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Molecular probe, colony structure and SEM of antennal sensillae substantiate intermediate workers of *Oecophylla smaragdina* (Fab.) as typical worker

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ABSTRACT: The polymorphic colony of arboreal weaver ant *Oecophylla smaragdina* Fabricius has three categories of workers designated as typical, major and minor. The colony structure fluctuated sharply with seasons but the numerical ratio of worker castes always remained as 65:25:10. Typical workers have highest number of sensillae/unit area on the terminal segment of antenna and all the above characters established their role in the colony different from major and minor workers. Microsatellite DNA analysis of worker castes indicated high degree of genetic diversity, heterozygocity and genetic polymorphism among three worker castes, reproductive males and females. Mitochondrial DNA analysis proved that all the three categories of worker castes were developed from eggs of a single queen.

KEY WORDS: *Oecophylla smaragdina*, typical worker, molecular probe, SEM antennal sensillae

INTRODUCTION

The weaver ant *Oecophylla smaragdina* Fab. forms large arboreal colonies in Tropical Asia and Australia. It represents a spectacular example for eusocial complexity and plays important role in our ecosystem as an aggressive predator, scavenger and symbiont (Holldobler and Wilson,1983). Adults and brood of these ants form an unconventional, cheap, highly nutritious food and a good medicine among ethnic communities all over the world (DeFoliart,1992) and also among tribes of Kerala (Vidhu and Evans, 2015). Their caste system contain three different types of apterous workers, winged males and winged females. Workers differ in their size, body proportion and in their tasks. The major workers do most of the external work for the colony, such as nest building, foraging, defending and exploring new territory. The minor workers are responsible for taking care of the eggs and young larvae (Holldobler and Wilson, 1990).

Both morphology and internal factors like physiological state and genetic makeup influence division of labour among colony and behavioural specialisations. Genetic studies on social insect groups strongly support the relationship between colony diversity, task specialisation and colony efficiency. Even though many reports have shown that *O. smaragdina* possessed only two types of worker castes (Holldobler,1983; Holldobler and Wilson,1977; Holldobler and Wilson1990; Lokker,1986) we could identify a third category of worker caste with clear difference in morphology and genetic makeup and it was termed as intermediates (Vidhu and Evans, 2011a). Body size, protein profile in SDS-PAGE, amount of formic acid in their poison gland (Vidhu and Evans, 2011b) and

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volatile compounds in their Dufour,s gland (Vidhu and Evans, 2015) have very well attested that the intermediates are unique and distinct from other two worker castes. Molecular probes, study of colony structure and SEM of antennal sensillae on the intermediate workers were under taken and the results are presented in this paper.

MATERIALS AND METHODS

Nests from a single colony were collected from the University College Campus, Thiruvananthapuram and anaesthetised. Three types of workers were separated, washed in distilled water, blotted with tissue paper and used for the study.

Colony structure: Large permanent nests of *O.smaragdina* of approximately 20 cm diameter were plucked from the tree with the branch itself and immersed in a wide mouthed jar containing cotton soaked in chloroform. After10 minutes, the dead ants were transferred in to a tray. Each type of ants was separated and counts were recorded.

Antennal sensillae studies of different worker castes: Morphology of antennae of all different individual types of the colony was observed using binocular dissection microscope with magnification of 4x. (Magnus, MS 24,India) and photographic images were recorded. Scanning Electron microscopic pictures of terminal segment of antennae of the three types of workers were taken. Sensillae identification was made as described by Gullan and Craston, 2005; Martin *et al.,*2011; Hartenstein, 2005 and Euzebio *et al.,* 2013. Distribution of sensillae on terminal segment of antennae was studied in all the castes by counting number of each type of sensillae per 60 µm × 60 µm area.

Scanning Electron Microscopic analysis (SEM): Antennae were cut and fixed in 3% glutaraldehyde buffered with 0.1 M phosphate buffer at room temperature or 0-4^0 C (minimum 2-4hours or maximum 24-48hours). The fixed sample was immersed in 1-2% Osmium tetroxide in 0.1 M phosphate buffer pH 7.2 (2-4 h) at room temperature and in an opaque container. Washed in 0.1 M phosphate buffer pH 7.2 (3 x 10 min.). Dehydrated in grades of ethanol (15-30 min each).Critical Point Drying was done. The antennae were then mounted on a brass stub and Gold sputtered for 2 min (SPI-Module Gold Sputter Coater).Observations were made using a JEOL JSM-5800VL SEM.

