. nov. 2

Holotype : Female (worker): Body length 3.4 mm (3.35–3.40 mm); forewing length, including tegula, 4.15 mm (4–4.15 mm); head length from anterior margin of clypeus to summit of vertex, in facial view 1.55 mm (1.52–

1.85 mm); head width 1.77 mm (1.64–1.85 mm); length of scape 0.624 mm (0.624-0.64); length of 3rd flagellomere 0.115 mm; metatibia length 1.54 mm (1.49–1.59 mm); forewing diagonal from base of vein M to base of crossvein r-rs at margin of pterostigma 1.12 mm (1.01–1.12 mm).

Length of compound eye almost twice (1.9 times) the length of scape, the ratio of inter ocellar distance to the ocellar diameter less than 2.5 (2.22-2.38 mm). Ocello orbital distance over half the inter ocellar distance. Interalveolar distance 1.1 times long than that of Alveolar diameter. Metasoma broad (narrower than head), pointed at the apex and well telescoped in to the preceding segments.

Ratio between length and width of head 1.07 mm (0.86-1.19 mm), length and diameter of scape 6.53 mm (6-6.95 mm), length and width of mandible 3.23 mm (3.19-3.33 mm), length and width of pterostigma 4.41 mm (3.78-4.63 mm), length and width of 3rd tibia 2.78 mm (1.93-2.21 mm), length of basitarsus and head width 0.34 mm (0.31-0.39 mm), WL2/HW 0.55-0.64 mm, HTL/HW 0.83-1 mm, HTL/WL2 1.37-1.58 mm, IOD/OOD 1-1.73 mm, malar space/F3 0.36-0.5 mm, interalveolar distance/alveolar diameter 1-1.3 mm, alveolorbital distance/alveolar diameter 1.45-2 mm, IOD/OD 2.22-2.38 mm, length of mesoscutum/width of mesoscutum 0.78-0.85 mm, length of eye/scape length 1.85-1.9 mm.

Vertex and frons with punctuation which are not closely spaced. Distal area of clypeus straight. Bees are medium sized. The inner extension of point of attachment of mandible and second teeth forms an angle.

Color: Black integument. Head black. Compound eyes orange brown. Clypeus black with yellowish brown strand apically. Labrum yellow brown with white short and long hairs. Mandibles golden yellow with apical black border. Ocelli light yellow. Scape dorsally dark brown to black and ventrally testatious. Pedicel dark brown. First flagellomere yellow brown to dark brown dorsally and

yellow brown ventrally. Flagellomeres with minute hairs and pits, dorsally dark and ventrally testatious. Mandibles are brownish yellow and point of attachement black, mandibles are bordered by brown color. Malar space black. Gena black.

Thorax black. Plumose hairs on thorax yellow brown, stout hairs black. Tegula and wing sclerites dark brown, hind wing veins dark brown. Pterostigma dark brown. Wings hyaline and iridescent. legs black. Trochanter yellowish brown with long white hairs. Femer, tibia and basitarsus black. Tarsal segments brown and become lighter towards the tip. Penicillium dark brown. hairs on tibia dark brown. plumose hairs on tibia dull colured. Arolium black and distal part white. Pleuron black with minute punctures. Mesoscutum black. Scutellum black. Metanotum black.Propodium black with punctures.

Abdomen black with hairs on dorsal segments except on the first two. First abdominal segment light brown. Distal half of the apical segment light.

Pilosity: Head with dense plumose hairs. Plumose hairs on lateral side of clypeus are longer, denser than that that on clypeus. Frons covered with sparse plumose hairs. Labrum with long and short golden hairs. Malar space black and with out hairs. Fine dull white hairs are present in gena. Hairs on vertex are dark brown. Hairs in neck.

Hairs on tegula are dark brown. Plumose hairs on mesoscutum forms hair bands which are not much distinct, long stout black hairs are covered all over mesoscutm. Anterior border of mesoscutem with dense plumose hairs and with more stout hairs.scutellum with dark stout hairs along with yellow brown plumose hairs on the upper margin and light hairs on the lower margin. Anterior meso pleuron with plumose hairs. Metapleuron with dense silvery hairs. Trochanter with white long spurs. Fore tibia with short white,stout black hairs on the anterior side, posterior side with lamellate hairs. Middle tibia with mixture of white hairs,

stout long black hairs, yellow brown pumose hairs.Hind tibia with long black stout hairs, outer margin with brown plumose hairs, Keriotrichea on hind tibia silver white in color. Posterior region of hind basitarsus with yellow brown hairs on the medial line, basal seraceous area varied (less than half, more than half).

Male: Unknown

Material examined: Holotype: F: INDIA, KERALA, Kumbaya, P. Faseeh, 22-X-2017; **Paratypes**: 5 F: Same data as that for Holotype.

Distribution: INDIA (Kerala)

4.3. NEST ENTRANCE

The genus *Tetragonula* is the most widely distributed stingless bee in Kerala. During the study period of 2016 to 2018, we have come across two new bees of *Tetragonula* genus along with the most common stingless bee of the Indian subcontinent *T. iridipennis*. The distribution and characters of genus *Lisotrigona* are not well known from Kerala and Indian subcontinent. The nest entrance characteristics of these bees are discussed as follows.

The habitats of stingless bees vary widely. The feral colonies were collected from different habitats such as, tree hollows, bamboo, stone walls, mud walls, fountains of buildings, roof, lateritic rocks, hollow brick walls, meter boards, iron pipes, bridges, and raincoat.

The trees which harbored nests of stingless bee *T. iridipennis* were, *Tectona grandis*, *Artocarpus heterophyllus*, *Ficus religiosa*, *Lagerstroemia microcarpa* (Ven teak), and *Mangifera indica*. All the trees recorded with stingless bee nests were live.

The nest and nesting habitats of the genus *L. cacciae* are not well known. The stingless bees nests of *L. cacciae* are recorded from *Tectona grandis* (Teak), *Artocarpus heterophyllus* (Jack tree), bamboo, stonewall, laterite wall, hollow bricks, and brick walls.

The nest entrance of the stingless bees differed in various locations. The parameters such as length of entrance tube, length and width of entrance mouth, the design of entrance tube, and height from the ground level recorded. Each of these characters varied in different colonies. The variation of nest entrance of the two genera *Tetragonula* and *Lisotrigona* are more specific and they can be easily distinguished from each other by their nest entrance. The observations on nest entrances of *T. iridipennis* (Table 4A, Table 4B) *Tetragonula* sp. nov. 1, *T etragonula* sp. nov. 2 (Table 5), and *L. cacciae* (Table 6) are provided.

4.3.1. Multiple Entrances in *Tetragonula iridipennis*

Out of two not seven *T. iridipennis* samples collected from various location in Kerala, six colonies were characterized by the presence of more than one nest entrance opening for a single colony. Out of six colonies identified, four were from the hilly region and two were from plains. Colonies were located from Manakkadavu (Kannur), Peechi (Thrissur), Vellayani (Thiruvananthapuram), Kadannamanna (Malappuram), Ambanad (Kollam), and Thenmala (Kollam).

The shape of multiple entrance tubes were noted as either round, oval or slit. Three of the nest entrances was oval and two of them were round and one was with slit entrance. It is also observed that shape of entrance mouth was same for all entrance tube from the same colony.

The number of guard bees varied in each colony. The number of guard bees was same in each active entrance tube of the same colony but one colony

Table 4A. Nest architectural variation of *Tetragonula iridipennis*

Location	Altitude	Design					Number of guard bees	Length of entrance tube		
		Round	Slit	Heart	Oval	Others		<1cm	1-3cm	>3cm
Kakkanad 1	8			+			3		+	
Kakkanad 2	8	+					4	+		
Arimboor	14			+			7		+	
District farm Alappuzha	14	+					3	+		
Alappuzha	14	+					4	+		
Mavelikkara	14					+	5			+
East fort	15			+			4		+	
Eastfort 1	15			+			3		+	
East fort 2	15				+		3	+		
Pandalam	16		+				8		+	
Pandalam 1	16		+				8		+	
Vellanikkara 1	19	+					3		+	
Vellanikkara 2	19		+				5		+	

Vellayani 1	29				+		4		+	
Nilambur	30	+					3			+
Kuttichira	36		+				12		+	
Kumbaya	38		+				18			+
Paipra	38					+	5		+	
Kadannamnna	39			+			5		+	
Kadannamanna	42				+		8		+	
Kadannamanna	42		+				4		+	
Nilambur	42		+				5		+	
Kadannamanna	42					+	2		+	
Nilambur	42					+	4		+	
Nilambur	44				+		4		+	
Nilambur	44					+	5		+	
Kulanada 1	44		+				5		+	
Kulanada 2	44				+		2	+		

Veliyam	49				+		6		+	
Kalluvathukkal 1	55			+			6		+	
Kalluvathukkal 2	55				+		6			+
Kalluvathukkal 3	55					+	10			+
Peechi	55				+		5		+	
Peechi 1	67				+		5			+
Balaramapuram 1	79					+	4			+
Balaramapuram 2	79					+	5		+	
Balaramapuram 3	79		+				6		+	
Balaramapuram 4	79					+	5		+	
Thachampara 1	82			+			8			+
Thachampara 2	82			+			5			+
Thachampara 3	82			+			8			+
Kalladikkod	87					+	6			+
Thirumeni	87		+				16			+

