CHAPTER 4

MATERIALS AND METHODS

4.1. SURVEY, COLLECTION AND IDENTIFICATION OF TERMITE PEST

Northern part of West Bengal has eight districts (Alipurduar, Cooch Behar, Jalpaiguri, Kalimpong, Darjeeling, Uttar Dinajpur, Dakshin Dinajpur and Malda) and three important ecological zones (Hills, Terai foothills and Plains) which cover a total area of 21,763.0 sq km. Tea is grown mainly in the foothill regions of North Bengal comprising districts of Alipurduar, Jalpaiguri, Kalimpong and Darjeeling. Even in the district of Uttar Dinajpur people cultivate tea, however, their share in total tea production is meager. Therefore, out of above mentioned eight districts, Darjeeling, Jalpaiguri, Kalimpong and Alipurduar were taken in to consideration in the present study due to their larger share of plantation area and tea production.

4.1.1. Study area:

The study was carried out in the tea plantations of the Dooars, Terai and Darjeeling hill slope which is spread over in an area of 10,629 sq km. Collections were made from various plantations (Table 4.1). The tea plantations cover an area with distinct geographical variations. Plantations like Kalchini Tea Estate (T.E.), Hantapara T.E. or Sunkosh T.E. with lush green tea bushes of Assam variety are located in vast

stretch of plain of the Dooars dissected by several rivers. Whereas, plantations like Fagu T.E., Ambiok T.E., Longview T.E., Putharjhora T.E. and Samsing T.E. which are located at the foothills and lower elevations has a mixture of China and Assam variety of tea plants. The exclusive China variety of tea plants can only be seen in typical hill slope like plantations of Makaibari T.E., Castleton T.E., Ambootia T.E. or Happy Valley T.E.

4.1.2. Survey and Specimen Collection:

Surveys were undertaken during 2009-2017 in the tea plantations of Terai and the Dooars (Figure 4.1) of North Bengal comprising of four districts namely Darjeeling, Kalimpong, Jalpaiguri and Alipurduar. Termites were collected based on the technique described by Su and Schafferahn (1986) with some minor modifications. Infested areas of tea plantations were dug to locate underground termite colonies. For abundance study collections were made randomly. However, for pesticide tolerance study two commonly occurring termite species namely *Odontotermes obesus* and *Microtermes obesi* were collected selectively from different tea gardens. Since these two species belong to two discrete feeding guilds, they required specific collection methods as discussed below.

Modified Bucket Traps (MBT): A plastic bucket roughly measuring about 20 cm of diameters and about 30 cm of height (Figure 4.2) were placed underground and covered by a lid. Buckets were tightly filled with moistened carton (paper) pieces. The cellulose content of the carton pieces worked as the trap and attracted termites from surrounding, which congregated at large numbers to feed on the cellulose content of the carton. MBT was mainly used to collect live-wood eating termite *Microtermes obesi*