Microsatellite DNA Fingerprinting: Microsatellite DNA finger printing for 5 microsatellite loci (Table.1) was done (Shluns *et al*., 2011; Shimizu *et al*., 2002; Schuelke, 2000) in three types of workers collected from 10 permanent nests in a single colony of *O. smaragdina*. Genomic DNA was isolated from single specimens using DNeasy® Blood and Tissue Kit (Qiagen) following manufacturer's instructions. Agarose Gel Electrophoresis for DNA Quality check was done.PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (contains 1.5 mM $MgCL_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 10ng DNA, 0.4 µl of PhireHotStart II DNA polymerase enzyme (Thermo scientific), 0.1 mg/ml BSA, 1pM of M13-tailed forward primer, 5 pM of reverse primer and 5pM of FAM-modified universal M13 primer. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). For capillary Electrophoresis of PCR Products ,one micro litre of the PCR product was added to 10 ìl Hi-Di formamide (Applied Biosystems) and 0.5 ìl Gene Scan 500 Liz - Size Standard (Applied Biosystems) and run on the ABI 3500 Genetic Analyzer. Data was analysed using Gene Mapper ID-X v1.4 software.

Allele frequency including allele number, inbreeding coefficient heterozygosity, gene diversity, polymorphism information content (PIC), frequency based genetic distance and stepwise patterns for microsatellite data were calculated using Power Marker® software.

DNA barcoding using universal primers of Cytochrome oxidase 1 (Cox1): Genomic DNA was isolated from single specimens using DNeasy® Blood and Tissue Kit (Qiagen) following manufacturer's instructions. Thorax of 3 types of workers were taken as sample by removing the head and abdomen using a sharp blade .The tissue were cut into small pieces and placed in a 1.5 ml

micro centrifuge tube. 180 µl of ATL buffer and 20 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 AL buffer and 200 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm for 1 minute. The DNeasy mini spin column was transferred to a new 2 ml tube and washed with 500 µl of AW1 buffer. Washing step was repeated using AW2 buffer. After washing the DNeasy mini spin column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of AE buffer. Agarose Gel Electrophoresis for DNA Quality check was done. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (150mM TrisHCl , pH-8; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.0mM $MgCl_2$, 20ng DNA, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.15 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5 pM of forward and reverse primers (Folmer *et al.,* 1994).

After Agarose Gel electrophoresis of PCR products, ExoSAP-IT Treatment was done. 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 sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1.

Statistical analysis: Analysis results were done using Microsoft excel tools. Microsatellite data analysis was done using Power Marker soft ware.

RESULTS

Major workers are the largest among the workers with body length ranged from 10.0 to 11.0 mm with mean body weight of 11.8 mg and it will be clear from Table.II and Figure.1a. They constituted 25% of total number of worker ants in a permanent nest.

Table.1.Primers used for Microsatellite Analysis

No.	Name	Sequence
1	MS6.7F	**TGTAAAACGACGGCCAGT**AGAGGGCACACATCCAACTAC
	MS6.7R	CATCGTAAGGAGAATTTTCGT
2	MS7.4F	**TGTAAAACGACGGCCAGT**ATTGCCGAGTGAAAGAGGAAC
	MS7.4R	AACTTCGCAGAATGACGAGTC
3	Osma101F	**TGTAAAACGACGGCCAGT**ACCTTACGATCGTGGCAG
	Osma101R	AATACTCCGTGACAATCC
4	Osma37F	**TGTAAAACGACGGCCAGT**GAATCCAGACCCGACGAACG
	Osma37R	CGAGAATCCGCCGCAATGAC
5	Ccon70F	**TGTAAAACGACGGCCAGT**GCATTAAAGTCGGGACGGAC
	Ccon70R	CAGATGCGAAGAGCTCGC