Karimba	91	+					2	+		
Poovam	91					+	5			+
Mullaringhad	96				+		9			+
Alakkode 3	114				+		4			+
Alakkode 1	115				+		7			+
Alakkode 2	115			+			8			+
Kuranghonchula	132		+				8		+	
Erinjeri 1	150					+	8			+
Erinjeri 2	150					+	3	+		
Erijeri 3	150		+				5		+	
Karadukka	165		+				7		+	
Kokkadavu	171	+					6		+	
Manakkadavu 1	184	+					5		+	
Thenmala	189	+					8			+
Braimore 1	257			+			4			+

Braimore 2	257	+					7			+
Braimore 3	257		+				4			+
Braimore 4	257		+				5			+
Braimore 5	257		+				4			+
Ambanad 1	580					+	3		+	
Ambanad 2	580		+				8	+		
Ambanad 4	580					+	5	+		
Ambanad 6	580					+	6		+	
Ambanad 7	580		+				14	+		
Percentage (%)		**14.70**	**26.47**	**16.17**	**17.64**	**23.52**		**14.7**	**50**	**35.3**
Average / Total		**10**	**18**	**11**	**12**	**16**	**5.87**	**10**	**34**	**24**

Table 4B. Nest entrance architecture of *Tetragonula iridipennis*

Location	Altitude (m)	Entrance width			Entrance length			Height from ground level		
		<1 cm	1-2 cm	>2cm	<1 cm	1-2 cm	>2 cm	<1m	1-2m	>2m
Kakkanad 1	8		+			+			+	
Kakkanad 2	8	+			+				+	
Arimboor	14		+			+			+	
District farm Alappuzha	14		+			+			+	
Alappuzha	14		+			+			+	
Mavelikkara	14			+		+		+		
East fort	15		+			+		+		
Eastfort 1	15		+			+		+		
East fort 2	15		+			+		+		
Pandalam	16		+			+		+		
Pandalam 1	16		+			+		+		

Vellanikkara 1	19		+			+		+		
Vellanikkara 2	19		+		+				+	
Vellayani 1	29		+		+				+	
Nilambur	30			+		+			+	
Kuttichira	36			+	+			+		
Kumbaya	38			+	+				+	
Paipra	38	+			+				+	
Kadannamanna	39		+			+			+	
Kadannamanna	42		+			+		+		
Kadannamanna	42		+			+			+	
Nilambur	42		+			+			+	
Kadannamanna	42	+			+					+
Nilambur	42	+			+				+	
Nilambur	44		+			+		+		
Nilambur	44		+			+		+		
Kulanada 1	44		+		+				+	

Kulanada 2	44	+			+				+	
Veliyam	49		+		+				+	
Kalluvathukkal 1	55		+		+				+	
Kalluvathukkal 2	55		+			+			+	
Kalluvathukkal 3	55		+			+			+	
Peechi	55		+			+		+		
Peechi 1	67		+		+			+		
Balaramapuram 1	79		+			+		+		
Balaramapuram 2	79		+		+			+		
Balaramapuram 3	79		+		+			+		
Balaramapuram 4	79		+		+			+		
Thachampara 1	82			+		+			+	
Thachampara 2	82		+			+			+	
Thachampara 3	82		+			+			+	
Kalladikkod	87	+				+			+	
Thirumeni	87			+	+					+

Karimba	91	+				+				+
Poovam	91		+			+			+	
Mullaringhad	96		+			+				+
Alakkode 3	114		+		+				+	
Alakkode 1	115		+			+			+	
Alakkode 2	115		+			+			+	
Kuranghonchula	132			+	+			+		
Erinjeri 1	150		+			+			+	
Erinjeri 2	150	+			+	+				+
Erijeri 3	150		+			+			+	
Karadukka	165		+		+					+
Kokkadavu	171	+				+			+	
Manakkadavu 1	184	+			+			+		
Thenmala	189		+		+			+		
Brimore 1	257		+			+		+		
Brimore 2	257	+			+				+	
Brimore 3	257		+		+				+	

Brimore 4	257		+		+					+
Brimore 5	257		+		+					+
Ambanad 1	580		+		+			+		
Ambanad 2	580			+	+			+		
Ambanad 4	580		+			+			+	
Ambanad 6	580		+		+			+		
Ambanad 7	580	+				+			+	
Percentage (%)		**17.65**	**70.6**	**11.76**	**45.56**	**54.44**	**0**	**36.76**	**51.47**	**11.76**
Average		**12**	**48**	**8**	**31**	**37**	**0**	**25**	**35**	**8**

Table 5. Nest entrance architecture of *Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2

Location	Altitude (m)	Design	No. of guard bees	Length of entrance tube (cm)	Nest entrance		Height from ground level (cm)
					Width (cm)	Length (cm)	
A. ***Tetragonula* sp. nov. 1**							
Chullikkara	111	Slit	7	3.4	2.3	0.8	158
Chullikkara	111	Square					
Changanasseri	11	Square					
B. *Tetragonula* sp. nov. 1							
Kumbaya	38	Heart	5	4	1.1	0.6	176
Poovam	91	Round	6	0.5	1.1	1.3	60
Ayarote round	111	Round	6	0.8	1.9	1.8	110
Karadukka 1	165	Round	11	3	3.2	4.2	250
Karadukka 2	165	Round	10	1.3	2.9	1.9	180
Karadukka 3	165	Round	8	1.4	1.8	2.1	195
Manakkadavu	184	Round	12	4.2	1.8	1.8	279
Ambanad 5	580	Round	8	3	1.8	2.3	82.5
Trivandrum	30	Round					
		Average	**8.25**	**2.275**	**1.95**	**2**	**166.56**

Table 6. Nest entrance architecture of *Lisotrigona cacciae*

Location	Altitude (m)	Design	No. of guard bees	Length of entrance tube (cm)	Nest entrance mouth		Height from ground level (cm)
					Width (cm)	Length (cm)	
Nilambur	44	Round	2	0.5	0.4	0.5	187
KFRINilamboor	44	Round	2		0.4	0.5	110
Peechi park	54	Round	2	0.8	0.4	0.5	75
KFRI Thrissur	79	Round					
Poovam	91	Round	2	2.3	0.3	0.4	183
Kokkadvu 1	171	Round	4	0.3	0.6	0.5	270
Kokkadavu 2	171	Round	4	0.4	0.4	0.3	220
Average			**2.66**	**0.86**	**0.42**	**0.45**	**174.16**

showed considerable difference where in, out of five active entrance tubes, one entrance tube was with 4 guard bees and the remaining entrance tubes were guarded by 2 bees.

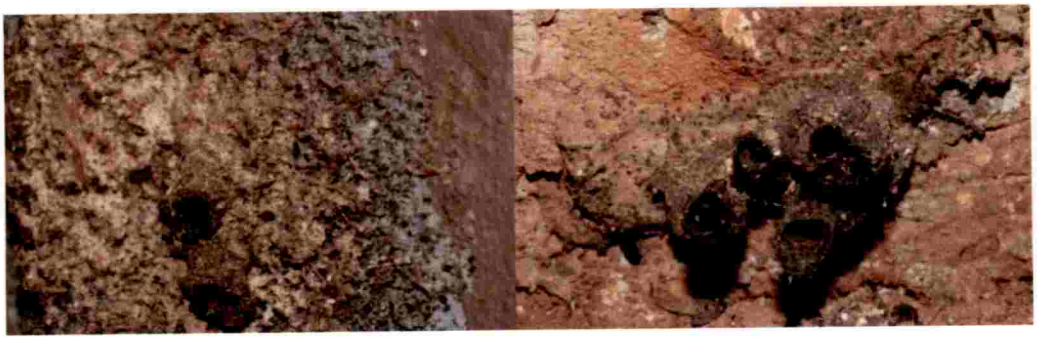
The number of entrance tube was two for colonies identified from Vellayani (Plate 11A), Thenmala (Plate 11B), Manakkadavu (Plate 11C), Peechi (Plate 11E), and Ambanad (Plate 11F) whereas five entrance tubes found in a single colony from Kadannamanna (Plate 11D). However, the number of active entrances differed in each colony. Colonies from Thenmala, Ambanad, and Manakkadavu were with both the entrances active, whereas colonies from Vellayani and Peechi had only one active entrance tube and the inactive one was closed entirely (Peechi) or joined with the active entrance tube mouth (Vellayani). It was observed that, all the five entrance tube were active in the the colony from Kadannmanna.

The length and width of entrance mouth were almost same for different entrance tubes in a single colony. Length of entrance mouth varied from 1 cm to 2.5 cm and width varied from 0.2 to 1.2 cm in different locations.