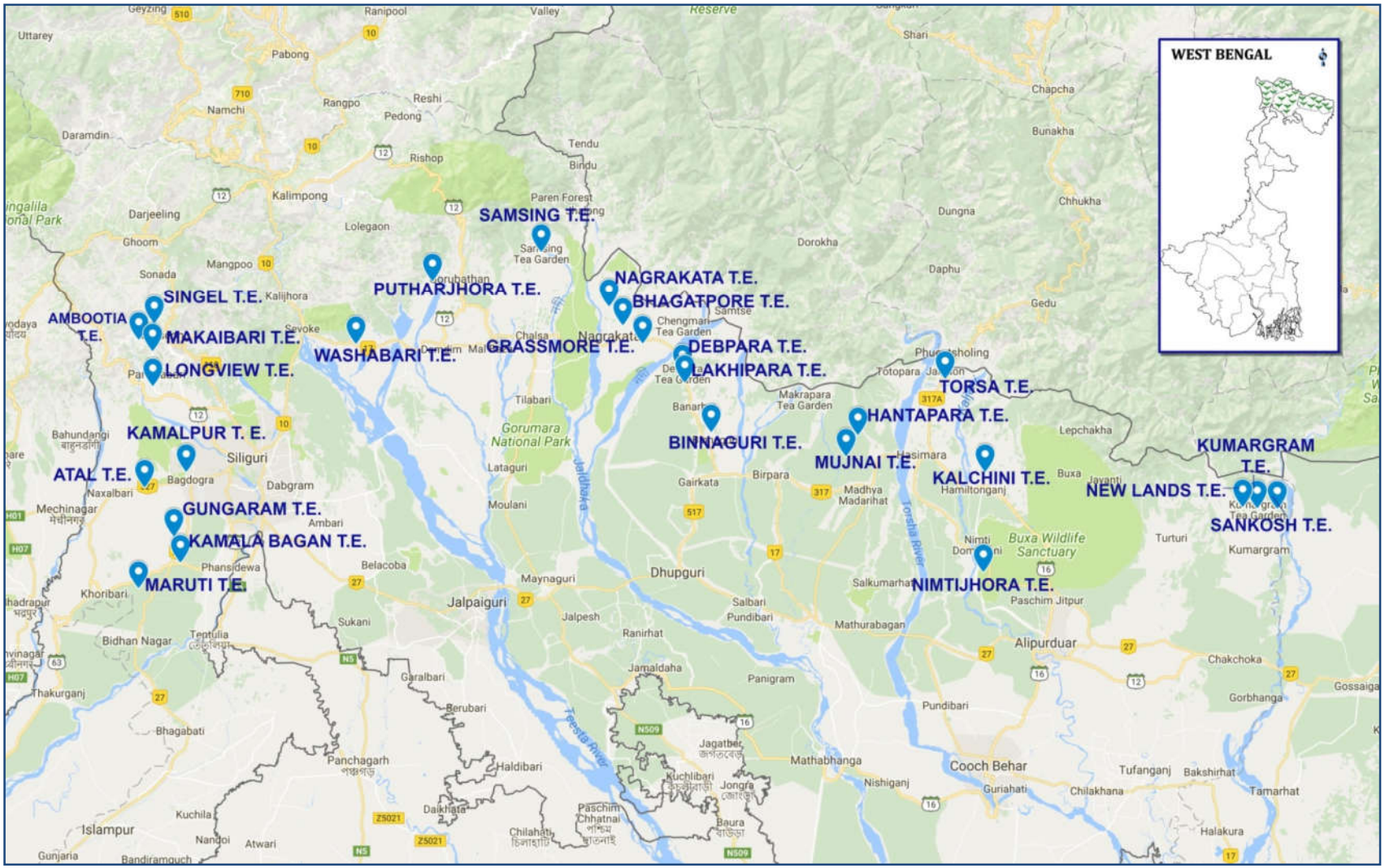

Figure 4.1: Tea Estates of Darjeeling Terai and the Dooars region visited for collection of termite specimen

Table 4.1: Tea Estates (T.E.) visited for collection of termite specimens during the study period

Ecological Zones	Tea Plantations	Management type	District	Geographical coordinates
HILLS	Ambootia T.E.	Organic	Darjeeling	26°87' N, 88°24' E
	Singel T.E.	Organic	Darjeeling	26°53' N, 88°15' E
	Makaibari T.E.	Organic	Darjeeling	26°51' N, 88°15' E
	Longview T.E.	Organic/Conventional	Darjeeling	26°48' N, 88°15' E
TERAI	Maruti T.E.	Conventional	Darjeeling	26°37' N, 88°10' E
	Atal T.E.	Conventional	Darjeeling	26°40' N, 88°15' E
	Kamalpur T.E.	Conventional	Darjeeling	26°42' N, 88°18' E
	Gungaram T.E.	Conventional	Darjeeling	26°37' N, 88°17' E
	Kamala bagan T.E.	Conventional	Darjeeling	26°34' N, 88°17' E
	Matigara T.E.	Conventional	Darjeeling	26°42' N, 88°23' E
	Tirrihannah T.E.	Conventional	Darjeeling	26°45' N, 88°16' E
	Panighata T.E.	Conventional	Darjeeling	26°45' N, 88°15' E
	Simulbari T.E.	Conventional	Darjeeling	26°47' N, 88°18' E
DOOARS	Washabari T.E.	Conventional	Jalpaiguri	26°52' N, 88°32' E
	Putharjhora T.E.	Organic	Kalimpong	26°56' N, 88°49' E
	Samsing T.E.	Organic/Conventional	Kalimpong	26°59' N, 88°48' E
	Nagrakata T.E.	Conventional	Jalpaiguri	26°54' N, 88°54' E
	Bhagotpore T.E.	Conventional	Jalpaiguri	26°53' N, 88°55' E
	Grassmore T.E.	Conventional	Jalpaiguri	26°52' N, 88°57' E
	Debpara T.E.	Conventional	Jalpaiguri	26°50' N, 89°00' E
	Lakhipara T.E.	Conventional	Jalpaiguri	26°49' N, 89°00' E
	Chamurchi T.E.	Conventional	Jalpaiguri	26°52' N, 89°04' E
	Binnaguri T.E	Conventional	Jalpaiguri	26°45' N, 89°03' E
	Hantapara T. E.	Conventional	Alipurduar	26°45' N, 89°14' E
	Dalmore T.E.	Conventional	Alipurduar	26°45' N, 89°09' E
	Mujnai T.E.	Conventional	Alipurduar	26°43' N, 89°14' E
	Torsa T.E.	Conventional	Alipurduar	26°49' N, 89°22' E
	Kalchini T.E.	Conventional	Alipurduar	26°42' N, 89°26' E
	Nimtijhora T.E.	Conventional	Alipurduar	26°34' N, 89°26' E
	Kumargram T.E.	Conventional	Alipurduar	26°39' N, 89°49' E
	New Land T.E.	Conventional	Alipurduar	26°39' N, 89°48' E
	Sankosh T.E.	Conventional	Alipurduar	26°39' N, 89°51' E