*Note: Forward primers are M13-tailed (**in bold**)*

Table 2. Primers used

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

The ratios of the length of head with thorax or head with abdomen were around the value of 0.5. (Table.3).Almost 65 % of worker ants in a colony was found to be intermediate forms (typical worker) and were found actively engaged in colony maintenance along with major workers. The typical workers are significantly shorter than the major workers. Their body length ranged from 7.9 to 8.7 mm with mean body weight of 6.3 mg. The ratio of length of head with thorax was below the value of 0.5, but the ratio of the length of head with abdomen was above the value of 0.8 (Table.3). Typical workers were almost double the length of minors and four times the weight of minors. (Table.3, Fig.1b.). Minor workers were very small when compared to major workers. They were mostly confined within the nests and rarely observed in the field. They constitute less than 10% in total number of workers. The body length of minor worker was found to be 4.3 to 4.9 mm (Table.3, Fig.1c).

The colony individuals showed marked difference in the shape, size and number of segments in their antennae (Fig.2). Antennae of three worker castes consisted of total 12 segments such as scape, pedicel and 10 flagellomeres. In major workers antennal length was 7.0 ± 0.2 mm and scape was very long compared to other workers. Intermediate workers possessed a medium sized antennae and length was 5.1 ± 0.1 mm. Minor workers possessed short stout antennae and the shape of terminal segment differed from other worker castes and it was rounded. Its length ranged between 1.9 mm to 2.1mm (Fig.2).

Three different worker castes showed significant difference in the number and distribution of sensillae on the terminal segment of the antennae .Scanning electron microscopic studies on different types of sensillae on the terminal segment of antennae of different castes of *O.smaragdina* has resulted in the identification of four types of sensillae (Fig.3) and they are,

1. Sensilla trichoidea type 1- ST_1
2. Sensilla trichoidea type 2- ST_2
3. Sensilla basiconica- SB
4. Sensilla ampullacea - SA

The shafts of Sensilla trichoidea type 1 (ST_1) are long, and narrow and tapering terminally. They vary in thickness, with diameter (near the base) of about 2-3µm and length of 11-13 µm. The Sensilla trichodea type 2 (ST_2) were long, tapered like curved hairs possessing an encircling and a middle

Table 3. Body dimensions of colony individuals of *O.smaragdina*

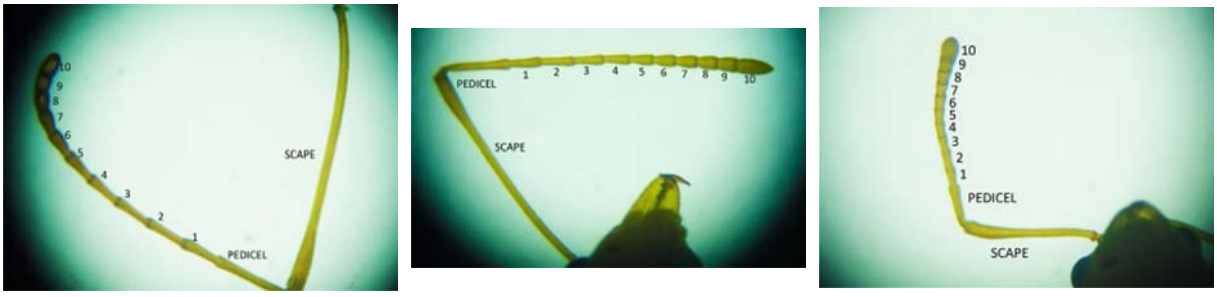
Sample	Worker castes		
	Major	**Intermediate**	**Minor**
Body length *			
Whole body	10.5 ± 0.5	8.3 ± 0.4	4.6 ± 0.3
Head	2.2 ± 0.2	2.2 ± 0.1	1.2 ± 0.06
Thorax	4.4 ± 0.3	4.2 ± 0.2	2.2 ± 0.1
Abdomen	4.1 ± 0.3	2.6 ± 0.2	1.9 ± 0.1
Body Width *			
Head	1.9 ±0.04	1.6 ± 0.03	1 ± 0.02
Thorax	1.1 ± 0.02	1.1 ± 0.01	0.7 ± 0.01
Abdomen	2.1 ± 0.05	1.7 ± 0.03	1.5 ± 0.01
Body Weight **			
Whole body	--11.8 ± 0.8	6.3 ±0.4	1.3 ± 0.08