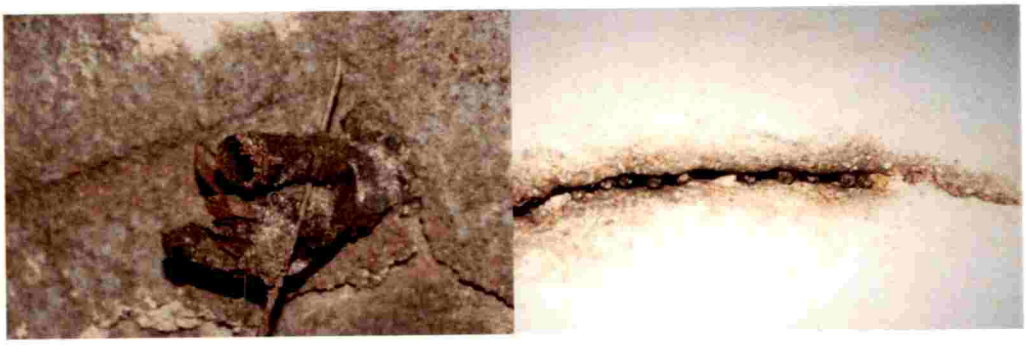
The height of colony from ground level varied in different locations but except one from Ambanad, all colonies were located at a height below 50 cm, and the colony from Ambanad was found at a height of 138 cm from the ground level. Five colonies were found associated with foundations of buildings made of stones and one colony was observed from a building wall.

All the five colonies were having one entrance tube just below the other entrance tube of the same nest and it gave an appearance of vertical arrangement of entrance tubes. The colony which has 5 entrances had them arranged in a different manner. Two sets of two entrance tubes were arranged vertically, one

A. Vellayani B. Thenmala



C. Manakadavu D. Kadannamanna



E. Peechi F. Ambanad

Plate 11. Multiple entrances in *Tetragonula iridipennis*

below to the other and the remaining one entrance tube was in line with the upper entrance tube (three tubes were in one line and two tubes were in another line).

In addition to these six colonies with multiple entrances, one more colony was identified from Thachampara (Palakkad dist) with multiple openings for nest entrance wherein, three to four small openings were observed in addition to the apical opening (Plate 12). Except for the apex, all openings were nonfunctional and without any guard bees. The main entrance was guarded by 4-5 bees. The colony was located 186 cm from the ground level on a fig tree.

4.3.2. *Number of Guard Bees*

4.3.2.1. Genus*: Tetragonula*

The number of guard bees of *T. iridipennis* varied with the lowest number of 2 (Nilamboor, Karimba, Kadannamanna, Kulanada) and highest number of 18 (Kumbaya) followed by 16 (Thirumeni) and 14 (Ambanad). The mean value of 68 different colonies of *T. iridipennis* was found to be 5.87. Only two colony of *Tetragonula* sp. nov. 1 could be located wherein guard bees observed were seven in number.

Tetragonula sp. nov. 2 were collected from 9 different colonies. The number of guard bees in them varied between 5 to 12 with a mean value of 8.25.

It was observed that the guard bees of *Tetragonula* sp. nov. 2 were more aggressive when disturbed, compared to that of the *T. iridipennis* and *Tetragonula* sp. nov. 1.

4.3.2.2. Genus*: Lisotrigona*

The behavior of these bees was quite different from that of the *Tetragonula* genus. A slight disturbance made the bees retract into their nests.

Plate 12. Single entrance tube with multiple opening from Thachampara

They were timid and very difficult to collect without destroying the colony. On continuous disturbance, few bees could be observed coming out from a colony located at Nilambur. The bees collected from KFRI displayed the habit of drinking sweat from hands and tears from the eyes.

The number of guard bees ranged from 2 to 4 in *L. cacciae* nests. Out of seven colonies located five of them were with 2 guard bees (KFRI Nilambur, KFRI Thrissur, Peechi, Poovam, Nilambur teak museum), whereas other two colonies (Kokkadavu) had four guard bees.

4.3.3. *Design of Nest Entrance.*

4.3.3.1. Genus: *Tetragonula*

Out of 68 colonies of *T. iridipennis* observed, the nest entrance design varied in shape from oval, heart, slit, round, toad mouth, a triangular, arch, square and tapetum. The most common design observed was slit (18 nos) (Plate 13A) followed by oval (12 nos) (Plate 13B) heart (11 nos), and round (10 nos) (Plate 13C). The most uncommon designs were tapetum (1 nos), toad mouth (1 nos) and triangle (2 nos) (Plate 14). Other shapes observed were Semicircular, arch, and cryptic. Two nests were noted from Ambanad (Kollam) werein one of the nest did not display any entrance tube (cryptic) and it was also smeared with yellowish material whereas another nest with very small extension and entrance was found smeared with resinous substances.

Two colonies (Chullikkara, Kasaragod) of *Tetragonula* sp. nov. 1 were observed. One colony had a slit entrance and another one had square-shaped entrance apex (Plate 15). Out of 9 colonies of *Tetragonula* sp. nov. 2 located, 8 colonies were with round entrance whereas one had heart shape (Kumbaya, Pathanamthitta) (Plate 16).

A. Slit

B. Oval

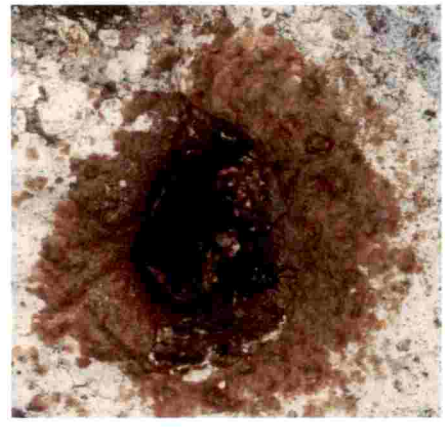
C. Round

Plate 13. Variation in nest entrances of *Tetragonula iridipennis*

A. Triangular

B. Tapetum

C. Toad mouth



D. Cryptic

Plate14. Most uncommon nest entrances recorded in *Tetragonula iridipennis*

A. Square entrance

B. Slit entrance

C. Broods

D. Layered arrangement of broods

D. Honey

E. Queen

Plate 15. Nest entrance and internal view of *Tetragonula* sp. nov. 1

A. Heart

B. Round

C. Round

D. Round

E. Round

(A. Kumbaya, B. Karadukka, C. Karadukka, D. Chullikkara, E. Poovam)

Plate 16. Nest entrance of *Tetragonula* sp. nov. 2 from different locations of Kerala

The nest entrances of *Tetragonula* sp. nov. 1 was similar to that of *T. iridipennis* nest whereas the nest entrance of the *Tetragonula* sp. nov. 2 with large round nest entrances differed considerably. The entrances were without any dust particles, black colored and with creamy wax and propolis. One colony had heart shaped entrance and yellowish muddy material was used for the construction of the nest entrance part.

4.3.3.2. Genus: *Lisotrigona*

All the six colonies of *L. cacciae* had round nest entrance (Plate 17). They were perfectly round and made of well-finished propolis. The color of nest entrance varied in different locations, from light yellow white at Poovam, reddish brown at Kokkadavu, to black at KFRI Thrissur, KFRI Nilambur and Peechi.

4.3.4. *Length and Width of Entrance*

4.3.4.1. Genus: *Tetragonula*

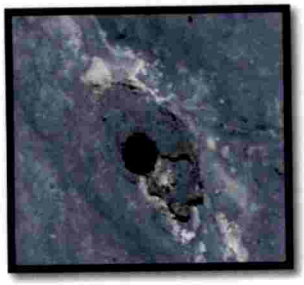
Out of 68 *T. iridipennis* colonies observed, 33 colonies were with the length of nest mouth less than 1cm, 37 colonies came in between 1-2 cm and one colony from Nilambur was with 2 cm. The mean value of entrance mouth length obtained was 1.08 cm.

Entrance mouth width varied from 0.5 cm to 3 cm with an average of 1.44 cm. 13 colonies were having entrance mouth width less than 1 cm, 50 colonies had between 1-2 cm, and 8 colonies had greater than 2 cm entrance mouth width. The highest value observed was recorded from Chullikkara with an entrance width of 3 cm. The maximum (70.6%) of nests came under the category of 1-2 cm, 17.65 % comes under the category less than 1 cm and only 11.6 % of the total nests were having nest entrance more than 2 cm.

A. Teak plant

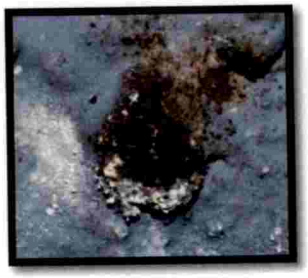
B. Jack fruit tree

C. Stone wall

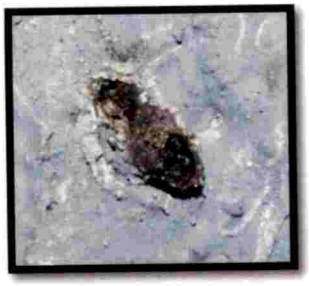


D. Stone wall

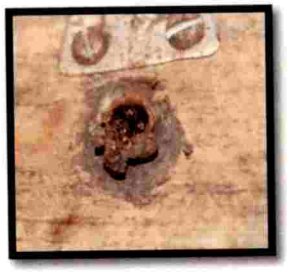
E. Stone wall



F. Stone wall



G. Stone wall



H. Hive

Plate17. Nest entrance of *Lisotrigona cacciae* recorded from various habitats

The entrance mouth width of *Tetragonula* sp. nov. 1 observed 2.3 cm and entrance mouth length as 0.8 cm. Out of 10 colonies of *Tetragonula* sp. nov. 2, six colonies were having entrance width in between 1-2 cm. Two colonies were having entrance width greater than 2 cm (both colonies were obtained from Karadukka). The average entrance mouth width noted as 1.96 cm. The length of entrance mouth varied from 0.6 cm to 4.2 cm with an average value of 2 cm. one colony was with entrance mouth length less than 1 cm, four colonies come in between 1-2 cm and three colonies were with more than 3 cm entrance mouth width.