(Figure 4.3). This technique of collection requires termites to get attracted to the bait, hence was time-taking. Once traps were set, they were visited and observed at regular intervals for collection. Even though the process seems time consuming, yet, with good observation and some experience one could collect good number of healthy individuals for experiments avoiding mechanical injuries to the termites that usually occur in other forms of collection methods. Success of the technique depends on many factors, of which the most important one is the distance of collection site from one another. Firstly, it is very difficult to regularly observe the traps set up at distant collection sites. Secondly, termites with specific food preference can only be collected with this. Termites, like soil feeding, humus feeding or some other species needed more specific collection techniques. In case of distantly located plantations termite infested stem of tea were collected. These stems were later on opened in laboratory to collect termite workers and soldiers.

In case of scavenging termite *Odontotermes obesus* collection was mainly done either by digging up the mounds or from mud galleries. This species build large above ground mounds known as Termitarium, often ranging up to a height of 4-5 ft on surface (Figure 4.4). Under the outer mud fortification of termitarium, lays the fungal garden. This is the site where worker termite grows fungus (*Termitomyces* sp.) for colony's consumption. In order to access fungal garden, portion of the termitarium was dug out exposing the inside of the colony [Figure 4.4(a)]. Fungal gardens containing large number of soldier and worker castes could be collected as a whole. Such collection ensured good catch of assayable individuals. *O. obesus* fed on dead cells of plant making mud galleries on tea stems and branches to reach out to food sources. These galleries also contained good number of individuals which were collected for pesticide bioassay and other purposes [Figure 4.4(g)].

