Mycodiversity in the combs of *Odontotermes* sp.

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Certificate of Examination

This is to certify that the dissertation titled "**Mycodiversity in the combs of *Odontotermes* sp.**" submitted by Ms. Nimisha.E.S (Reg. No. MS12053) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Dated: April 21, 2017

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Rhitoban Ray Choudhury at the Indian Institute of Science Education and Research, Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a confide record of original work done by me and all sources listed within have been detailed in the bibliography.

Nimisha.E.S
April 21, 2017

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Rhitoban Ray Choudhury
(Supervisor)
April 21, 2017

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Abstract

Termites are eusocial insects, originating from cockroach-like ancestors having evolved eusociality about 100 Mya. Eusocial behavior of termite colonies help them to get access to otherwise inaccessible niches and thus higher fitness of the colony. Interaction between termites and fungi range across both higher and lower termites. Most of these interactions are facultative with one exception, the fungus-growing termites of the family Macrotermitinae. This mutualism originated in Africa about 30 Mya. This fungus growing termites use symbiotic fungi (*Termitomyces*) as an external- rumen for plant degradation. In this symbiotic relationship, termites provide substrate for the growth of *Termitomyces*, maintain the fungus garden by continuous addition of predigested plant material and consumption of the older comb material. *Termitomyces* are maintained as monoculture in termite nests. During the establishment of a new colony, the fungal partners are collected from the environment by termite workers. Agricultural symbiosis between termites and fungi is symmetrical because both termites and fungus have single origin and both obligatorily depends on this relationship. *Termitomyces* acts as a food source for termites. Many other fungi like *Xylaria, Trichoderma, Penicillium* etc are also present inside the termite nests. In the absence of termites, antagonistic fungi, like *Xylaria*, starts to grow over the combs. This indicates that termites or *Termitomyces* are using some mechanisms to prevent the growth of contaminating fungi. The present study tries to explore the Mycodiversity in the combs of *Odontotermes* termites and also attempts to see how these antagonistic fungi interact with termites and *Termitomyces*.

Chapter 1

Introduction

a) i. What are termites?

Termites are small soft-bodied, light-colored insects of the order Blattodea. Based on their evolution, behavior and anatomy they are divided into two broad groups: lower and higher termites. The termite families Hodotermitidae, Mastotermitidae, Rhinotermitidae, Serritermitidae, Kalotermitidae and Termopsidae consists of lower termites, which have protozoans in their hindguts, while higher termites do not (Krishna & Weesner, 1969). There are approximately 2300 species of termites known worldwide (Kambhampati & Eggleton, 2000). Most of them live in the tropical, subtropical and temperate regions of the world (Krishna & Weesner, 1969). Termites are hemimetabolous insects, where the insect larvae metamorphose to winged and reproductive adults directly without any intermediate pupal stage. They feed primarily on the cellulose and lignin found in plants. Based on what food they consume and where they live, termites are grouped into four ecological groups: dry wood termites, damp wood termites, harvester termites and subterranean termites. Higher termites have the ability to utilize much broader range of substrate than lower termites. This includes soil feeding, litter-consuming life styles in addition to wood and grass feeding. Since termites cannot completely digest lignocellulose, they rely upon symbiotic microbes and protists present in their hindgut to supply most of the enzymes necessary for cellulose digestion. Lower termites have bacteria and archaea along with protozoa in their gut, while higher termites have bacteria and more elaborate gut anatomy, while lacking the protozoa. Therefore, these higher termites

have different gut components with increased gut compartmentalization and alkalinity (more basic pH level). Higher termites with different feeding habits are associated with different varieties of symbiotic bacteria (Miyata *et al*., 2007; Schmitt-Wagner *et al*., 2003; Shinzato *et al*., 2007; Warnecke *et al*., 2007). Individuals in a colony exhibit 'trophallaxis' or exchange of rectal contents, which allows efficient use of nutrients and transfer of gut symbionts (Bignell *et al*., 2011). This particular behavior also helps in recognition of colony members and in distribution of chemicals involved in caste regulation (Honghog, 2010).

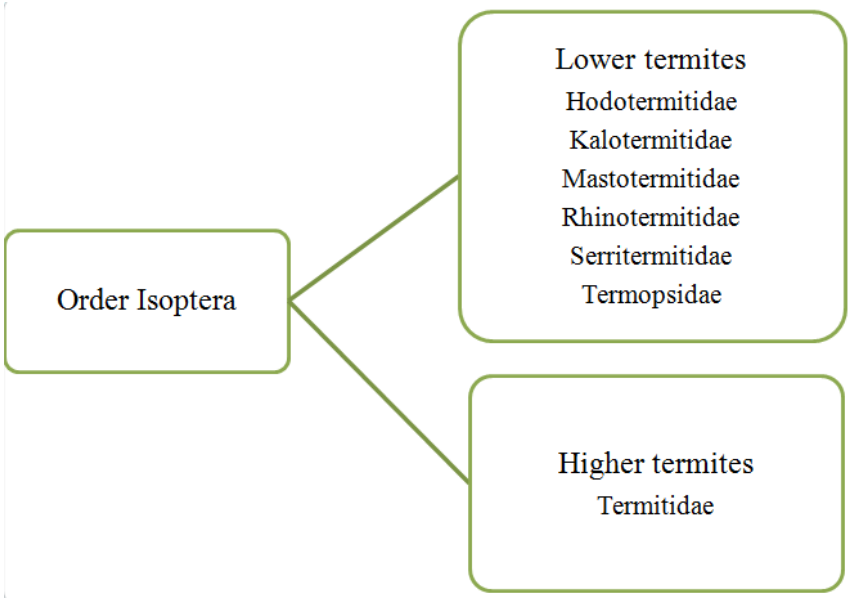


Figure.1.1 Classification of termites

ii. Eusociality in termites

Eusocial termites evolved during the upper Jurassic or lower cretaceous period from an ancestor which probably ate and also nested in wood (Thorne *et al*., 2000). In a termite colony, all members of a family live together. Because of the extent to which individuals appear to operate as a unit for perpetuation and reproduction of the colony William Morton Wheeler (1911) described the social insect colony as an organism or super organism (Queller & Strassmann, 1998). Eusociality is a highly elaborate social system with three basic characters (Honeycutt, 1992; Crespi & Yanega, 1995; Gadagkar, 1993; Wilson, 1971, 2005)

- Cooperative care of young (even when they are not directly their Offspring).

- Overlapping of generations.
- Division of labour.

Eusociality is mostly observed and studied in Hymenopterans (ants, bees and wasps) and in termites. Eusocial behavior is also seen in other species (Nowak *et al*., 2010).