4.3.4.2. Genus: *Lisotrigona*

Out of six samples of the *L. cacciae* collected from different location of Kerala, five were having width and length of entrance mouth less than or equal to 0.5 cm and one of the colony was with entrance width 0.6 cm. The mean value obtained for length and width of entrance mouth was 0.45 cm and 0.41 cm respectively. The length of entrance mouth ranged from 0.3 cm to 0.5 cm and width of entrance mouth width ranged from 0.3 cm to 0.6 cm.

4.3.5. *Length of entrance tube*

4.3.5.1. Genus: *Tetragonula*

The length of entrance tube ranged from 0 to 13.5 cm in *T. iridipennis*. Three nests were without an entrance tube and the longest entrance tube observed from Mullaringhad (Idukki) with 13.5 cm long entrance tube, followed by 12.5 cm from Thachampara (Palakkad), 10.5 cm from Mavelikkara (Alappuzha) and 10 cm from Braimore estate (Thiruvananthapuram). Seventeen colonies were with entrance tube length less than 1 cm, 29 colonies came under category 1-3 cm and 22 colonies were with entrance tube length more than 3 cm.

The nest entrance tube length of *Tetragonula* sp. nov. 1 observed as 3.4 cm, whereas the length of nest entrance tube varied from 0.5 cm to 4.2 cm in *Tetragonula* sp. nov. 2 with a mean value of 2.28 cm.

4.3.5.2. Genus: *Lisotrigona*

The entrance tube length of *L. cacciae* nests ranged from 0.3 cm to 2.3 cm with a mean of 0.86 cm. The longest tube (2.3 cm) was obtained from Poovam (Kannur) followed by 0.8 cm from Peechi (Thrissur).

4.3.6. *Height from Ground Level*

4.3.6.1. Genus: *Tetragonula*

Out of 54 feral colonies of *T. iridipennis* observed 24 were located at a height less than 1 m from ground level, 25 were a height between 1-2 m and 5 colonies obtained from a height more than 2 m from ground level. The colony found at the maximum height was from Kuranghan Chola (Malappuram) which was at a height of 5.28 m from ground level (Nest was located at the top of a tree) followed by 3.29 m from Kadannamanna (Malappuram) and 3.10 m from Braimore estate (Thiruvananthapuram).

The hive of *Tetragonula* sp. nov. 1 was observed at a height of 1.58 m from the ground level. The hive of *Tetragonula* sp. nov. 2 has kept at different heights 60 cm (Poovam), 1.10 m (Chullikkara), 1.76 m (Kumbaya), 1.80 m (Karadukka), 1.95 m (Karadukka), 2.50 m (Karadukka) and 2.79 m (Manakkadavu). One feral colony of it was obtained from a height of 0.83 m (Ambanad) from ground level.

4.3.6.2. Genus: *Lisotrigona*

Out of five feral colonies of *L. cacciae* one colony (Peechi) was at a height of 0.75 m (less than 1 m) from ground level and three colonies (Poovam, KFRI Nilambur, and Nilambur teak museum) were in the category 1-2 m from ground level and one colony was at a height of 2.7 m (more than 2 m from ground level).

The *L. cacciae* nest recorded from Nilambur teak museum was on a Teak plant (*Tectona grandis*) at a height of 1.87 m from the ground level and colony recorded from the Poovam was located on a Jackfruit tree at a height of 1.83 m from ground level. The mean height of feral colonies of *L. cacciae* was recorded as 1.65 m height from the ground level.

Discussion

5. DISCUSSION

The results obtained through an extensive study on stingless bees of Kerala (2016-2018), with an objective to know the diversity of stingless bees in Kerala and the variation of their nest entrance are discussed in this chapter.

5.1. STUDY OF STINGLESS BEE DIVERSITY

The current study brings out two new species of stingless bees along with *T. iridipennis,* and *L. cacciae* which are already described stingless bees from Kerala. The stingless bees of Indian subcontinent are not much explored as like neo-tropical stingless bees and studies on their diversity and distribution are lacking from Kerala as well as India. The studies of Mohan and Devanesan (1999), Devanesan *et al.,* (2003), Jayalekshmi (2015), Rahman *et al.,* (2015), Divya (2016) showed the presence of *T. iridipennis* in the Kerala. We have also recorded the *T. iridipennis* as the most widely distributed stingless bee in Kerala.

The Kani tribes of Western Ghats were rearing stingless bees belonging to *T. iridipennis* (Kumar *et al.*, 2012). Rasmussen (2013) summarized stingless bees are widely distributed all over the Indian sub continent except at the higher elevations and drier interior regions. In our study we have sampled stingless bees from an altitude of 8 m to 1064 m from mean seal level and found that these bees are well distributed in the Kerala.

Jobiraj and Narendran (2004) reported a tiny stingless bee *L. mohandasi* from KFRI (Kerala Forest Research Institute), Peechi. Viraktamath and Jose (2017) recorded *L. chandrai* from Kasragod and Kannur district of Kerala. The current study considered both *L. mohandasi* and *L. chandrai* as new synonyms (Rasmussen, 2017) of *L. cacciae*. The present study extended the distribution

range of *L. cacciae* to Malappuram (KFRI Nilambur) district in addition to previous records of distribution.

The present study introduces two new stingless bees (*Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2) to the existing bee fauna of India. The requirement of additional collection of stingless bee from Indian subcontinent is mentioned by Rasmussen (2013), the author also stated the group has the potential to raise the species from Indian sub continent and more native workers are needed to solve the problem of this group. More extensive study can come across with new species from Indian sub continent (Rathore *et al.*, 2013). The geometric morphometric analysis on wing patterns clearly showed the existence of various clusters within the *Tetragonula* complex (Francoy *et al.*, 2016).

The differences in the morphometric observation, RAPD analysis and differences in the nest entrance characters reveals the existence of two new species of stingless bees other than the *T. iridipennis* in populations of Siitlingi and Coimbatore population of Tamil Nadu (Sriram *et al.*, 2004). The novel taxonomic tools (Frietas *et al.*, 2009), morphological and molecular data (Francoy *et al.*, 2016) will be useful for discovery of new species.

5.2. IDENTIFICATION OF STINGLESS BEES

5.2.1. Morphological Characterization

Identification of the *Tetrgonula* complex by morphological characterization of workers is difficult. Distinct morphological characters are rare in this group and most of the previous workers used body ratios, size, color, and hair pattern to distinguish these bees (Sakagami, 1978; Rasmussen, 2008; Rasmussen, 2013; Rahman *et al.*, 2015; Viraktamath and Jose, 2017; Engel *et al.*, 2017; Silva *et al.*, 2018).

The *Tetragonula* complex regarded as the notorious group without a proper key to identify them at species level (Rasmussen, 2008). Rasmussen (2013) reviewed the stingless bees of Indian sub continent without giving a species level key to *T. iridipennis* complex and mentioned lack of comprehensive publication to solve the species complex of the *Tetragonula* in India. The availability of literature from India is scarce in stingless bees (Vijayakumar and Jayaraj, 2014). The present status of knowledge on diversity, biology, nest characters are inadequate in stingless bees, and to understand the different attributes of these bees, systematic investigation is needed to be carried out in this group (Makkar *et al.*, 2016). Many of the bees in the genus *Tetragonula* are very close in external appearance, so authors have to relay up on the characters such as color, size and setation on the body to distinguish the species of stingless bees (Engel *et al.*, 2017).

It was observed that, the bees displayed variation in their body color, body size and pilosity in the present study. There were no clear cut morphological differences in the four species *T. iridipennis, T. praeterita, T. ruficornis,* and *T. bengalensis* and they were mentioned as *iridipennis* species group (Rasmussen, 2013).

The notable difference recorded by Rasmussen (2013) in *T. ruficornis* was, white hairs in the hind tibia and basal seraceous area less than half of the basitarsus which make it distinguishable from other bees in *iridipennis* group. He also recorded *T. ruficornis* as the lightest and *T. praeterita* as the darkest among the four bees in the *iridipennis* species group.

The *T. iridipennis* and *Tetragonula* sp. nov. 1 was appeared to the similar size whereas former was darker than the other. The *Tetragonula* sp. nov. 2

appeared robust and bigger than the other two bees and were darker than the *T. iridipennis* and *Tetragonula* sp. nov. 1.