* Value are expressed in millimetre n=10, ± SD

**Value are expressed in milligram, n=10, ± SD

a) Major worker (1x) b. Intermediate worker (1x) c) Minor worker (1.5x)

Figure 1. Three types of worker castes of *O.smaragdina*



a. Major b. Intermediate c. Minor

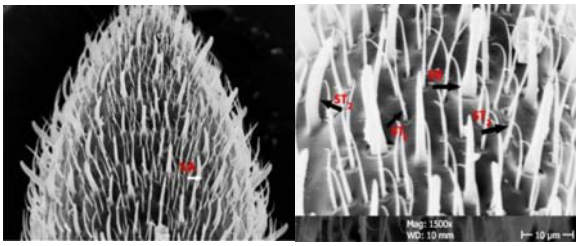
Figure 2. Antennae of three worker castes

cuticular ledges. They vary in thickness, with diameter (near the base) of about 1 to 2 µm and length of 13-14 µm. Sensilla basiconica (SB) type always consisted of two parts, a peg and a socket. The peg was porous on the distal end. They vary in thickness, with diameters (near the base) of about 3-4 µm and length of 13-15 µm. Sensilla ampullacea were characterized by prominent elliptical depression and a central opening and approximately 2 µm diameter.

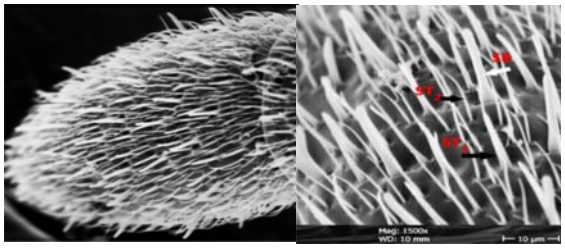
Among the three categories of workers, the most abundant sensilla on the tip of antenna was ST_2 followed by ST_1 and SB and number of sensilla type SA was the least one. The number and distribution of all the above four types of sensilla in three categories of workers, present in unit area, at the terminal antennal segment is shown in Table 4.The three categories of workers showed a significant difference in the number of sensillae among one another with highest density in typical workers. Shape of the terminal segment of antennae of major and typical workers were almost same but the density of sensilla distribution was very high in typical workers than that of major worker (Table.4). The shape of terminal segment of the antennae of minor worker was almost round (Fig.3) but that of other two worker categories, it was pointed.

Microsatellite DNA Fingerprinting: Microsatellite DNA finger printing was done in different colony individuals such as three types of workers, winged males and females of single nests of *O.smaragdina*. Five short sequence repeat markers were used for this study. Based on the microsatellite sequence analysis results, intra nest relatedness and frequency based genetic distance between five colony individuals in the colony were analysed using Power marker soft ware and the results were shown in Table.5 and Table.6. The results have clearly indicated significant genetic diversity and genetic polymorphism among three categories of workers and also between winged females and males.

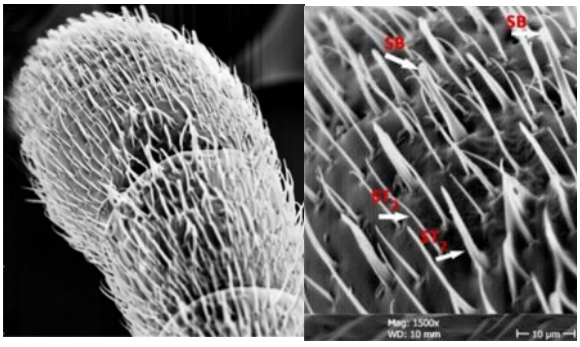
DNA Barcoding using universal primers of Cytochrome oxidase 1 (COX1): Amplified PCR products of universal primers of mitochondrial Cytochrome C oxidase 1 gene from three worker castes showed no significant difference. The Cytochrome oxidase gene sequences of three



a. Major



b. Intermediate



c. Minor

Figure 3. Sensillae on the terminal antennal segment of major, intermediate, minor workers of *Oecophylla*

workers were shown as Fig.4 which showed only single nucleotide substitution in minor workers.