- *Synalpheus* snapping shrimp (Duffy *et al*., 2007).
- Halicted bee *Lasioglossum duplex* (Sakagami & Hayashida, 1960)
- Adult erotylid beetles of the genus *Pelaphacus* (Costa, 2006)
- Ambrosia beetle *Austroplatypusincompertus* (Kent & Simpson, 1992)

Eusociality in haplodiploids can be explained by inclusive fitness theory and kin selection (Strassmann *et al*., 2011). Kin selection explains how self sacrifice or altruism can evolve if it is sufficiently beneficial to relatives. It provides explanation for the evolution and maintenance of worker sterility. Inclusive fitness explains the total proportion of genes an organism contributes to next generation. Many eusocial insects including ants, bees and wasps are haplodiploid but termites are diplo-diploid insects. Haplodiploid insects contain females with two alleles at a locus and males with single allele at a locus. Because of this, they exhibit different kin relatedness than that of diplo-diploid insects like termites. Haplo diploid female is related to its sister by ¾ whereas diplo-diploid female is related to its sister by ½. Based on kin selection the eusocial female would prefer to help their mother to raise their sisters rather than helping to increase their direct fitness by raising their own offspring to which they related to ½. This explains the reason behind sterile castes in eusocial insects in which workers prefer to increase their indirect fitness than direct, which is less beneficial (Hamilton, 1964). However, such theories describing the origin, evolution and spread of eusociality in diploid groups like termites is still lacking.

Termite colony has an animate and an inanimate part. Animate part consists of the individuals living within the colony and inanimate part is the structure built by them in which they live. Animate parts of the colony consist of castes, which are groups of individuals that can become irreversibly behaviorally distinct at some point prior to reproductive maturity. Individuals of one caste have higher mean life time reproduction and at least one other caste exists that help individuals of other caste to reproduce

(Crespy & Yenega, 1995). Reproductives, workers, soldiers, nymphs are the major castes of termites.

iii. Termites and wood damage

Many species of termites have become pests in agricultural areas and housing structures, causing extensive damage to wood and wood-derived products. They live in colonies in rotting wood, ground and construct mud galleries or tubes to protect workers which forage for food. Undigested lignin is largely excreted and used as a colony construction material (Amelung *et al*., 2002). Termites harbor microbes that can digest lignocellulose. These hindgut microbes in termites break down the cellulose into short-chain fatty acids molecules like acetic acid (Karasov & Duglas, 2013). Termite's cells use these acids as nourishment. These microbes also produce gases during this breakdown process. Methane gas is a major by-product and termites are a significant source of methane in our atmosphere (Rasmussen & Khalil, 1983). Major groups of microbes present inside termite gut are bacteria, archaea and protozoa. Anatomically evolved gut with increased compartmentalization in the hindgut and increased alkalinity (more basic pH level) also helps in digestion (Brune, 2014; He *et al*., 2013). The microbes residing in the termite gut demonstrate various relationships including pathogenicity to obligate mutualism (Rosengaus *et al*., 2011). To prevent these pathogens and parasites, termites have accordingly developed several defensive strategies (Mueller *et al*., 2005). Worker termites are able to control infection from pathogens or parasites by secreting antimicrobial substances in defensive glandular secretions, faeces, and body exudates (Rosengaus *et al*., 2004, 2011).

iv. Insect agriculture

Agriculture in insects has evolved independently many times. Most prominent examples are termites, ants and ambrosia beetles (Muller *et al*., 2005). In Ambrosia beetles it has evolved seven times (Farrel *et al*., 2009) but in termites and ants, agriculture has evolved only once (Aanen *et al*., 2002; Mueller *et al*., 2001). The members of the subfamily Macrotermitinae, which are higher termites, have symbiotic association with *Termitomyces* fungus (Aanen *et al*., 2007). Agriculture behavior in these insects includes obligate nutritional dependency on a particular crop, inoculation

of cultivars on particular habitats or particular substrates and cultivation aimed at improvement of growth conditions of crop (Muller *et al*., 2005).

Termitomyces fungus present in the nests of Macrotermitinae is important for lignocellulose digestion and as a food source for termites. *Termitomyces* in turn gets suitable growth substrate provided by worker termites (Lefèvre *et al*., 2006).

v. Termites in India

Historical mention of termites as wood destroyers first appeared in ancient Sanskrit texts, the *Rig Veda*, ca. 1350 B.C (Snyderv, 1956). *Mahabharata* (ca. 100 B.C to early A.D) also mentions about termites. In early parts of 20th century, entomologists in India were content with repeating generalized control measures. Today almost 300 species of termites are known from the Indian region. India is one of the 12 megadiverse countries of the world. This diversity can be attributed to vast variety of landforms and climate, resulting in habitats ranging from tropical to temperate and alpine to desert. All the seven families of Isoptera have been in existence from the period of late Mesozoic (Das & Alfred, 1998:220). A region with a number of taxonomic units is said to be biologically diverse and if contains more species, genera, families than normal expectation is known as hyperdiversive. As a taxonomic group this order does not contain numerous species, genera and families in all habitats (Das & Alfred, 1998:222). The most diverse family is Termitidae with 145 genera under four subfamilies (Das & Alfred, 1998:221). Over all, these termite species are distributed in about 286 genera. **Figure 1.2.** Shows the species diversity of termites of the world (Varshney, 1987:2).