Hairs on the clypeus and frons were dense in *T. iridipennis*, in *Tetragonula* sp. nov. 1 clypeus was rich with plumose hairs than frons which didn't obscure the integument, whereas in *Tetragonula* sp. nov. 2 the plumose hairs on the lateral sides appeared thick and larger than that on the clypeal region. Rasmussen (2013) inferred that, except *T. praeterita*, all other bees in the *iridipennis* group from India had abundant hairs on clypeus as well as the frons.

The hair bands on the *Tetragonula* sp. nov. 2 were not distinct as in the *T. iridipennis* and in *Tetragonula* sp. nov. 1, it had more dark stout hairs even in the interspaces. Similar hair pattern was observed by Sakagami (1978) in *T. gresitti*, but the frontal hairs on that specimen were not plumose as in *Tetragonula* sp. nov. 2.

In mesoscutm of *T. laeviceps* mixture of dark hairs were scarce and were not banded well (Sakagami, 1978). Hairs on thorax with glabrous interspaces considered as the key for the *iridipennis* group (Rasmussen, 2013).

A closer examination of the permanent slides showed considerable difference in the mandible of the *Tetragonula* sp. nov. 1 where it is curved at the inner margin of mandible. But in *T. iridipennis* and *Tetargonula* sp. nov. 2 it is angulated at the inner margin of the mandibles. The point of attachement of mandibles also varied in 3 bees. The *Tetragonula* sp. nov. 1 showed considerable difference from *T. iridipennis* and *Tetragonula* sp. nov. 2 in curvature of antennal comb. Novel taxonomic tools can bring out new species of stingless bees (Frietas *et al.*, 2009).

The latest classification of Indo-Malayan stingless bees by Rasmussen (2017) considered *L. mohandasi*, *L. chandrai*, and *L. revanai* as new synonym for *L. cacciae*. The *Lisotrigona* bees identified during our study couldn't be distinguished with the key provided by Viraktamath and Jose (2017) and thus, is considered as *L. cacciae* in present study. The color of integument varied from brown to black in these bees. Rasmussen (2013) observed that, the head of *L. cacciae* is near black in some specimens.

A distinct behavior was observed in *Lisotrigona* bees collected from KFRI Thrissur where, they were attracted towards the eyes as well as sweaty body, whereas bees located from KFRI Nilambur hovered behind the head. Similar experience was reported by Karunarathne (2017) in *L. cacciae* from Sri Lanka.

5.2.2. Micrometry

All the body measurements and body ratios were taken from previous works of Sakagami (1978), Rasmussen (2013), Viraktamath and Jose (2017) and Engel (2017).

The mean head width of observed was 1.6 mm, 1.53 mm and 1.7 mm in *T. iridipennis*, *Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2. The head width observed by Danaraddi and Viraktamath (2009), Danaraddi *et al.*, (2012), Rasmussen (2013), Vijayakumar and Jayaraaj (2014), Makkar *et al.*, (2016) and Tej *et al.*, (2017) in *T. iridipennis* support the current observation.

The head width of *Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2 was closer to that of *T. praeterita* and *T. bengalensis* which were reviewed by Rasmussen (2013). The mean head width recorded by Silva (2018) in *T. praeterita* is larger than that of Rasmussen (2013) observed. The mean head length of *T. iridipennis* was closer to the measurements given by Rasmussen

(2013) and Makkar *et al.*, (2016) for the same species. Head length of *Tetragonula* sp. nov. 1 came closer to the *iridipennis* group. But head length of *Tetragonula* sp. nov. 2 was a larger value which doesn't match with any of the bees in *iridipennis* group.

Almost all measurements of *T. iridipennis* recorded in the present study are in conformity with the measurements documented by Rasmussen (2013) for the same species except the slight variation in the wing measurements. The comparative body measurements of different species from Indian subcontinent in the current study and the values given by Rasmussen (2013), Rathore *et al.*, (2016), Silva *et al.*, (2018) are provided in Table 7.

5.2.3. Statistical Analysis

The results of scree plot analysis grouped the stingless bees *viz.*, *T. iridipennis, Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2 in to three distinct clusters. Similar analysis was conducted by Silva (2018) to separate *T. iridipennis* and *T. praeterita* from Sri Lanka.

5.2.4. Molecular Characterization

The attempts at partial sequencing of Mitochondrial COI gene of the three species of bees, viz., *T. iridipennis, Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2 collected from Kerala resulted in considerable variation in their nucleotide base pairs. The phylogenetic tree constructed using gene sequence data group them into three distinct clusters. Sriram *et al.*, (2004) concluded the possibility of existence of two different stingless bee species based on morphometry and molecular analysis (the results of RAPD-PCR analysis of two populations of stingless bees from Tamil Nadu displayed much variation at genetic level). Four nuclear gene fragments and 16S rRNA sequence data were compared for 24 taxa

Table 7. Comparative body measurements of stingless bees from Indian subcontinent

Body parts	Current findings			Rasmussen 2013				Rathore *et al.* 2013	Silva *et al.* 2018	
	T. sp. nov. 1	*T.* sp. nov. 2	*T. iridipennis*	*T. iridipennis*	*T. praeterita*	*T. ruficornis*	*T. bengalensis*	*T. grisetti*	*T. praeterieta*	*T. iridipennis*
Width of head	1.53	1.7	1.6	1.6	1.52	1.66	1.7	1.92	1.91	1.79
Length of head	1.33	1.54	1.33	1.3	1.32	1.32	1.34	1.65		
Length of compound eye	1.11	1.19	1.06	1.1	1.04	1.13	1.13	1.32	1.21	1.27
Width of compound eye	0.38	0.46	0.37	0.4	0.4	0.42	0.43	0.48		
Upper inter orbital distance	1	1.14	1.05	1	1.04	1.02	1.02	1.12	1.19	1.31
Lower inter orbital distance	0.88	1.02	0.89	0.82		0.79	0.87	1.01		
Diameter of median ocellus	0.14	0.19	0.15	0.15	0.14	0.14	0.15	0.21		
Inter ocellar distance	0.35	0.46	0.36	0.33	0.35	0.38	0.38	0.39	0.41	0.47

ocellorbital distance	0.21	0.28	0.22	0.23	0.24	0.24	0.23	0.28		
Inter alveolar distance	0.14	0.19	0.16	0.15	0.15	0.17	0.17	0.17	0.11	0.21
Alveolloribital distance	0.27	0.22	0.32	0.3	0.27	0.31	0.26	0.37		
Alveolocellar distance	0.69	0.75	0.68	0.71		0.67	0.67	0.82		
alveolar diameter	0.16	0.18	0.15	0.14	0.15	0.12	0.12	0.16		
Length of clypeus	0.32	0.35	0.31	0.33		0.39	0.33	0.4		
Maximum width of clypeus	0.71	0.78	0.71	0.7		0.71	0.65	0.69		
Inter tentorial distance	0.47	0.51	0.45	0.48		0.48	0.51	0.54		
Clypeocellar distance	0.93	0.97	0.89	0.96		0.06	0.14			
Length of malar space	0.04	0.06	0.05	0.03		0.05	0.06	0.11		
Length of scape	0.57	0.63	0.55	0.57	0.59	0.57	0.57	77		
Diameter of scape	0.08	0.11	0.09	0.1	0.08	0.1	0.1	0.11		
Diameter of 3 flagellomere	0.14	0.13	0.12	0.12	0.13	0.13	0.13	0.9		

Length of pedicel+flagellomere	1.06	1.14	0.97	1.24		1.25	1.3			
Length of first flagellomere	0.07	0.08	0.06	0.09	0.05	0.07	0.11			
Length of 2nd flagellomere	0.12	0.11	0.11	0.07	0.12	0.11	0.11			
Length of 3rd flagellomere	0.12	0.12	0.11	0.1	0.1	0.11	0.11			
Length of mandible	0.6	0.65	0.62	0.62		0.62	0.6			
Width of mandible	0.21	0.21	0.16	0.19		0.21	0.18			
Length of forwing excluding tegula	3.28	3.7	3.33	3.44	3.44	3.2	3.5	4.36		
Length of forwing including tegula	3.55	4.1	3.49	3.8	3.7	3.5	3.8	4.81		
WL2	0.92	1.07	0.97	1.01	0.95	0.95	0.95			
Width of forwing	1.19	1.33	1.06	1.32		1.28	1.25			
Length of pterostigma	0.54	0.59	0.52	0.48	0.48	0.51	0.48			

width of pterostigma	0.13	0.13	0.13	0.11	0.11	0.13	0.13			
Length of marginal cells	1.22	1.35	1.17	1.21	1.21	1.23	1.21			
Width of marginal cells	0.31	0.35	0.31	0.3		0.3	0.29			
Length of abscissa of Cu	0.74	0.81	0.7	0.71	0.7	70	0.71			
length of abscissa of M	0.59	0.75	0.56	0.58	0.6	0.52	0.55			
Hamuli	5	5	5	5	5	5	5			
Length of mesoscutum	0.86	0.93	0.86	0.87	0.85	0.83	1.00			
Width of mesoscutum	1.05	1.11	1.02	1.01	1.00	1.05	1.13			
Width of scutellum	0.75	0.8	0.77	0.57	0.61	0.69	0.79			
Length of scutellum	0.3	0.33	0.32	0.33	0.24	0.33	0.28			
Length of tibia 3	1.41	1.51	1.42	1.55	1.47	1.43	1.51	1.61		
Width of tibia 3	0.49	0.54	0.5	0.54	0.54	0.52	0.54			
Length of basitarsus 3	0.49	0.55	0.46	0.5	0.48	0.48	0.5			