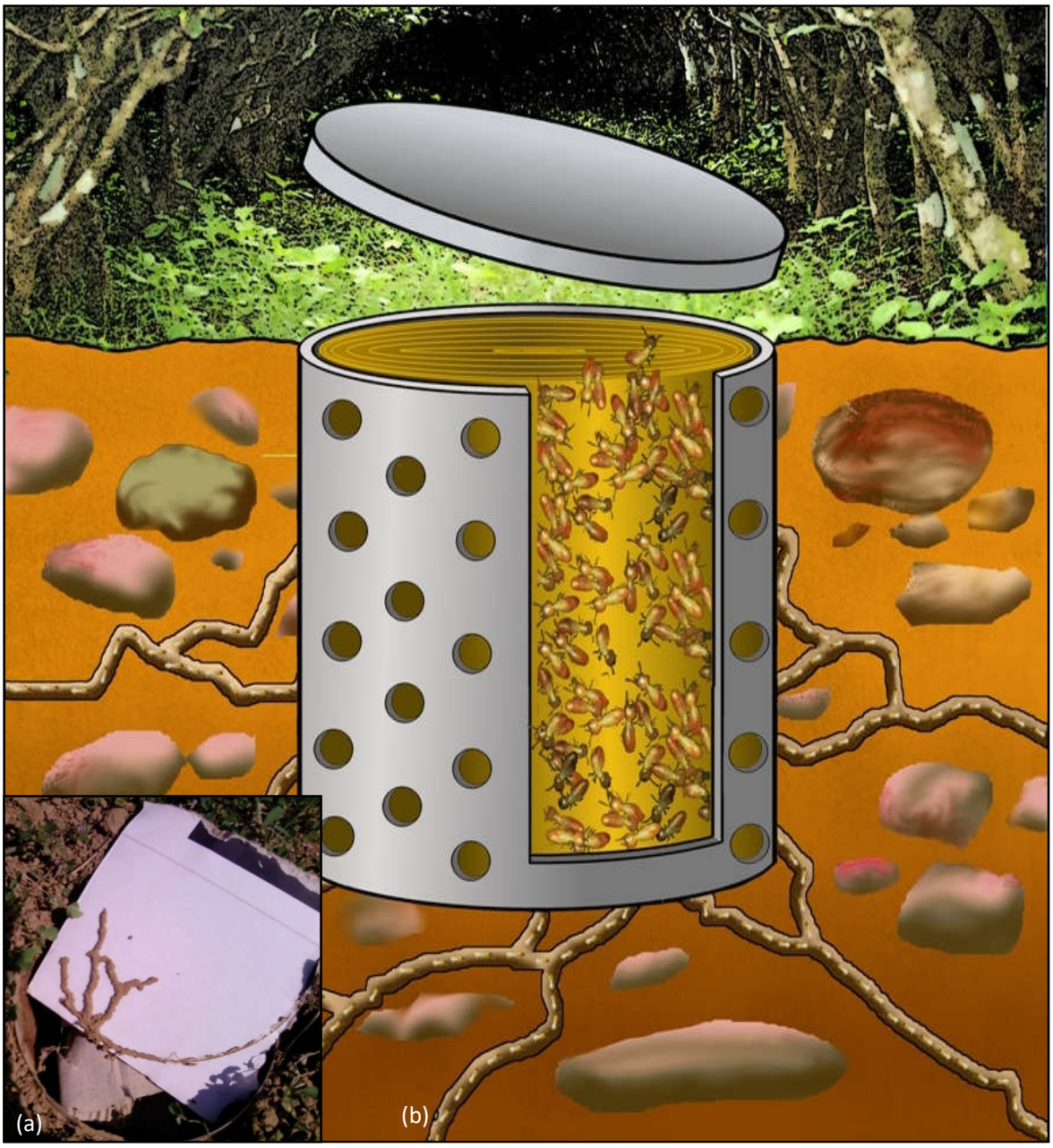

Figure 4.2: (a) A modified Bucket Trap. Termite mud galleries can be seen underside of the lid, indicating termites being attracted to the trap, (b) diagrammatic cross-section of the trap

4.1.3. Laboratory rearing and acclimatization of termites:

Rearing and acclimatization in the laboratory were mainly done for two species of termites namely *Odontotermes obesus* and *Microtermes obesi*. Both of these species are subterranean in nature, primarily feeding on wood. *O. obesus* feeds on dead-wood, whereas *M. obesi* on live-wood. Collected samples were reared in BOD under 25°C ± 2°C of temperature and RH 80% ± 5% and kept for at least a week or more. After acclimatization they were subjected to bioassay of different pesticides.

4.2. TAXONOMIC IDENTIFICATION

Soldier castes of a colony are considered ideal for identification because of their distinct mandibles which most of the cases provide a unique taxonomical character state. Although imagoes and worker castes are also considered for species level identification, yet soldier caste remains pivotal for final identification. Preliminary identifications were done under the WILD stereobinocular microscope following literature like Fauna of India (Isoptera) – Vol. I by Roonwal and Chhotani (1989) and Fauna of India (Isoptera) – Vol. II by Chhotani (1997). For species level identification and confirmation, termite specimens were first sent to Zoological Survey of India, Kolkata and later on to the laboratory of "Insect Identification Service", Division of Entomology, Indian Agricultural Research Institute (IARI), New Delhi. Some of the species which were identified during May, 2012 have also been registered under RRS No. 1677-1702/12 and determined by the authority in-charge of the "Network Project on Insect Biosystematics (NPIB)".