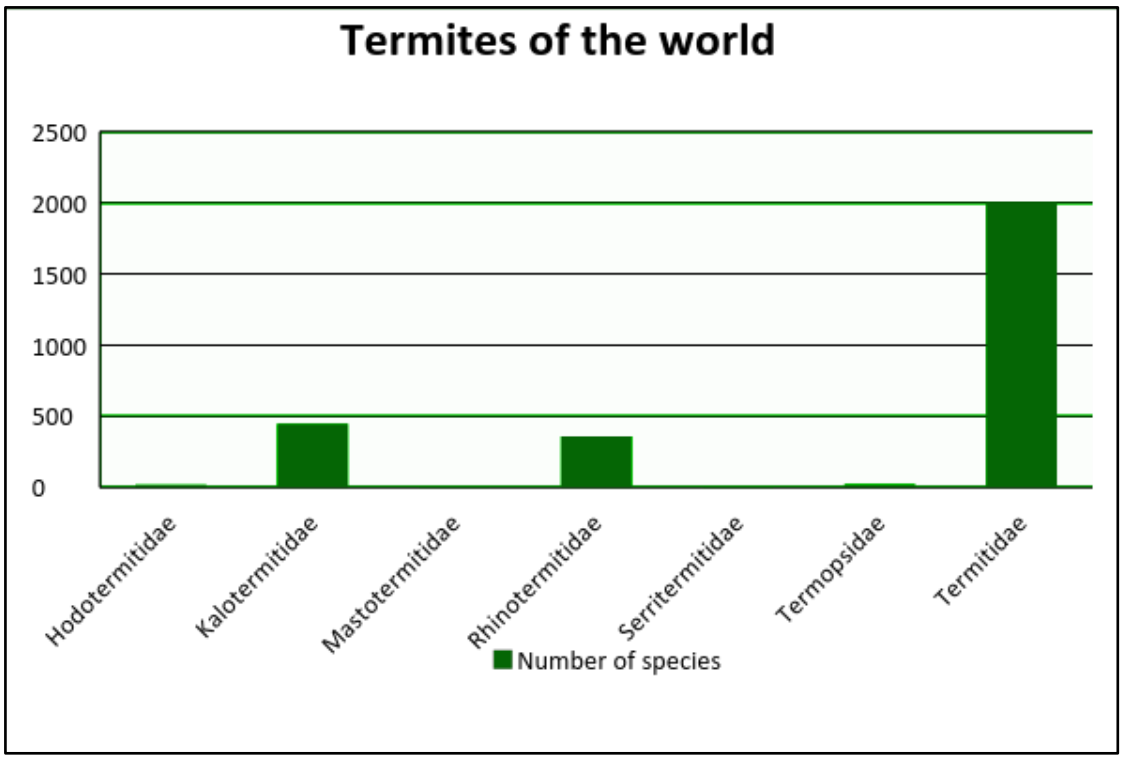


Figure 1.2 Diversity of termite species

Hyperdiversity of termites occurs in tropical rain forests of Western Ghats in the peninsular and in the north- eastern borderland, as well as in the moist deciduous forest tracts in the foothills of Himalaya. Wide distribution of termites can be seen in regions with red, sandy loam, lateritic and red loam soils. Gujarat and Rajasthan provide an interesting field of study on the Isopteran fauna because of the diverse ecosystems varying from the arid region in the east up to the salty marsh land of Rann of Kutch in the west and above the Aravallis.

Local species richness and regional generic richness are strongly influenced by altitude and climate, asymmetries in latitude and longitudinal gradients, with the highest diversities of termites found in wet low land tropical forests. Moisture is the most important factor for survival of the termites. They try to avoid extreme arid and cold regions. Species richness gradually decreases towards the higher altitudes of the Himalaya (temperate regions), arid stretches of the Thar Desert in the west, but it increases towards the north-eastern areas. Taxonomic diversity of Isoptera known from India is shown in **Table 1.1.**

Taxonomic diversity of Isoptera in India

Species	Genus	Taxonomic category in India	
		Subfamily	Family
2	1	Hodotermitinae	Hodotermitidae
1	1		Indotermitidae
40	9		Kalotermitidae
7	1		Stylotermitidae
1	1	Psammotermittinae	Rhiotermitidae
8	2	Heterotermitinae	
7	1	Coptotermitinae	
8	3	Rhinotermitinae	
50	8	Amitermitinae	Termitidae
36	10	Termitinae	
48	6	Macrotermitinae	
44	10	Nasutitermitinae	
1	1		Termopsidae

Table 1.1 Taxonomic diversity of Isoptera in India (Alfred & Das, 1998:221)

b) i. Biology of termites

Termite colony consists of functional reproductives, workers, soldiers and immature individuals. Immature individuals are known as nymphs. Two kinds of functional reproductives are present in termite colonies: primary and secondary reproductives. Primary reproductives include king and queen. They are highly pigmented, round headed, sclerotized and develop from winged adults. Primary reproductives have long antennae, chewing (mandibulate) mouthparts, and well-developed eyes. They have two pairs of similar, long, semi-transparent wings with many veins. In almost all species, there is one pair of king and queen per colony. Queen is the caste which is responsible for most of the reproduction in the colony. The reproductives, especially the queen,

have an extended lifetime when compared to the other castes. Queens of termite species have life span of over 25 years while kings substantially have shorter life cycle (Lewis, 2008).

Secondary reproductives are individuals of the colony other than the king and queen, where reproductive organ development takes place if the king and/or queen of the colony die. They are bigger in size than the workers, light in color and never develop wings. The functions of reproductives include production of new individuals, distribution of the species by swarming, choice of the site for new colony.

Workers that care for the nest are blind and sterile. They are wingless and soft bodied. They are responsible for all things in the colony other than egg laying and defense in the colony, that includes making tunnels and chambers, and repairing it when necessary, and taking care of young individuals and the queen of the colony. In the case of higher termites they are responsible for tending the fungus gardens.

Soldier's duty is to ensure protection to the colony. Soldiers of nearly all termite species have strong and large muscular jaws which they use to tear an enemy into pieces. Besides using their sharp jaws to attack the enemy they use chemicals to paralyze the enemy by exploding themselves on the top of enemy and secreting chemicals which are poisonous and can damage the attacker's physical structures (Yaha ,1999).

Nymphs resemble adults but are smaller and are the most numerous stage in the colony. They resemble their parents in general body shape. They have pigmented eyes and more or less developed wing pads. All the nymphs, after a few moults, can be separated into the following two types.

- Large headed sterile forms (workers)
- Small headed reproductive forms

Nymphs are quite active throughout the initial stages of their life of about one year, but they become relatively inactive for very short duration. During this period they undergo the process of moulting and exhibit marked morphological changes and growth of their body. They can be categorized into different castes on the basis of their morphological differences after moulting.

Some nymphs develop wing buds, become longer, and finally develop into the fully winged adults becoming the future kings and queens. They can vary in color from black to pale brown with opaque grey to black wings. As these adults are winged, they fly away in groups of thousands of individuals during their breeding season (usually soon after the first rain). This phenomenon is commonly referred to as swarming, which is also known as nuptial flight (Dial & Vaughan, 1987). These swarmers are relatively poor fliers as most of them flutter for only a few yards before they fall to the ground. After finishing this short flight, the swarmers drop their wings and the males begin search for compatible mates.

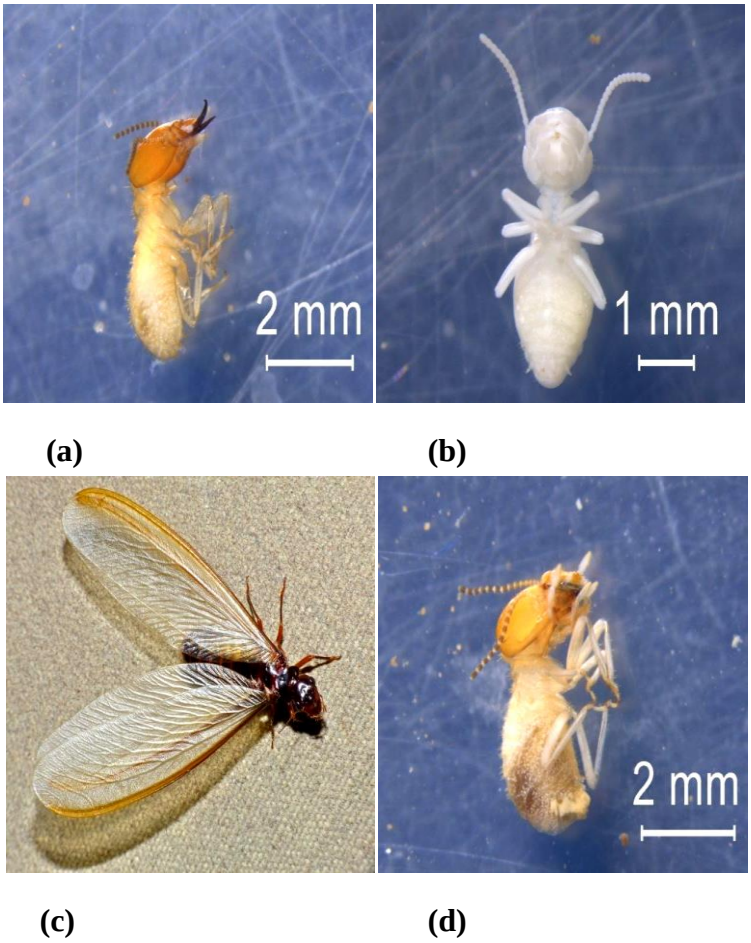


Figure 1.3. (a) Termite nymph (b) Soldier (c) Winged alate (d) Worker

Hemimetabolous development in termites causes high degrees of polymorphism and some degree of polyphenism, where two or more distinct phenotypes are produced by the same genotype.Because of this hemimetabolous development an undifferentiated

larvae can develop into nymph, workers and more advanced stages can develop into soldiers or neotenics (Bagnères & Hanus, 2015). The size of termite colony range from a few thousands in primitive Kalotermitidae (Kalshoven, 1930) to numbers in the order of 10^6 (Gay & Greaves, 1940).