Width of basitarsus 3	0.26	0.27	0.25	0.29	0.27	0.27	0.29			
Width of tergum 3	1.26	1.32	1.15	1.36	1.28	1.32	1.28			
Length of hairs on clypeus	0.05	0.05	0.05	<0.01	<0.01	<0.01	<0.01			
Length of hairs on vertex	0.09	0.13	0.1	0.13	0.11	0.07	0.05			
Length of hairs on frons	0.06	0.05	0.05	<0.01	<0.01	<0.01	<0.01			
Length of hairs on scutellum	0.06	0.48	0.1	0.12	0.12	0.1	0.15			
Length of body	3.23	3.37	3.6	3.55	3.33	3.45	3.55	6.12		
Ratio between length and width of head	1.16	1.1	1.2	1.23	1.15	1.26	1.27			
Ratio between length and diameter of scape	7.07	6.25	5.75	5.7	7.38	5.7	5.7			
Ratio between length and width of mandible	2.88	3.19	3.95	3.26		2.95	3.33			

Ratio between length and width of pterostigma	4.22	4.65	4.01	4.36	4.36	3.92	3.69			
Ratio between length and width of tibia 3	2.9	2.82	2.85	2.87	2.72	2.75	2.8			
Ratio between length and width of basitarsus 3	1.87	2.02	1.88	1.73	1.77	1.77	1.72			
Ratio of length of basitarsus 3 and head width	0.319	0.33	0.29	0.313	0.32	0.289	0.294			
WL2/HW	0.6	0.63	0.61	0.631	0.63	0.57	0.56			
HTL/HW	0.92	0.89	0.89	0.97	0.97	0.86	0.89			
HTL/WL2	1.53	1.42	1.46	1.5	1.55	1.50	1.59			
IOD/OOD	1.68	1.64	1.64	1.43	1.46	1.59	1.65			
Malar space/F3	0.27	0.43	0.41	0.25		0.384	0.461			
Inter alveolar/alveolar	0.92	1.09	1.07	1.07	1.00	1.42	1.42			

diametr										
Alveolorbital/alveolar diameter	1.74	1.25	2.13	2.14	1.8	2.6	2.16			
Inter ocellar distance/ocellar diameter	2.46	2.38	2.35	2.2	2.51	2.71	2.53			
Length of mesoscutem/width of mesoscutum	0.82	0.84	0.84	0.86	0.85	0.79	0.88			
Length of eye/scape length	1.96	1.88	1.93	1.93	1.76	1.98	1.98			

to construct phylogennetic hypothesis on Neotropical stingless bees of genus *Trigona* (Rasmussen and Camerago, 2008).

5.3. NEST ENTRANCE ARCHITECTURE

The nests of stingless bees differ from honey bees as they construct nests in closed habitat rather than open areas, and their nest is also provided with a characteristic entrance for the landing of foraging bees. The present study from Kerala during 2016-2018 reveals the presence of stingless bees in all the districts of Kerala. The bees were sampled from the coastal areas, plains, and hilly regions of the state to obtain maximum diversity.

The nesting habitats of *T. iridipennis* observed during the study were in stone walls, mud walls, tree hollows, iron pipes, meter boards, laterite walls, hollow bricks and building foundations. Similar results were reported by (Pavithra *et al.*, 2013; Ramya, 2014; Virkar *et al*, 2014; Roopa *et al*, 2015; Jose, 2015). One of the colonies were obtained from an unused raincoat from a car porch, (Jose, 2015) also observed the same habitat in the offseason during his study.

The nesting habitat preference of *T. iridipennis* was more towards the wall cavities than that of tree cavities (Danaraddi *et al*, 2012; Pavithra *et al*, 2013; Patel and Pastagia, 2016). Jose (2015) identified 18 trees which harbor stingless bee nests including *Tectona grandis, Artocarpus heterophyllus,* and *Mangifera indica*. The current study also reports Ven teak (*Lagerstroemia microcarpa*) as a nesting site for *T. iridipennis*.

Willi and Michener (1973) reported that, two species of stingless bees *Tetragonisca angustula* and *Trigona fulviventris* rarely forms nests with two or three entrances. Roubik (2006) stated the existence of multiple entrances in some genera such as *Lepidotrigona, Scaptotrigona, Plebia, Tetragona*, and

Hypotrigona, and these multiple entrances cannot consider as a consistent character in these genera. Benziger *et al*., (2009) observed two exceptional nests of minute stingless bees, *L. cacciae* and *L. furva* having two nest entrances for a single colony. The nest entrances of *Pariotrigona klossi* obtained from calcareous rocks were having several tubelets (highest reported were with 300 tublets) arranged on interconnected clumps (Benziger *et al*, 2011). Such clumps were observed only in one of the five colonies (colony which was with five entrance tubes had originated from a clump) during the current study. There were no clumps observed for the nests with two entrances but each tube was very closely constructed or merged partially at the base. It may be due to increase in the stability of the nest entrances as the number of entrance tubes increase. Divya *et al*. (2016) observed two nests of *T. iridipennis* each with two entrances from Poojappura and Tholicode of Kerala. Jose (2015) observed two separate entrance funnels in some *T. iridipennis* nests.

The behavior of constructing multiple entrances can be considered as a defensive strategy of the stingless bees as a mechanism to avoid the huge foraging traffic in the strong colonies. The building of large entrance may result in a variation in the microclimate of the bee nest, which may adversely affect the colony and that may serve as the reason behind why they construct more than one entrance instead of one big entrance. It is also difficult to manage the intruders as the size of the nest entrance increases and at the same time, an efficient distribution of guard bees can be done by dividing the nest entrance and can make a colony which is more defensive. All the nests mentioned were obtained from stone walls of the base of the building and all of them were free from harsh weather and less disturbed. Benziger (2011) stated in order to construct complex nest entrances they need to be protected well and the nests of *Pariotrigona klossi* obtained from crevices of limestones were protected by overhanging rocks.

Multiple exit holes and platforms were observed in nest entrance of *Tetragona clavipes* and the species also varied with broad and lamellate nest entrances (Roubik, 1979). Roubik (2006) reported construction tubercles or hollow tubes around the nest entrance of *Lestrimelitta* and occurrence of dual entrances in colonies of *Meliponula ferrugenia* and *L. ventralis*. Jose (2015) reported a *Tetragonula* colony entrance from an artificial hive which is having two openings in a single entrance tube and guard bees were present in both openings. In the current study, we observed single entrance tube with multiple opening in a natural condition (in the feral colony), but none of them were functional except the opening at the apex of the tube (Fig.9).

The shape of nest entrance of *T. iridipennis* varied from slit-like, circular, oval, funnel-shaped and the feral colonies are dominant with slit and circular nest entrance apex (Pavithra *et al*., 2012; Jose, 2015). The shape of entrance apex was similar for each and every opening of an entrance tube of a colony, and it varied among colonies observed in this study from slit-like and oval to circular.

The nest entrance length of *T. iridipennis* was previously reported in a range of 0.6 cm to 1.8 cm by Roopa *et al*. (2015), 1.0 cm to 10.26 cm by Jose (2015) and 0.5 cm to 9 cm by Ramya *et al*. (2015). *Tetragonula* colonies which did not have an entrance tube was also observed (Jose, 2015) from Kerala. The length of the entrance of colonies observed here varied from 2.1 cm to 7.6 cm and one colony displayed only a slit-like opening.

In addition to environmental cues, the pheromone-regulated nest mate interaction also acts as a stimulus for nest building, when these stimuli become more complex and vigorous it changes the nest building behavior and results in the formation of novel building action (Pavithra *et al*, 2013).

The nesting habitats of the genus *Lisotrigona* are not well known. Jobiraj (2004) recorded *L. mohandasi* from the flower of *Tectona grandis*. *L. carpenteri* nested in brick walls, crevices of rocks, trees and human constructions (Chinh *et al.*, 2005). Viraktamath and Jose (2017) recorded nests of *L. chandrai* from hard laterite rocks, tree cavities, and foundation walls. The current study records nest of *Lisotrigona* bees on Teak (*Tectona grandis*), Jack tree (*Artocarpus heterophyllus*), bamboo, brick walls, hollow bricks, and stone walls which are identical to the findings mentioned in previous records. All the habitats of *L. cacciae* collected from Kerala were closer to the forest ecosystem with considerable anthropogenic activity. These findings are supported by (Banziger *et al.*, 2009) who recorded *L. cacciae* and *L. fulva* from primary forest, secondary forests and from synanthropic habitats.