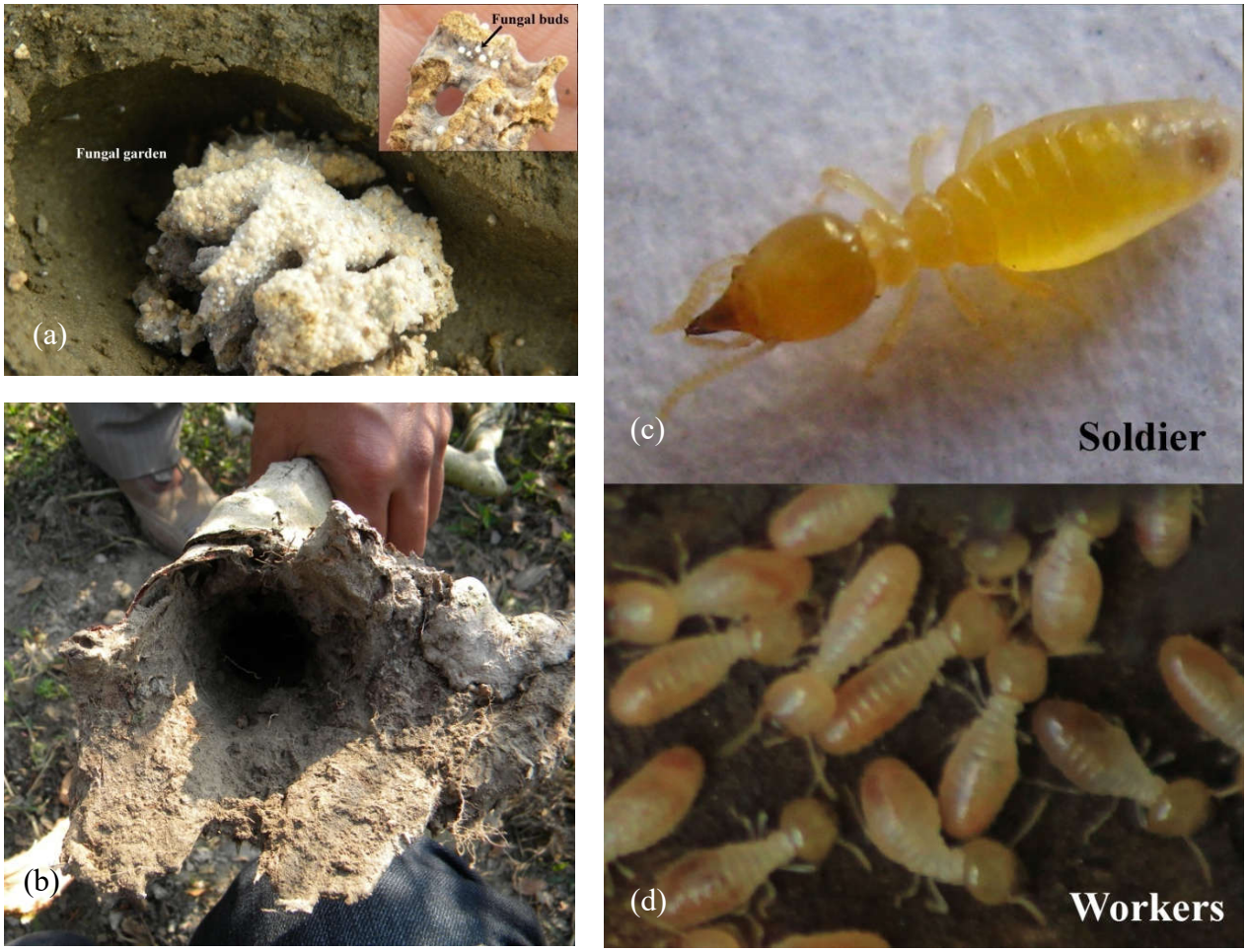

Figure 4.3: *Microtermes obesi* (a) fungal garden (inset: close up of fungal garden showing fungal buds); (b) a severely damaged tea stem showing hollowed pith; (c) soldier caste and (d) worker castes