ii. Termite life cycle

Most termites swarm in late summer, usually associated with the onset of heavy rain. During this swarming, pairing takes place. The founding of a new colony begins when a mated pair finds a suitable nesting site near or in wood and constructs a small chamber in which they enter and seal themselves. Soon afterwards, the female begins to lay egg. The eggs produced by the colony queen hatch after 30 days. Upon hatching the larvae resemble small white adults. Through the series of moulting (where they shed their skin and increase in size) young individuals develop into their respective castes. Different castes take a different number of moults to achieve maturity, with workers having the shortest moulting stage and reproductives having the longest (Varma *et al*., 1994; Pearce, 1997). Early colonies will not produce any alates as there is a population threshold that must be reached before the queen will produce them. Once the alates are produced they leave the nest in search of new territory, they usually land a few hundred meters away. The release of alates often coincides with fresh rainfall (Lewis, 2008). Once male and female alate pair, they burrow into the nearest potential nesting area, develop a chamber for them and begin a transformation from alate to royal pair.

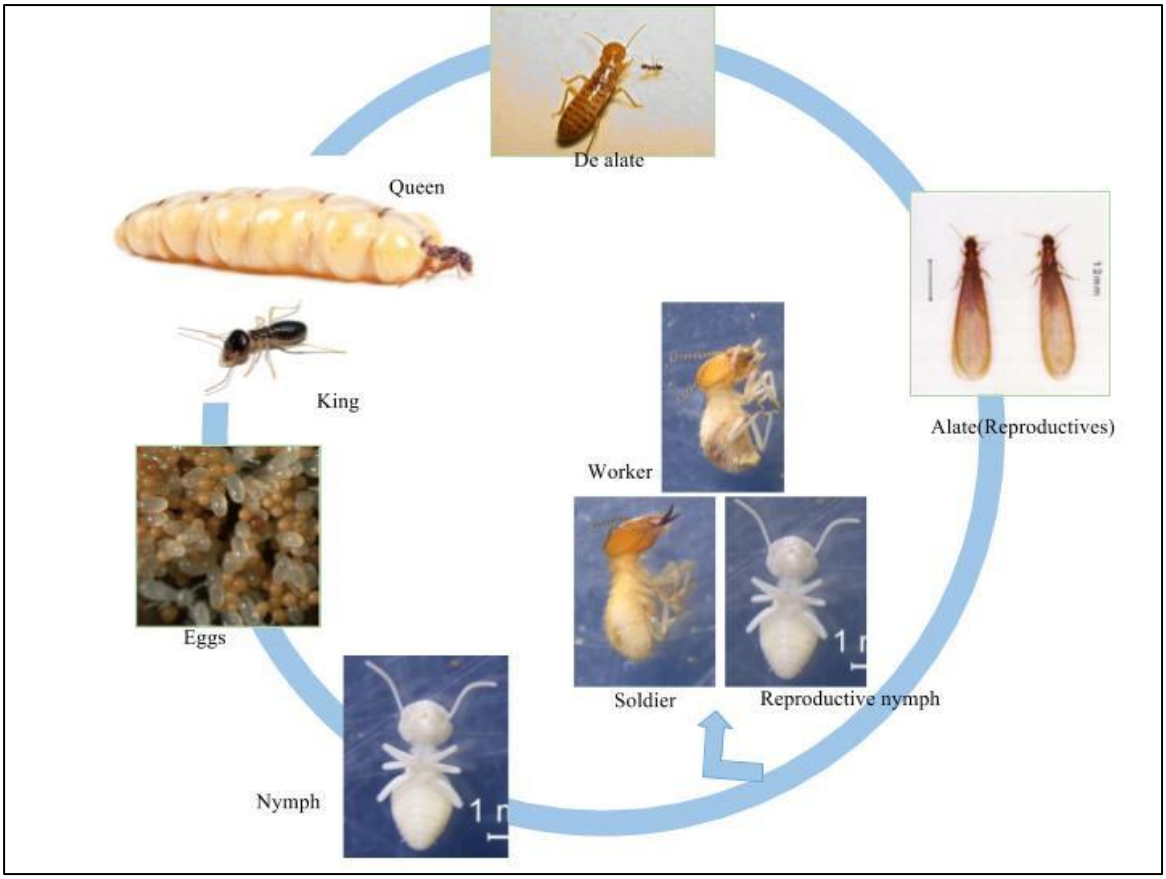


Figure1. 4. Life cycle of termites.

iii. Termite swarming

Swarming is a natural part of reproductive cycle of the termite colony. When a colony reaches maturity, in order to increase the distribution of the species, they develop male and female reproductives with wings, which are called alates. Exterior weather conditions are the major determinants for triggering swarming. Early spring with warmer weather followed by rain is the most suitable condition for swarming (Minnick, 1973). After the emergence, these male and female swarmers do a small flight, and then they seek a suitable environment in which they can begin a new colony. Other insects such as ants also practice such kind of swarming.

iv. Systematic position and phylogenetic relationships

Termites evolved from non or subsocial wood feeding cockroaches (Lo *et al*., 2000). Cockroach genus *Cryptocercus* forms the sister taxon of this group (Inward *et al*. 2007). These wood eating cockroaches belong to the order Blattodea in the super order Dictyoptera. Rhinotermitidae marks the difference between higher and lower termites, and thus they play key position in the evolution of the order Isoptera. DNA sequencing results have revolutionized the areas of systematic and phylogenetic studies. The degree of genetic relationship between species forms the base of phylogenetic studies.

c.i. Termites and fungi

Mutualistic interactions between species have played a central role in diversification of species (Thompson, 1999). It represents major and ecologically highly successful transition in evolution (Smith & Szathmary, 1995). Fungus growing social insects are good examples of mutualistic symbiosis. Growing fungi for food has evolved independently in new world Attine ants and old world Macrotermitinae termites (Batra & Batra, 1979; Darlington, 1994; Poulsen & Boomsma, 2005; Mueller *et al*., 1998). Some termites have symbiotic association with *Termitomyces* fungus. This association helps in the degradation of large amount of plant matter. Warm, moist and suitable conditions of the mound enhance this symbiotic association. Individual nests harbor *Termitomyces* in monoculture (Aanen *et al*., 2009; Katoh *et al*., 2002; Moriya *et al*., 2005). Termite genus *Odontotermes* belongs to the family Termitidae. Most of the species are mound building and very few are subterranean. They also have symbiotic association with *Termitomyces*. They construct complex mounds with extensive system of tunnels and conduits provide ventilation to underground parts of mounds. Mounds are present above the subterranean nest which is a spheroidal structure consisting of numerous gallery chambers. Due to the continuous digging and decomposition of plant material, the mound soils are generally more fertile than other soil (Gosling *et al*., 2012; Dangerfield *et al*., 1998). This soil is heavier in structure and poorer in organic matter than the surrounding soil (Joachim & Kandiah, 1940). The mounds are sub-cylindrical structures which are wider at the base with thick walls. Inside these mounds many small and big chambers are present. Symbiotic fungus *Termitomyces* are grown on the fungus

comb constructed by the termites. Termites water their fungal gardens with their excretions and thus maintain the necessary humidity for fungal growth.