The distinct nature of nest entrances of genus *Lisotrigona* can be used to differentiate it from normal nest entrances of stingless bees of the genus *Tetragonula*. This is supported by the findings of Lima *et al.* (2013). Kelly *et al.* (2014) mentioned stingless bees have distinct nest entrance at the genus level. The variations observed in the nest entrance of stingless bees of Kerala in their design and various metric values such as length of entrance tubes, length and width of entrance apex, number of guard bees, the height of nest entrance from mean sea level is similar to a study of Suriwanto *et al.* (2017).

The number of guard bees of *T. iridipennis* varied considerably from one colony to the other. Similarly, guard bees of *Tetragonula* sp. nov. 2 also varied from 5-12. The mean value of guard bees observed in *T. iridipennis* nest entrance was 5.9 and that of *Tetragonula* sp. nov. 2 were 8.25, whereas Jayalekshmi (2015) reported that the mean value of guard bees of *T. iridipennis* ranged from 6 to 9. The findings of Jose (2015) confirmed that, the number of guard bees varied with season and different time of a day. They were recorded in more numbers at honey

flow season compared to dearth periods. Divya (2016) recorded a maximum number of 20, and a minimum number of 4 guard bees from the midland location of Kerala.

Couvillon (2007) reported that there is a direct relationship between aggressiveness of colony and the width of entrance opening with respect to the number of guard bees. The mean value of guard bees of *Tetragonula* sp. nov. 2 noticed was more than that of *T. iridipennis* and this may be the reason for their more aggressive nature.

The guard bees of *L. cacciae* were shy enough to come out and they moved back into their nest when slightly disturbed. The number of guard bees ranged from 2 to 4 in seven colonies located. Couvillon (2007) categorized bees based on a number of guard bees as, few guards (1-2), several guards (3-5) and many guards (6 and more), and recorded those colonies with few guards as quite timid. Viraktamath and Jose (2017) observed 2-3 guard bees in the nest entrance of the *L. chandrai*. He also noticed the calm behavior of bees while disturbed.

The shapes of nest entrance of stingless bee colonies have a great role in managing the foraging traffic as well as defending the colony from various intruders. The shapes with larger entrances can avoid overlapping of foraging bees and they also show increased defensivity (Beismiejer *et al.* 2007). The most frequent nest entrance design observed in *T. iridipennis* during the study was slit-like entrance (26.47 %) followed by an oval (17.64%), heart (16.17%) and round shapes (14.70%). Similar findings were reported by Pavithra *et al* (2013) where she mentioned *T. iridipennis* from Karnataka preferred elliptical oval shape than that of round entrance. Jayalekshmi (2015) found that, stingless bees of southern Kerala preferred round entrance more than that of any other shapes. The findings of Divya (2016) is also similar to the current finding wherein she reported that,

slit entrance (73.33%) are more preferred by stingless bees of Kerala followed by round entrance (20%). However, percentage margin of the slit to round entrance is very large in findings of Divya (2016) than that of the present work. It may be due to the limited study area of the former work. Jose (2015) also observed the dominance of slit entrance over circular and oval shapes. Similar nests were also recorded during the current study but there was no report of toad mouth, triangular, and tapetum shaped nest entrances of the *T. iridipennis* from Kerala. The toad mouth shape considered as an extraordinary design for stingless bees nest entrance (Couvillion, 2007) was also recorded for *T. iridipennis* during our study. The nest entrances of *Tetragonula* sp. nov. 1 observed was of slit and square shape. Slit-like nest entrances are noted to be the most common type of design in *T. iridipennis* (Pavithra *et al*, 2013; Jose 2015, Divya, 2016), whereas square entrances were not mentioned anywhere in previous studies. The shape, rigidity, and color of nest entrances varied within the species of *Tetragonula* (Suriwanto *et al*., 2017).

The nest entrance characters of *Lisotrigona* are not much known except the report on *L. carpeteri* by Chinh *et al.,* (2005) and report on *L. chandrai* by Viraktamath and Jose (2017). The nest entrance *L. carpenteri* was observed as brownish and brittle (Chinh *et al.,* 2005), and that of *L. chandrai* was recorded as having black resinous material on entrance tube (Viraktamath and Jose, 2017). The findings the of our study matches these studies wherein, the shape of entrance tube were cylindrical, short and with round apex having variation in color of entrance tube (pale yellowish white, reddish brown and pure black from a different location).

The length and width of entrance tube greatly varied in different colonies of *T. iridipennis*. An average length and breadth of entrance mouth observed was 1.08 cm and 1.44 cm, respectively in *T. iridipennis*. This result is in line with

findings of Pavithra *et al.*, (2013) where she reported that, *T. iridipennis* preferred an entrance opening of 0.8 cm to 1.4 cm over small and large openings. Jose (2015) reported that, the diameter of nest entrance of *T. iridipennis* ranged from 1.03 cm to 3.13 cm. Observations of Roopa *et al.* (2015) was also in agreement with the current results wherein, length of entrance mouth ranged from 0.6 cm to 1.8 cm and width ranged from 0.3 cm to 1.4 cm. The width of entrance apex ranged from 0.3 cm to 1 cm in Karnataka (Ramya *et al.*, 2016). The length and width of entrance tube of *Lisotrigona* bees are not much aware, the studies on their nests are very limited (Chinh *et al.*, 2005). Chinh *et al.* (2005) stated the outer diameter of nest entrance of *L. carpenteri* ranged from 0.48 cm to 1.1 cm. The findings of Viraktamath and (Jose 2017) indicate the nest entrance apex of *L. chandrai* were small enough to hold 2- 3 guard bees.

In the present study, we got similar results, wherein the length and width of nest entrance apex ranged from 0.3 cm to 0.5 cm and 0.3 cm to 0.6 cm. The size of nest entrance varies according to the defensive nature, foraging traffic, climatic condition, and strength of colony (Beismiejer *et al*, 2007).

The lengths of entrance tube was found to vary from one colony to other. Few of the colonies observed were without an entrance tube (Cryptic). These type of nest entrances were previously recorded from the genera *Austroplebia, Celetrigona, Dolichotrigona, Freisella, Geotrigona, Hypotrigona, Leurotrigona, Liotrigona, Lisotrigona, Melipona, Meliponula, Mourella, Paratrigona, Plebia, Schwarziana, Schwarzula, Trigonisca* (Roubik, 2006), and *T. iridipennis* (Jose, 2015). The range of length of entrance tube observed in *T. iridipennis* nests was 0 to 13.5 cm. The results of Jayalekshmi (2015), Jose (2015) and Ramya *et al.*, (2015) revealed that nest entrance length of *T. iridipennis* varied from1.56 cm to 5.48 cm, 1.06 cm to 10.26 cm and 0.5 cm to 9 cm respectively.

The length of entrance tube reported by Danaraddi (2009) was 10.88 cm and 9.66 cm. Danaraddi (2007) and Roopa *et al.* (2015) reported that, the entrance tube length ranges from 0.56 cm to 1.45 cm and 0.6 cm to 1.8 cm which was found to be different from the current results ontained. This may be due to the existence of wide range of plasticity in the construction of nest entrance (Sakagami, 1983), even though stingless bees shows some degree of specificity towards a particular taxon (Willie and Michener, 1973).

The nest entrance length of *Tetragonula* sp. nov. 1 was observed as 3.4 cm and that of *Tetragonula* sp. nov. 2 ranged from 0.5 cm to 4.2 cm. The differences in entrance tube length of three species supported by findings of Kelly *et al.*, (2014) where he found entrance tube length of *T. itama, T. thoraccica, T. terminata* and *T. laeviceps* varied as 7.84±7.39 cm, 7.38±3.65 cm, 7±2.02 cm, and 4.25±1.75 cm, respectively. Similar findings were given by Suriwanto *et al.* (2017) in nest entrance of *T. biroi*, *T. sapiense*, *T. fuscobalteta* and Silva *et al.* (2018) in the nest entrances of *T. iridipennis* and *T. praeterita*.

The nest entrance length of *Lisotrigona* in the present study ranged from 0.3 cm to 2.3 cm which shows close conformity with findings of Chinh *et al.*, (2005) who observed entrance length of *L. carpenteri* in a range of 0.5 cm to 5 cm. The description of Viraktamath and Jose (2017) on *L. chandrai* entrance also supports the present findings.

The height at which nests constructed are very important, as factors such as wind, rain, parasites, and predators have a great effect on the existence of a colony. The finding of Pavithra *et al.* (2017) shows that *T. iridipennis* preferred middle elevation for construction of nest and 47 % of the colonies were observed in a range of 11-15 feet (3.048 m- 4.572 m). However, our results show that, 51 per cent of the colonies found at a height of 1- 2 m above the ground level and

36.47 per cent of the colonies are at a height below 1 m. This may be due to the change in physiographic condition, climate and difference in the incidence of intruders in the two study areas.

The four species *T. hockingsi, T. carbonaria, T. mellipes* and *T. devenporti* of *Tetragonula* complex were identified based on the difference in their brood combs and nest architecture (Brito, 2014).