DISCUSSION

Even though previous investigators have described only two categories of workers such as major and minor workers (Holldobler,1983; Holldobler and Wilson,1977; Holldobler and Wilson1990; Lokker,1986),careful observation has revealed that there are three categories of workers such as major, intermediate and minor, and they are exhibiting significant difference in morphology, biochemistry and genetic constitution and are not at all exhibiting, overlapping of body dimension such as length of the body and antennal length. During all seasons the distribution of workers in the colony remained constant with a numerical ratio of 25:65:10 as major, intermediates and minor respectively. Even though the colony structure such as presence of brood and reproductive forms are closely related with rainy season the numerical ratio of the worker castes remained constant throughout the year (Vidhu,2015).The previous investigators have described the intermediate category of workers together with major workers(Holldobler,1983; Holldobler and Wilson,1977; Holldobler and Wilson1990).

All major workers were with body length ranging from 10 to 11 millimetres, intermediate categories were between the length of 7.9 to 8.7 millimetre and minor workers were too much smaller than the other two categories. The three categories of workers showed clear difference in size and body proportions. Poison gland secretion of the three categories of workers showed sharp difference in

Table 4.

Density and distribution of Sensillae on the terminal antennal segments of different colony individuals of *O.smaragdina*

Different castes in the colony	Sensilla trichoidea		Sensilla basiconica SB	Sensilla ampulaceae SA
	ST_1	ST_2		
Major	11 ± 0.5	54 ± 3.2	5 ± 0.03	1 ± 0.01
Intermediate	17 ± 1.5	65± 4.3	8 ± 0.02	1 ± 0.09
Minor	11 ± 0.8	50 ± 3.0	4 ± 0.04	1 ± 0.01

All the values are mean ± SD, n=6.
Number of sensillae at unit area of 60µm×60µmwas presented