(a) (b)

Figure1 .5. (a) and (b) are *Odontotermes* mound in IISER Mohali campus

In *Odontotermes* mounds there are very few diurnal fluctuations, and temperature and humidity vary between a range of 4˚C and 4% (Agarwal, 1980). The complex internal structure of Macrotermes mounds are designed to dissipate heat in the hot season and retain it in the cool season (Rajagopal, 1982). The low pH of the fungus comb helps to prevent the growth of bacteria, otherwise comb appears to be a favorable substrate for microbial activity (Thomas, 1987).

Chapter 2

2.a)i. Fungus growing termites

Fungus growing termites originated in African rain forest and occur throughout the old world tropics (Aanen & Eggleton, 2005). The fungus growing termites (Macrotermitinae) started cultivating *Termitomyces* (Phylum: Basidiomycota) for food about 30 Mya (Aanen DK & Eggleton, 2005*). Termitomyces* as edible termite associated fungi is reported to be common in various parts of the world. Only 30 species of *Termitomyces* from Asia and Africa have been reported so far (Kirk *et al*., 2008). The name *Termitomyces* was given by Roger Heim in 1941 to a group of agaricus associated with termite nests in Central Africa and Asia. This association between *Termitomyces* and its obligate mutualist termites of the subfamily Macrotermitinae seems to be the result of a co-evolutionary process since the early tertiary with a single origin in the African rainforest (Aanen *et al*., 2002; Aanen and Eggleton, 2005). Macrotermitinae often dominate the termite fauna in tropical savannas and forests (Abe & Matsumoto, 1979; Abe & Vatanabe, 1979), whereas the large number of fungus growing termite species can be found in rainforest habitats (Nobre *et al*., 2011; Jones *et al.,* 2011).

2. ii. Geographical Distribution of *Termitomyces*

Termitomyces have been reported from various parts of the world. So far around 30 species of *Termitomyces* have been reported from Asia and Africa (Krik *et al.,* 2008). Low species diversity of *Termitomyces* compared to large diversity of termites indicates that small numbers of *Termitomyces* are associated with their hosts (Aanen *et al*., 2002). Earlier descriptions of novel *Termitomyces* species are based on fruiting bodies. Some *Termitomyces* lineages rarely form fruiting bodies and some of them not even form fruiting bodies.

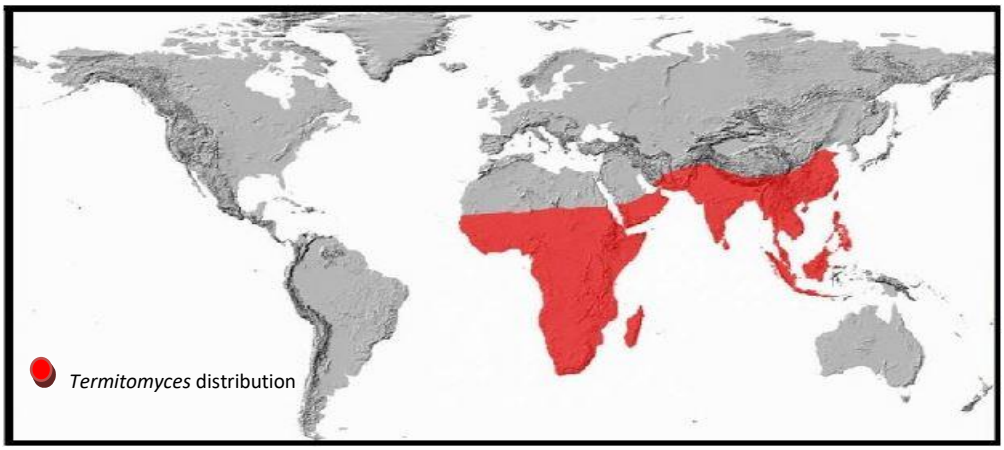


Figure.2.1. Geographic distribution of *Termitomyces*

Termitomyces - Edible termite associated fungi is reported to be common in various parts of India. Two-thirds of the species of the genus *Termitomyces* recorded worldwide occur in six states of the Western Ghats and on the west coast of India. There are 15 species of *Termitomyces* present in Kerala and 10 species present in Goa, Karnataka, Maharashtra and Tamil Nadu contain nine, three and two species of *Termitomyces* respectively (Natarajan & Raman, 1983). From Punjab three species of *Termitomyces* have been reported (Rawla *et al.*, 1983).

Figure.2.2. *Termitomyces* distribution in India

2.iii. Termites and *Termitomyces.*

Fungus growing termites make use of obligate mutualism with *Termitomyces*, which are part of extracorporeal digestive system. Termites acquire this fungus through vertical transmission (by alates) or horizontal transmission in which *Termitomyces* is acquired from the environment during the formation of new mounds (Korb & Aanen, 2003, Nobre *et al.*, 2010). Symbiotic relationships have had an essential role in termite evolution and it helped them to occupy previously inaccessible niches that have abundant resources (Waller *et al*., 1988). *Termitomyces* degrade the plant material and produce nodules from the faecal deposits of termites. These faecal pellets are then shaped into comb by the termites. The plant substrate is efficiently digested via the combined efforts of fungal and gut bacterial enzymes (Liu N *et al*., 2013; Darlington, 1994). Combs are present in specially constructed chambers, either inside a mound or dispersed in the soil. Combs vary in size and shape in different genera of termites.

Figure.2.3. (a)Termite mound (Chattbir Zoo) (b) *in situ* image of fungus comb

Inside the termite gut this wood slurry is inoculated with a variety of fungal spores. Once these spores are deposited in the comb they germinate and spread as white nodules which are conidia, rich in Nitrogen form through the comb. As these *Termitomyces* grow, they digest cellulose, converting it to simpler sugars and nitrogen. The termites then consume this enriched fodder and old parts of comb for food. Inside termite gut

these will be digested by termite's intestinal symbionts and intestinal enzymes (Traniello & Leuthold, 2000).