Summary

6. SUMMARY

The investigation on "Biosystematic studies on stingless bees (Apidae: Meliponini) of Kerala" was implemented in three stages-collection of stingless bees from various parts of Kerala state; identification of specimens through morphological characterization, micrometry and molecular analysis; documentation of nest entrance architecture. The project was carried out in College of Agriculture, Vellayani with an objective of studying the stingless bee diversity of Kerala and documenting their nest entrance architecture. A total of 225 colonies were sampled from all districts of the state and specimens collected were critically examined for morphological differences by preparing permanent slides. The bees sorted out through morphological characters were subjected to micrometric analysis as well as DNA sequencing.

The results of the study are summarized as follows. A total of 225 stingless bee colonies were sampled from an altitude range of 8 m to 1064 m from mean sea level. The majority of the stingless bees recorded were the common stingless bee of Indian sub continent, *T. iridipennis*. This bee was widely distributed all over the state and recorded from all the fourteen districts of the Kerala state. The minute stingless bee species *L. cacciae* also recorded from Kasaragod, Kannur, Thrissur districts, and current study extended the range of distribution of this species to Malappuram district also. In addition to *T.iridipennis* and *L. cacciae*, two new species of bees were identified through morphological characterization, *Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2. They were described and illustrated by photographs. Major morphological differences observed in the general appearance of the bees were, color and banding on the clypeus, facial hairs, shape of antennal comb and hair pattern in the mesoscutum.

The results of Principle component analysis of morphometric data arranged the two new species (*Tetragonula* sp. nov. 1, *Tetragonula* sp. nov. 2) into distinct clusters supporting their new species status. The partial sequencing of the Mitochondrial COI gene also supported the identity of new species described, where, the inter-specific genetic variation of 554 DNA base pairs against *T. iridipennis* and was in a range of 9.02-9.94 per cent and 19.49-20.76 per cent in *Tetragonula* sp. nov. 1, and *Tetragonula* sp. nov. 2, respectively. Intra-specific variation observed was 0.36 per cent, 0.54-1.62 per cent and 2.71 per cent for *Tetragonula* sp. nov. 1, *T. iridipennis* and *Tetragonula* sp. nov. 2 respectively.

A total of 3302 wings were examined from 69 different stingless bee colonies. The results confirmed 10.54 % variation from the normal number of hamuli (5 hamuli in each wings) in *T. iridipennis*. Asymmetric nature of wing hamuli was observed among colonies as well as within the colonies in *T. iridipennis*. The hamuli variations observed were in both the wings were 5*6, 6*5, 6*6, 4*5,5*4, and 4*4 hamuli. These four combinations show asymmetry in both right and left wing of the bees. The highest percentage of asymmetry observed were 5 hamuli in left wing and 6 hamuli in right wing. Highest percentage of hamuli variation recorded from colonies sampled from Ambanad estate (Kollam) followed by Balaramapuram (Thiruvananthapuram), and Kuranghan chola (Malappuram).

The feral nests of stingless bees are recorded from various habitats such as tree hollows, bamboo, stone walls, mud walls, foundations of buildings, roof, lateritic rocks, hollow brick walls, meter boards, iron pipes, bridges, and raincoat. All the nests harboring trees were live. *Tectona grandis*, *Artocarpus heterophyllus*, *Ficus religiosa*, *Lagerstroemia microcarpa* (Ven teak), Bamboo and *Mangifera indica* were recorded as nesting habitat for *T. iridipennis*. The *Lisotrigona* nests were recorded from *Tectona grandis* (Teak), *Artocarpus*

heterophyllus (Jack tree), bamboo, stonewall, laterite wall, hollow bricks, and brick wall.

The nest entrances of stingless bees varied widely throughout the state. The maximum variation was observed in the nests of *T. iridipennis*. The most preferred nest entrance design recorded was slit like entrance over oval, heart and round shape. The most unfamiliar nests recorded for *T. iridipennis* was triangular, toad mouth, and tapetum shaped nest entrances. The multiple entrances are also noted from six different locations of the state. Except one colony from Kadannamanna (Malappuram), all colony were with two nest entrances, whereas former one was with five entrance tube for a single colony.

The design of nest entrance recorded for *Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2 was square and round shaped, respectivly. The nest entrances of *L. cacciae* recorded were small round and was almost consistent in their dimensions, whereas color variation recorded in the nests of these bees may be due to use of different materials for nest construction.

The number of guard bees varied from 2 to 18, 5-12, and 2-4 in *T. iridipennis*, *Tetragonula*. sp. nov. 2, and *L. cacciae* respectively. The guard bees of *Tetragonula* sp. nov. 2 displayed more agressive behavior than that of *T. iridipennis*, and *Tetragonula* sp. nov. 1. The bees of *L. cacciae* behaved differently. When we approach the nest entrance and tap the nearby area of the nests, they retract inside the nests. The *L. cacciae* bees collected from KFRI Thrissur were attracted towards the eyes and sweaty body.

Considerable variation was observed in the nest entrance length, entrance mouth width and entrance mouth length in the bees of *Tetragonula* genus. *L. cacciae* showed more consistency in their nest entrance architecture. The entrance length preferred by *T. iridipennis* was less than 1 cm. The observation on the 54

feral colonies showed a preference of constructing nest at a height less than 2 m from the ground level. The average height of nest recorded for *L. cacciae* is 1.74 m from the ground level.

Internal nest architecture also varied in the four bees discussed above. The colony of *Tetragonula* sp. nov. 1 showed characteristic arrangement of brood cells in layers one after the other. The layers looked like arch like vertical combs connected by few pillars. Each layer could be separated out easily. The internal nest architecture of *L. cacciae* were differed from that of the *Tetragonula*, wherein, very small brood cells and honey pots were arranged and the density of bees were also very less. Their honey pots appeared white white. The current study doesn't focus on the internal architecture of the stingless bees.

Our study has opened a window towards the two new stingless bee species from Kerala. There is immense scope for undertaking studies on their biology and honey production potential, its quality analysis and medicinal properties. The pollination potential of these bees have to be explored and assessed. The observations on asymmetry in wing hamuli of *T. iridipennis* has to be studied, as the number of hamuli has a direct relation with flight capacity of insects.

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Abstract

BIOSYSTEMATIC STUDIES ON STINGLESS BEES (APIDAE: MELIPONINI) OF KERALA

by

FASEEH P

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ABSTRACT

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ABSTRACT

The study entitled "Biosystematic studies on stingless bees (Apidae: Meliponini) of Kerala" was conducted during the year 2016-2018 at the Department of Agricultural Entomology, College of Agriculture, Vellayani with the objectives of studying the stingless bee diversity of Kerala and to document their nest entrance architecture.

A total of 225 different colonies of stingless bees were sampled from all districts of the state. The sampling altitude ranged from 8 m (Kakkanad, Alappuzha district) to 1064 m (Pampadumpara, Idukki district) above mean sea level.

Two new species (*Tetragonula* sp. nov. 1, and *Tetragonula* sp. nov. 2) of stingless bees based on adult worker specimens are described and illustrated with the help of photographs. They are compared morphologically and molecularly with closely related species. Differences in morphology based on principal component analysis and genetic analysis based on partial sequences of the mitochondrial COI gene barcode region support the recognition of the two new species.

Along with the description, an analysis of their phylogenetic relatedness is provided. The genetic analysis reveals that, *Tetragonula* sp. nov. 1 is closely related to the common species *T. iridipennis* whereas *Tetragonula* sp. nov. 2 is distantly related. The inter-specific genetic variation observed between *T. iridipennis* was in a range 9.02-9.93% and 19.49-20.76% for *Tetragonula* sp. nov. 1 and *Tetragonula* sp. nov. 2 respectively.

Out of various morphological characters observed, the number of hamuli was found varying within the *T. iridipennis* colonies. The current study revealed that, there is 10.54% variation in the number of hamuli among the 1651 number of individual bees studied. The normal number of hamuli recorded in this species is 5 on each wing but it varied from 4 to 6 on both wings and also exhibited asymmetry between the left and right wings.

The nest entrances of stingless bees varied widely within the colonies of *Tetragonula* spp. The variation of nest entrance between the genera *Tetragonula* and *Lisotrigona* were more prominent. It was observed that, the nest entrance

shape of *Tetragonula* sp. nov. 1 was square whereas it was round in *Tetragonula* sp. nov. 2. The most common form preferred by *T. iridipennis* was slit like entrance over oval, heart, round, and arch shaped ones. Length of the nest entrance mouth preferred by *T. iridipennis* was less than 2cm and the nest entrance width was recorded in a range of 1-2 cm. Out of 54 feral colonies of the *T. iridipennis* studied, 24 were located at a height less than 1m from the ground level, 25 were located at a height of 1-2 m and 5 colonies were located more than 2 m above the ground.

Two stingless bee species. *viz.*, *T. iridipennis* (Smith) and *Lisotrigona cacciae* (Nurse) were the only records from Kerala before this study. The discovery of two new species during the present study elevates the total stingless bee fauna of Kerala from 2 to 4 species. As the number of hamuli shows 10.54 % variation, this character should be relied upon with caution while distinguishing species.