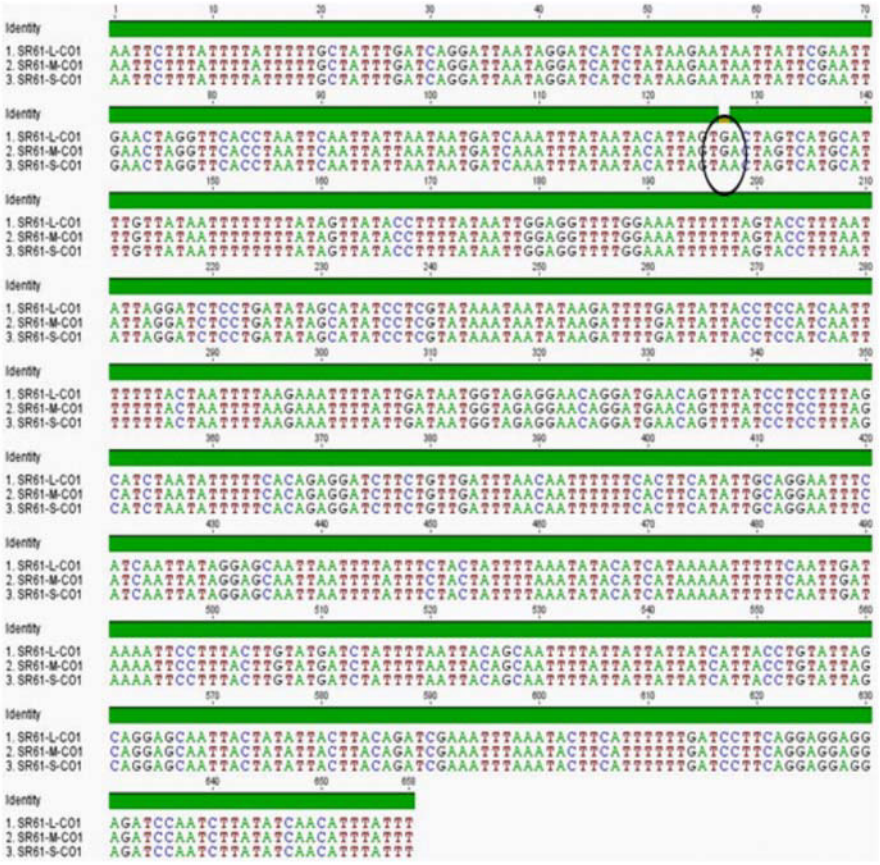


Figure 4. Sequence alignment- Cox 1

the formic acid and the amount of FA vary sharply under different ethological states (Vidhu and Evans, 2014). Analysis of the Dufour'sgland secretion of the three categories of workers proved the contrasting difference in the presence of 23 chemical compounds inintermediates,13 in major and 17 in minor workers (Vidhu and Evans,2015). Gel doc analysis of the protein profile of the head and thorax of three categories clearly showed marked difference in molecular weight of different bands. The head of major worker showed 13 bands, intermediates with 23 bands and minor with least number of bands as 11 (Vidhu, 2015). Presence of highest number of volatile compounds in intermediate category of workers showed their difference in role in the colony in communicating within colony mates and presence of additional enzyme system in then for the production of additional volatile compounds. The total protein content of three categories of workers showed significant variation and its content was highest in intermediates and lowest in minor workers. Electropheretic profile also showed sharp difference in head and thorax of three categories of workers. The intermediates even though appeared as miniatures of major workers they differed sharply in the quantity and quality of body proteins. Differential distribution of protein in the head and thorax of intermediates and major workers clearly indicated their genetic dissimilarity. The total content of protein was highest among intermediates and lowest among minor workers. This also supported the argument that the worker types in *O.smaragdina* colony are not two but three distinct categories (Vidhu and Evans,2011a). Lokkers (1986) has reported that the first few batches of eggs laid by the newly impregnated queen were developed in to small ants which are smaller than major workers and larger than minor workers. This was very well attested by the investigators that the dealated, green coloured, pregnant queen lived well camouflaged at the tip of the shoot, among tender

Table.5. Microsatellite DNA Fingerprinting- Power Marker Data

Marker	Major allele frequency	Allele no.	Gene Diversity	Heterozygosity	PIC	Inbreeding coefficient (f)
M1	0.4000	4.0000	0.7000	0.8000	0.6454	-0.0323
M2	0.7000	2.0000	0.4200	0.6000	0.3318	-0.3333
M3	0.4000	4.0000	0.7000	0.8000	0.6454	-0.0323
M4	1.0000	1.0000	0.0000	0.0000	0.0000	NaN
M5	0.7000	3.0000	0.4600	0.4000	0.4102	0.2381
Mean	0.6400	2.8000	0.4560	0.5200	0.4066	-0.0297

PIC- polymorphic information content

Table 6. Frequency based genetic distance among colony individuals

OTU	S1	S2	S3	S4	S5
S1	0.0000	0.4495	0.1273	0.5550	0.3521
S2	0.4495	0.0000	0.5322	0.2775	0.3521
S3	0.1273	0.5322	0.0000	0.6376	0.4347
S4	0.5550	0.2775	0.6376	0.0000	0.3750
S5	0.3521	0.3521	0.4347	0.3750	0.0000

leaves and the ants developed from first few batches were identical to intermediate category of workers in size and body proportion.

The antennae form the major sense organs for insect communication and survival, and the antennal sensillae receives stimuli for various behavioural modifications in the host such as mate selection, locomotion, foraging and defence which are in constant contact with the environment (Chapman, 1982). The antennae of all worker castes possessed scape, pedicel and 10 flagellomeres (total 12 segments) .Scapes with flagellomeres constitute antennomeres and thus females possessed 11 antennomeres. The type, abundance and distribution of sensillae on antennae depend on various behavioural aspects (Chapman, 1982). The terminal segment of antennae of different castes of *O.smaragdina* possessed two types of ST (ST_1, ST_2), SB, and SA. Sensillae density on the terminal segment of antennae was maximum in intermediate category of workers and the least in minor workers. The present study showed that minor workers were almost fully confined within the nest itself and were not participating actively with other two categories of workers for maintenance of territory, predation and defending invaders. Differential distribution of sensillae on the antennae of worker castes clearly indicated their dissimilar role in the colony. Different patterns of trichoid and basiconic sensilla numbers were described in different populations of *Rhodnius prolixus* sampled from east and west of the Andes Mountains. These differences suggest that the geographical isolation of the populations was associated with the numbers of antennal sensillae (Esteban *et al*., 2005).Variations in sensory organs between two populations of *Atta robusta* may indicate an adaptation of this species to different environmental conditions (Euzebio*et al.,* 2013).