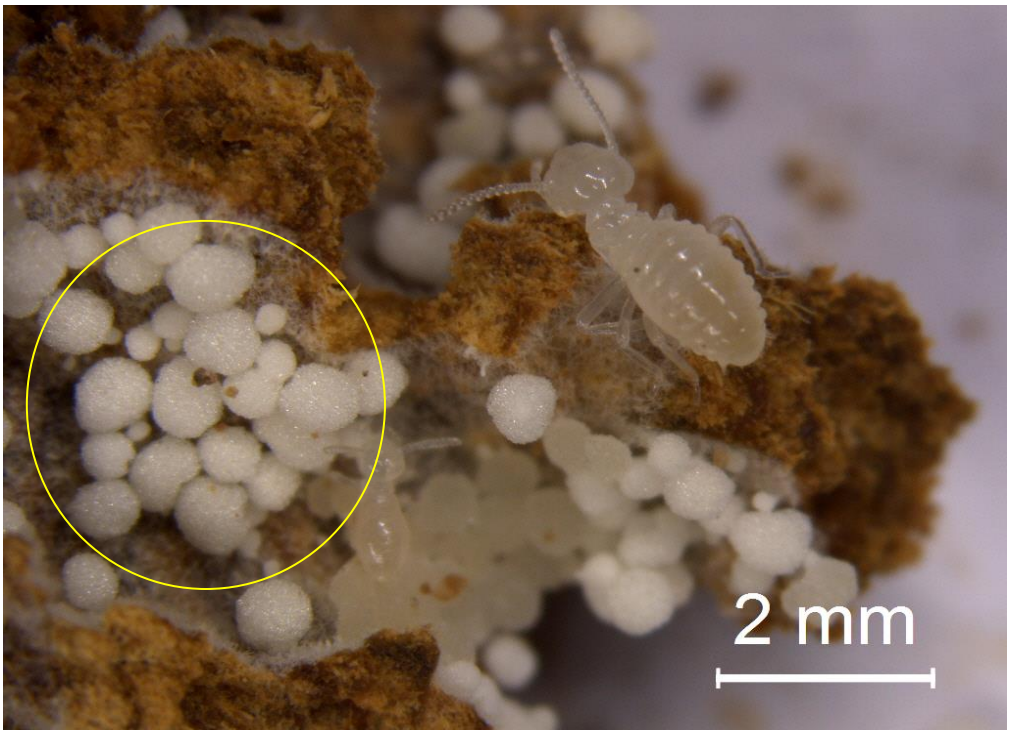


Figure.2.4. Comb with *Termitomyces* nodules (white ball like structures circled in yellow)

Termitomyces fungus is reared as a monoculture in termite colonies. Experiments have shown that the main reason behind the monoculture is positive frequency dependent selection, genetic drift and selection for rapid asexual spore formation (Aanen, 2006, 2009). The common microfungi that affect the fungus comb in the absence of the termites include genera *Aspergillus*, *Penicillium* and *Hypocrea* (*Trichoderma* sp.) (Mathew *et al*., 2011). *Termitomyces* provide cellulases and xylanases which can work synergistically or complementarily with endogenous termite-derived enzymes (Martin & Martin, 1978; Rouland-Lefèvre *et al*., 1991). Colonisation by other strains which are collected during horizontal transfer will thus be prevented.

There are several wild mushrooms that grow in the forests which form an integral part of human diet during monsoon months when these are abundantly available.

Termitomyces is the most popular edible mushroom and has unique and subtle flavor. They can be found during the rainy seasons (Devi & Singh, 2014). They usually grow in contact with termite nests in forest soil. *Termitomyces* species are generally rich in minerals such as potassium, calcium, magnesium, iron and manganese (Mattila *et al*., 2001). It is used as a medicine to lower blood pressure, to treat rheumatism, kwashiorkor, obesity and diarrhoea (Breene, 1990; Nabubuya *et al.*, 2010). Due to mutualistic symbiosis between termites and *Termitomyces,* neither of the two partners can exist without each other. Hence the artificial cultivation of *Termitomyces* is difficult. *Termitomyces* contain enzymes of industrial importance such as xylanase, amylase and cellulase (Khowala & Sengupta, 1992). Nitrogen content in termite's body is very less due to their consumption of dead plant material. *Termitomyces* also serve as a nitrogen rich food source for termites (Matsumoto, 1976; Collins, 1983). Fungus growing termites play a dominant role in litter removal (Abe, 1980; Buxton, 1981).Their contribution to ecosystem decomposition is highest in savannas, with up to 20% of total carbon mineralization (Wood & Sands, 1978). *Termitomyces* in combs have several functions in the nest. They may maintain high humidity required by termites and help in ventilating the nests (Escherich, 1911).

Chapter 3

Materials and methods

3.i. Study area

Figure.3.1. Map of IISER Mohali campus (study area)

This study was performed in Mohali, Punjab. The selected study sites were situated along IISER Mohali campus (Latitude: 30.7046486 m, Longitude: 76.7178726 m and elevation 316m (1037ft)). The campus spread across 126.024 acres. The map of the area was obtained from Google maps and it is scaled down to 1" = 100 m. Here the temperature may rise to a maximum of 47 °C in summer and it generally remains between 30 and 40°C. Average temperature in winter remains at 7 to 15 °C and (minimum) 1 and 5 °C.This study was started from May 2016. We have selected 8 different locations of termite nests from IISER Mohali campus (locations are marked in the **Figure.3.1**. Selection of different locations were done based on accessibility of that area and size of the termite mounds.

3.ii. Collection and identification of termites from mounds

Different castes of termites were collected from each termite mound. This includes workers, soldiers and nymphs. The samples were separated from debris with the help of brush. Samples were preserved in 100% ethanol and kept at -20˚C for molecular identification. The preserved samples were labelled carefully with all required information. Genomic DNA from workers was extracted using the following method:

Genomic DNA isolation

Lysis buffer	100 ml
5.0 M Tris HCl	2.0 ml
5 M NaCl	0.2 ml
0.5 M EDTA	2.0 ml

- Termite samples were taken in 1.5 ml Eppendorf tube and were washed 2-3 times with autoclaved water till ethanol is removed completely.

- 200µl lysis buffer, 5µl 22mg/ml Proteinase K and 5 µl SDS were added to this tube.
- Samples were crushed using pestle and placed in 37˚C water bath overnight.
- After incubation, 250 µl of Phenol was added to the tube and mixed by inverting. Centrifuge the sample for 5 min. at 4˚C, maximum speed.
- Aqueous layer was collected carefully and 250 µl of 24:1 Chloroform: Isoamylalcohol was added to this, followed by centrifugation for 5 min at 4˚C, maximum speed.
- The top aqueous layer was collected carefully and 150 µl of chilled Isopropanol with 0.1 volume of Sodium acetate.
- Incubation was done at RT for 30-40 minutes. Centrifuge the mixture for 5 min at 4˚C, maximum speed.
- The pellet was washed with 70% ethanol, followed by air drying.
- Pellet was dissolved in 100 µl TE.