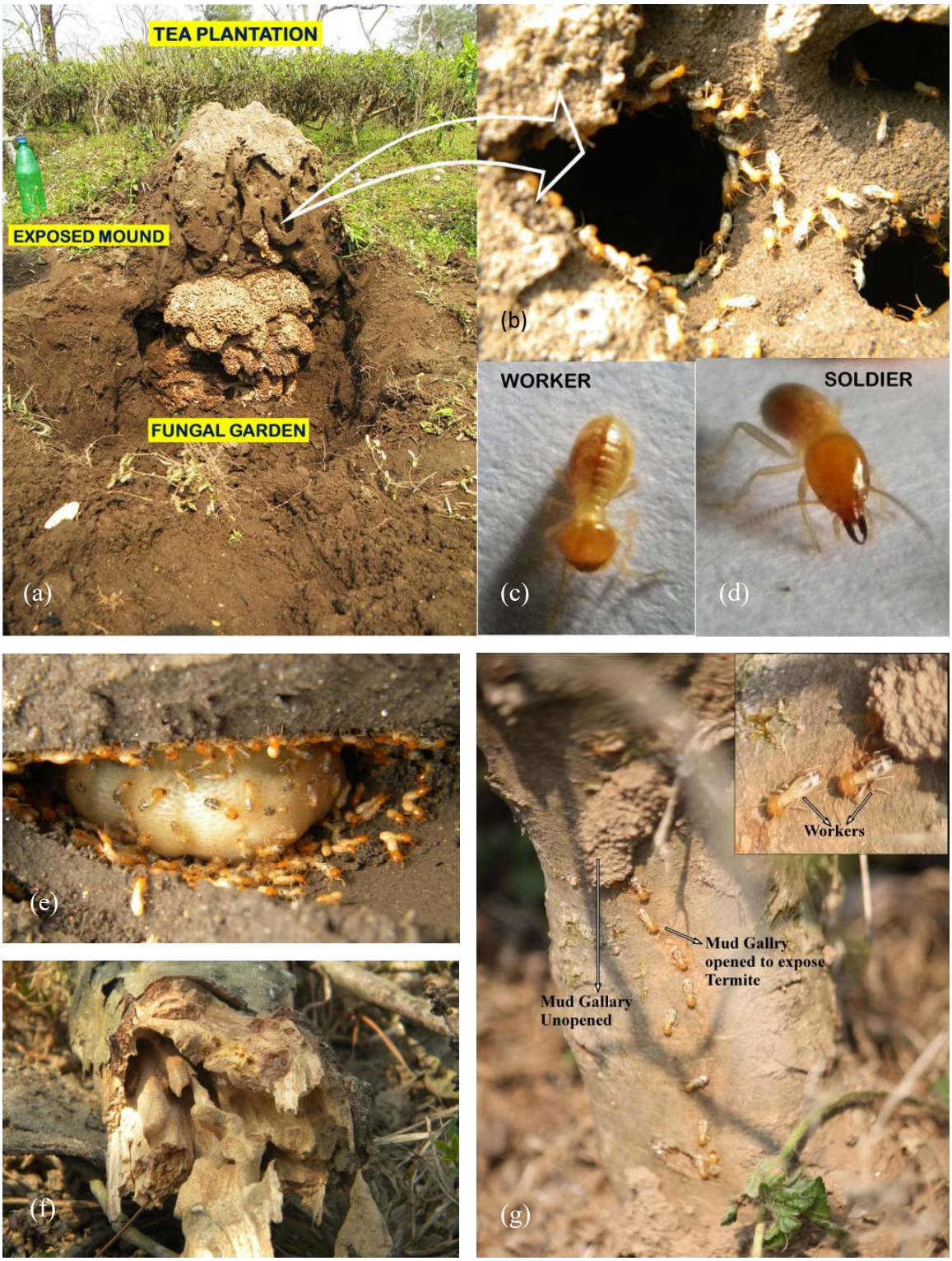

Figure 4.4: *Odontotermes obesus* (a) termitarium dug open to expose fungal gardens; (b) a set of workers repairing the broken mound; (c) worker caste; (d) soldier caste; (e) an opened royal chamber showing a part of Queens' physogastric abdomen; (f) damage symptoms on a infected bush frame, and (g) a mud gallery opened to expose worker castes (inset)

4.3. MORPHOMETRIC AND MOLECULAR PHYLOGENY

Traditionally, termites are identified based on morphological traits (character states). However, sometimes morphological identification becomes difficult among closely related taxa, or soldier-less genera. Now-a-days in addition to the phenetic taxonomy the Random Amplified Polymorphic DNA (RAPD) has come up as an easy and effective molecular tool for studying the variation at species level (Faragalla et al., 2015). RAPD-Polymerase Chain Reaction (RAPD-PCR) randomly amplifies many regions of genomic DNA using random primers and can be used for detecting polymorphisms at many loci between species and populations (Williams et al., 1990). The techniques followed are described as below-

4.3.1. Morphotaxonomy:

Morphological characters of soldier and worker castes were pooled in as per the methods of Donovan et al. (2000) and Engel et al. (2009). For phenetic studies, in total 25 taxonomically important characters and character states of worker and soldier castes were taken in consideration (Table 4.2). A morphometric character is a complex feature which unlike molecular scoring, can not be scored with just absence or presence of a character state. Few characters can be limited under this option, however, most need more than two set of scoring units or character states which were scored either – 0, 1, 2, 3, 4 or 5. The data matrix was pooled in Parsimony application of PHYLIP v3.695 to prepare a consensus tree.

Table 4.2: Characters and their character states of soldier and worker chosen for Phenetic studies