The different types of sensillae we have identified in *O.smaragdina* fully agreed with the previous studies (Martin *et al*.,2011). Sensilla trichoidea (ST_1,ST_2) was the most abundant sensillae in all the individuals within the colony of *O.smaragdina.* The adult major workers possessed ST_2 density of 60-66 numbers/3600 $\mu m^{2,}$ area at the terminal segment of antenna. Among three types of workers the density of ST_2 on the antennal tip was highest

among in intermediate category of workers among the trichoid sensillae, ST_1 (thick, curved at base) is considered as mechanoreceptors and ST_2 forms gustatory receptors (Baaren*et al*., 2007). The ampullacea sensilla of ant species *Atta* are considered to be associated with the detection of the CO2 concentration within nests (Kleineidam*et al.,* 2000) and these type of sensillae were found to be very less in *O.smaragdina* workers.

The relationship between colony task, body size and lineage appeared to be complex. Colony genetic diversity might improve division of labour by increasing the morphological or behavioural variation among workers (Crozier and Page 1985; Robinson 1992). Studies have been reported on a genetic component to worker size polymorphism observed in ant colonies such as *Formica, Acromeryrmex and Camponotus* (Frazer *et al.,* 2000; Hughes *et al*., 2003). Here the genotyping of three worker categories, for 5 microsatellite loci were done. Good levels of genetic diversity among 3 groups were obtained for 4 loci. The population diversity and allelic variability is indicated by polymorphic information content (PIC). The PICs ranged between 0.000 and 0.6454 with a mean of 0.4066 (Table.5). The data on allele frequency based genetic distance among 5 groups revealed that the typical or intermediate worker group has shown diversity from other groups such as major, minor, winged males and winged females as 0.4495, 0.5322, 0.2775, 0.3521 respectively. Interestingly the major, intermediate and minor groups display significant genetic distance from each other (Table.5, 6). High levels of gene diversity and heterozygosity also indicate genetic variability among each caste.

Cox 1 gene of mitochondrial DNA in *O.smaragdina* workers was more or less similar in mass and sequence data and it has revealed that the three categories of workers not exhibited any significant difference. These data will ultimately aid in investigations on dynamics of morphological and developmental evolution as well as biology of this social insect. Even though all the first few batches of eggs laid by the newly impregnated young queens developed in to small workers of intermediate size (Lokers ,1986),as the queens gradually matured, genetic polymorphism within the genes might have resulted to phenotypic polymorphism among workers.

On the basis of presence of secondary metabolite, formic acid secreted by the poison gland and also on the basis of the presence of volatile compounds in the Dufour's gland, the intermediate category of workers stood between the other two categories of workers such as major and minor as a unique one with special characters. Even though the three categories of workers are developed from the eggs laid by a single mother the intermediate category of workers maintained their own individuality among other workers by possessing certain peculiar features such as highest amount of body protein, significant variation in the amount of formic acid in their poison gland and highest number of volatile secondary metabolites in their Dufour's gland and highest number of sensillae (Vidhu, 2015; Vidhu and Evans, 2015). In our investigation it was well understood that the major population of workers are intermediates and it was always around 65% of the total number. The genetic variability within the different categories of workers has very well attested the existence of an intermediate category of workers which are not mere miniatures of major workers, but as a third category with different mode of chemical communication by Dufour's gland secretion. So it can be interpreted that the division of labour such as looking after the brood, colony maintenance and defence etc. develop only during the establishment of the new colony in to wider territory and original workers are intermediate category and major and minors are secondary modification through genetic polymorphism and differential expression of genes. So the intermediate category of workers can be designated as typical workers.

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