Quantification of genomic DNA was done by Nanodrop spectrophotometer. DNA extraction was followed by PCR for amplification of the DNA. PCR was performed using Primers specific for mitochondrial DNA encoding the *CO II* subunit. Since mitochondrial DNA evolves much more rapidly than nuclear DNA, phylogenetic studies are done using sequence variation in this mitochondrial DNA. Rapid changes in mitochondrial DNA results in the difference between populations that have diverged only for a short period of time (Brown *et al.*, 1979).
DNA extraction was done using **COII F1 (5'-GGDCAYCAATGRTRYTGAAG-3')** and **COII B2 (5'-AGTACTTGCTTTCAGTCATC-3')** primers (Ohkuma *et al.,* 2003). Samples were identified using NCBI BLAST and phylogenetic tree was constructed after Bayesian analysis.

3.iii. Collection and study of mycodiversity in combs

Eight different locations from IISER campus (**Figure.3.1**) were selected for this study. The selected areas were (1) Hostel-5 area (2) Near sewage treatment plant (East gate) (3) Shopping complex area (4) Chilla forest area (5) Guest house (6) Professor quarter (7) Health centre (8) CAF.

To explore the composition of fungus comb's mycobiota and microbiota in detail, combs from eight different locations were collected for six months. Mounds were excavated and, fungus combs were collected in sterile plastic boxes. These boxes with fungus combs were kept in incubator at 25˚C (RH-65). Combs were regularly observed for changes both in the presence and absence of termites. *Termitomyces* nodules present in the comb and contaminating fungus which appeared after few days of comb incubation were collected and preserved in ethanol at -20˚C.

3.iv. Isolation, morphological and functional studies of *Termitomyces* isolates.

From each fungus comb, the *Termitomyces* nodules were collected using sterile forceps. Nodules were inoculated into plates containing different cultivation media. Media used for cultivation were following: Potato Dextrose Agar (PDA), PDA+ Yeast Extract Agar and Yeast Malt Extract Agar (YMEA). Inoculations were done on plates containing each media followed by incubation at 30°C. Growth rates were monitored daily for two -three weeks. Based on colony morphology and growth characteristics, the fungal isolates were sub-cultured until they were axenic. Cultures were preserved in autoclaved water in culture tubes at 4˚C.

3.v. Isolation, morphological and functional studies of antagonistic fungi present in termite combs.

Fungus mycelia present on the combs were collected using sterile forceps and inoculated on PDA plates. Plates were incubated at 25˚C. Growth rates were monitored regularly for two weeks. Sub-culturing was done until culture appear to be pure, i.e., without any contamination. Cultures were preserved in autoclaved water in culture tubes at 4˚C.

3.vi. Identification of contaminating fungus

For the identification of different contaminating fungi, DNA was extracted. Extraction of genomic DNA is usually done with Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer (Doyle & Doyle, 1987) followed by purification through

phenol/chloroform extraction and precipitation with isopropanol or ethanol (Ashktorab & Cohen, 1992).

- The fungal mass from the culture plate was scraped out using sterile forceps.
- The fungal mass obtained from the culture plate or broth was placed in a 1.5 ml tubes.
- The sample was grinded and resuspended in 600µL modified CTAB buffer (at 65° C) using sterile pestle.

CTAB	2% W/V
NaCl	1.42 M
EDTA	20 mM
Tris	100 mM
β-Mercaptoethanol	1 µL

- 4µL fresh RNAase (20 mg/ml) was added in each tube and incubated at 65°C for 30min. Samples was then vortexed.
- To the resulting fungal tissue homogenate, 600 µL of Phenol: chloroform: Isoamyl alcohol (25:24:1) was added and mixed well, followed by centrifugation at 13,000 rpm for 15 min (Note: this step can be repeated once more to completely get rid of proteins/cell debris).
- The upper aqueous layer was taken in a fresh centrifuge tube and then 600 µl Chloroform: Isoamyl Alcohol (24:1) was added, followed by centrifugation at 13,000 rpm for 10 minutes.
- Supernatant was transferred to a fresh centrifuge tube.
- 0.7 volume (approx. 350 µL) of Isopropanol was added to the supernatant and the sample was then mixed by inversion followed by spinning for 5 minutes at 13000 rpm.
- The supernatant was discarded and pellet was dried by inverting on tissue paper.
- Pellet was washed using 1 mL of 70% ethanol followed by spinning down for 5 minutes at 13000 rpm.

- Pellet was then air dried and dissolved in 1× TE buffer.

The quantity of the extracted genomic DNA was determined by measuring the absorbance at 260 nm using Thermo Scientific Nano Drop spectrophotometer. The quality of extracted genomic DNA was assessed by subjecting them on 1% agarose gel electrophoresis after DNA amplification using PCR. DNA amplification was performed with universal fungal primer pairs ITS4/ ITS5 (White *et al.*, 1990). After sequencing, sequences were compared with closest sequences deposited in GenBank (NCBI) public database using the BLASTN software. To assign phylogenetic relationships, sequences showing maximum identity were selected to construct phylogenetic tree. All of the sequences were aligned using CLUSTALW software. Phylogenetic analysis was conducted using Bayesian methods.

3.vii. Staining of fungus samples

Lactophenol blue staining is the most widely used method for staining and observing fungi. The preparation has three components. Lactic acid for preserving fungal structures, phenol for killing living organisms and cotton blue for staining fungal cell walls.

Lacto phenol Cotton Blue Stain(LCB)	
0.05g	cotton blue
20g	phenol crystals
40ml	Glycerol
20ml	Lactic acid
20ml	Distilled water

Preparation of Lactophenol blue stain involves following steps:

- Cotton blue was dissolved in distilled water and was left overnight for eliminating insoluble dye.
- Phenol crystals were dissolved in lactic acid and glycerol was added to the solution.

- To this Phenol+Glycerol+Lactic acid solution filtered cotton blue solution was added.
- After mixing it properly, dye was stored at room temperature.

Staining of fungus samples

- Fungus mycelia were transferred onto glass slide using sterilized forceps and LCB stain was added to it.
- Tissue was smashed gently and mixed with the stain.
- Cleaned cover slip was placed over mycelia + LCB with the help of forceps and using blotting paper excess stain was removed.
- Samples were observed under low and high power microscope.

3.viii. Fungus interaction assay

We examined the interactions between *Termitomyces* and contaminating fungi *Xylaria in vitro.* This interaction experiment was performed to study whether the *Termitomyces* inhibited or promoted the growth of contaminating fungi. The experiment was conducted by the following steps.

- Separate cultures of *Termitomyces* and *Xylaria* were made on PDA + YMEA and PDA media respectively.
- *Xylaria* plug was placed on the plates containing *Termitomyces* culture. (As *Termitomyces* possess slow growth rate compared with *Xylaria*, 2 weeks old culture of *Termitomyces* was used for the above experiment.)