Sl. No.	Characters and the character states
1.	Ocelli of soldiers: present = 0; absent or vestigial = 1.
2.	Pigmentation of soldier compound eyes: present = 0; absent = 1.
3.	Soldier compound eyes: rudimentary = 0; absent = 1.
4.	Frontal gland developed into distinct fontanelle: absent = 0; present = 1.
5.	Postclypeal furrow of workers: absent = 0; present as shallow longitudinal furrow = 1.
6.	Clypeus (workers only) in profile: not keeled = 0; with keel = 1.
7.	Soldier mandible: marginal teeth: distributed along length, with 1–4 teeth along middle of margin = 0; lost except small teeth/serrations at base = 1.
8.	Soldier mandible: short to moderate in length, length ca. 2 or less 3 basal width = 0; greatly elongate and narrow, length ca. 3 or more 3 basal width = 1; vestigial = 2.
9.	Soldier mandibles: symmetrical, not clicking = 0; asymmetrical, clicking = 1.
10.	Soldier head capsule: rectangular in dorsal aspect 5 0; phragmotic, plug shaped = 1; nasutiform = 2.
11.	Diagonal grooves between fontanelle and postclypeus: absent = 0; present = 1.
12.	Soldiers: present = 0; lost = 1.
13.	Soldier nasus: short = 0; very long, length several times width = 1.
14.	Nasute fontanelle: rimmed or slit like = 0; minute, not rimmed or slit like = 1.
15.	First proctodeal segment: expanded = 0; tubular, not dilated = 1.
16.	Soldier labral apex: sclerotized = 0; hyaline = 1.
17.	Soldier heads: normal = 0; flattened = 1.
18.	Soldier labrum: well developed = 0; vestigial = 1.
19.	Soldier pronotum: flat = 0; saddle shaped = 1.
20.	Soldier fontanelle: normal, dorsal-facing = 0; enlarged and facing anteriad = 1.
21.	Nasute head capsule: not constricted = 0; slightly constricted = 1.
22.	Protibial apical spur number: three or more = 0; two = 1.
23.	Mesotibial apical spurs: four or five = 0; three = 1; two = 2.
24.	Metatibial apical spurs: four = 0; three = 1; two = 2.
25.	Soldier labral brush: absent = 0; present = 1.

4.3.2. RAPD-PCR

4.3.2.1. DNA Isolation:

DNA was isolated using Chelex 100 chelating resin (Walsh et al., 1991; Cano and Poinär, 1993). Chelex 100 is efficient than proteinase K and phenol-chloroform extraction for isolation of DNA from miniscule amount of samples. Although termite genomic DNA is extracted using Phenol-chloroform or other techniques, yet, these techniques demand quite good amount of sample size. As our sample size of some of the termite species was very limited, therefore, we resort to Chelex 100 technique for DNA extraction.

4.3.2.2. Primer Selection:

For RAPD a total of 25 decamer primers were initially chosen for screening. 20 primers were taken from 'OPA' Kit (Eurofin) and five were selected based on published information (Faragalla et al., 2015). These primers were then screened for strong bands, polymorphism and reproducibility. Based on these results, two Primers were selected from Kit 'OPA' (OPA-1 and OPA-2) (Table 4.3) for genomic DNA amplification.

Table 4.3: The nucleotide sequences of the primers used and their GC percentage

Primer No.	Sequence (5'-3')	GC %
OPA-01	CAGGCCCTTC	70%
OPA-02	TGCCGAGCTG	70%

4.3.2.3. RAPD:

Extracted genomic DNA was dissolved with 20 µl of PCR reaction buffer containing 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 20 pM of primer and 0.5 U of Taq polymerase. PCR was done according to the

methods of Williams et al. (1990) with initial heat step (94ºC for 5 min), 40 cycles of denaturation (94ºC for 1 min), annealing (36ºC for 1 min) and extension (72ºC for 2 min) and final extension (72ºC for 7 min). Application was performed using a programmable Eppendorf Mastercycler. The products of PCR and DNA size markers [λ DNA double digested with EcoR1 and Hind III (Bangalore Genei)] were loaded onto a 1.6% agarose gel in Tris-Borate-EDTA buffer (Sambrook et al., 1989) and were run for 4 h at 50 V. The gels were stained with ethidium bromide and photographed using UV transilluminator.

4.3.2.4. Data analysis:

The RAPD patterns of each isolates were evaluated, assigning character state '1' to indicate the presence and '0' for the absence of band in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair-wise comparison. All reproducible polymorphic bands were scored and analyzed following UPGMA cluster analysis method and computed *in silico* into similarity matrix using NTSYSpc-Numerical Taxonomy System Biostatistics, version 2.11W, (Rohlf, 1993). The SIMQUAL program was used to calculate the Jaccard's coefficients. The result generated in this analysis was then used to construct dendograms using the SAHN clustering programme, selecting the unweighted pairgroup methods with arithmetic average (UPGMA) algorithm in NTSYSpc. The termite species were then grouped with identical band patterns for a given primer.

4.4. BIOASSAY OF INSECTICIDES

4.4.1. Insecticide used:

Commercially available insecticides like, Imidacloprid (17.8% SL) and Chlorpryifos 20% EC (DURSBAN) are the major synthetic chemicals recommended against tea termites. These chemicals therefore, were primarily screened against the termites. There are reports of use of Endosulfan and synthetic pyrethroids against termites in recent past (Choudhury et al. 2005) therefore Endosulfan 10% EC (THIODAN), Cypermethrin 20% EC (RIPCORD), were also screened to check the tolerance status of termites against them. Concentration gradients prepared varied for different termite species depending upon the initial trials and the level of tolerance.

4.4.2. Bioassay:

After collecting termites from field, they were kept in BOD incubator at fixed temperature of 25^{o}C ± 2^{o}C and 80% ± 5% of RH. Due care was taken for acclimatization of worker termites before proceeding for the bioassay (Gurusubramanian et al., 1999). Bioassay involved specifically exposure of the worker caste of the termite species to insecticides (such as, Cypermethrin 10% EC, Endosulfan 35% EC, Cholrpyrifos 20% EC and Imidacloprid 17.8% SL) by "Surface coating method" after Kranthi (2005). Other then "Surface coating method" few other bioassay techniques like "Insect Dip Method", "Contaminated Soil essay" and "Topical spray method" were also tried however, these methods seem to have higher mortality due to handling in comparison to the "Surface coating method". Termites were treated with at least five concentrations of insecticides prepared by serial dilution in distilled water and each dilution was replicated thrice (Figure 4.5). A set of 30 termite workers were used in each replicate (n=30), that was subjected to a particular concentration of insecticide.

Figure 4.5: Procedure for bioassay of pesticide against *O. obesus* and *M. obesi*

A set of 90 worker termites divided equally into three replicate treated with distilled water were kept separately as control. Termites under evaluation were maintained at 25^{o}C ± 2^{o}C temperature and 80-85% of RH in BOD incubator. The mortality count was recorded after 24 hours. Bioassay data was pooled and concentration mortality regression was computed by Probit analysis (Finney, 1971) aided by computing software. Moribund insects were counted as dead (Gurusubramanian and Bora, 2007).

4.5. ENZYME ASSAY

4.5.1. General Esterase Activity:

General esterase activity was measured by using α-naphthyl acetate (α NA) as substrates after the method of van Asperen (1962) with few modifications. 20 µl of supernatant equivalent to one adult insect was taken in each well of the 96-well microplate reader in duplicate. 200 µl α NA (30 mM) was added to each well for reaction. The reaction was stopped after 10 minutes by adding 50 µl of staining solution containing 0.1% Fast BB salt and 5% SDS (2:5). The plate was left for five minutes for equilibration and absorbance was recorded at 590 nm. The change in absorbance was converted to end product formation from a standard curve of α naphthol (5-500 nM). Blanks were set at the same time using reaction mixture without protein extracts.

4.5.2. Glutathione *S*-transferase (GST):

The activity of GST was measured using 1-chloro-2, 4-dinitrobenzene (CDNB) and reduced glutathione (GSH) in conjugation reaction as described by Habig et al. (1974). The reaction mixture contained 150 µl of GSH (1.0 mM) and 50 µl CDNB (1.0 mM). The microplate was left for 3 minutes for equilibration and reaction. The absorbance was recorded at 340 nm continuously for 5 minutes. An extinction

coefficient 9.6 $mM^{-1}cm^{-1}$ was used to convert the change in absorbance per minute to rate of conjugate formation.

4.5.3. Cytochrome P450 monoxygenase:

As heme protein is a major constitute of the majority of Cytochrome P450, its activity was calculated by estimating heme peroxidase activity (Penilla et al., 2007; Tiwari et al., 2011). 20 µl of enzyme homogenate was incubated with 200 µl of TMBZ solution (0.01 g of TMBZ in 5 ml of methanol + 15 ml of 0.25 M sodium acetate, pH 5.0) and 80 µl of 0.0625 M PBS (pH 7.2) and 25 µl of 3% H_2O_2 for 30 minutes at 25°C. Absorbance was recorded at 630 nm on microplate reader. The standard curve of heme peroxidase activity was prepared using Cytochrome c from horse heart type IV. Total Cytochrome P450 was expressed as nmoles of Cytochrome P450 equivalent units (EUs) per mg protein per minute.

4.5.4. Protein estimation:

Protein in the tissue extract was estimated as described by Lowry et al. (1951) using bovine serum albumin (100 µg ml^{-1}) as standard to construct a standard curve.

4.5.5. Electrophoratic analysis of general esterases

Electrophoresis was carried out in 8% polyacryalamide gels using equal amount of protein in Tris-glycine (pH 8.3) at 200 V at 4°C. The gels were stained for esterase isozymes according to Georghiou and Pasteur (1978) with slight modifications. Densitometric analyses of the gels were performed using 'Image Aide Analysis software'.