The experimental set up is shown in **Figure3.2.**

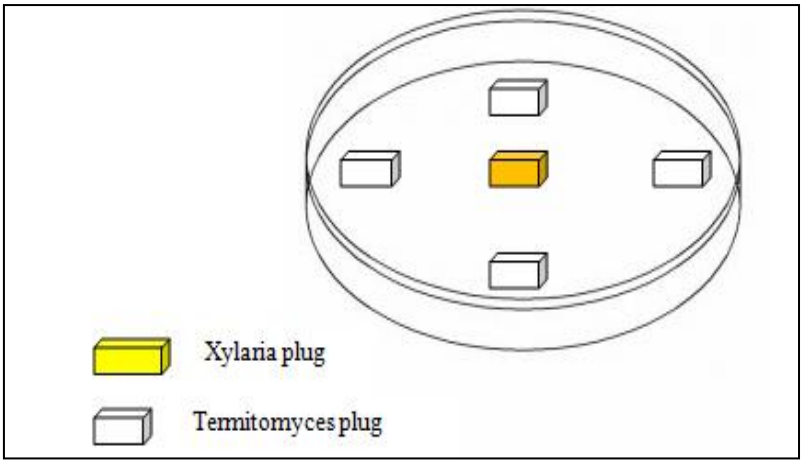


Figure.3.2.*Termitomyces* and *Xylaria* interaction assay

Culture plates were incubated at 30˚C in dark. Growth rates were monitored daily for one week.

Chapter 4

Results

4.i. Identification of termites

Phylogenetic tree of termites using Bayesian method was drawn on the basis of multiple sequence alignment of mitochondrial *CO II* gene. The constructed phylogeny revealed the formation of two major clusters, the genus *Microtermes* and *Odontotermes.*

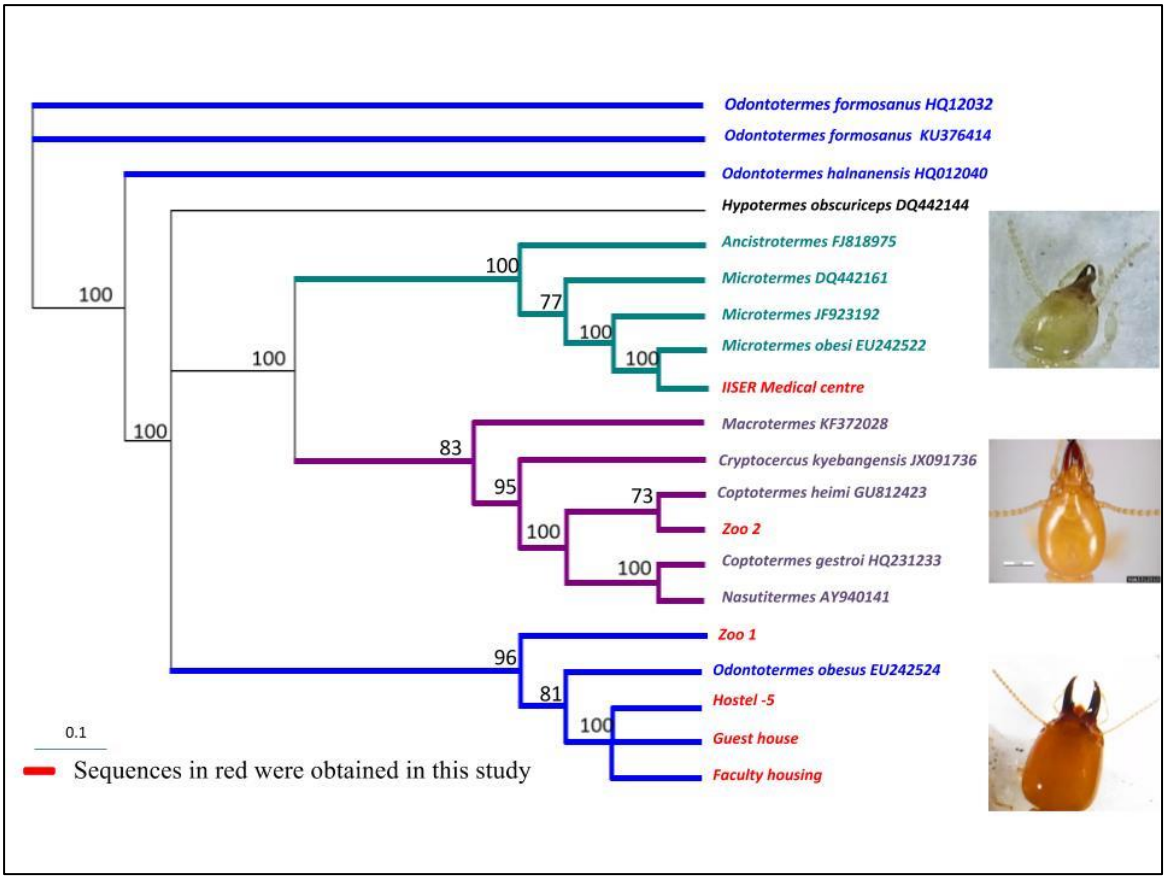


Figure.4.1. Bayesian phylogenetic analysis of *COII* fragments.

The samples collected from Zoo were identified as *Coptotermes*, which are lower termites (**Table.4.1**). Our results demonstrate that DNA sequences of genes which are not likely to vary in function are useful for analysing termite phylogeny.

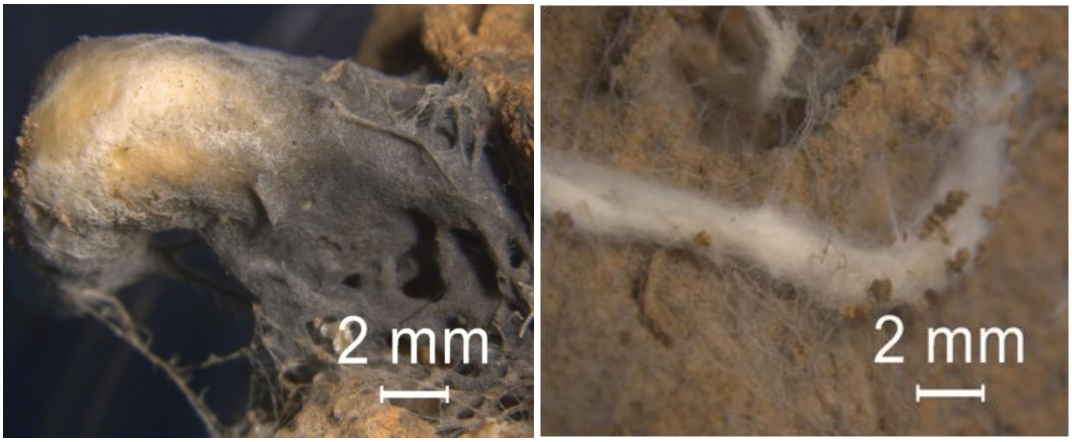
Location	**Termite genus**
Chilla forest	*Odontotermes*
Guest house	*Odontotermes*
Faculty housing	*Odontotermes*
Shopping complex area	*Odontotermes*
Hostel 5	*Odontotermes*
Sewage treatment plant	*Odontotermes*
NIPER Campus	*Odontotermes*
CAF area	*Microtermes*
IISER Medical centre	*Microtermes*

Table.4.1. Termite genera identified from IISER campus

4.ii. Mycodiversity in *Odontotermes* combs

Our aim was to characterize the Mycodiversity within the combs of *Odontotermes* to identify what are the different fungi that are interacting with *Termitomyces* and *Xylaria*. Active termite combs are dominated by monoculture of *Termitomyces*. Foraging behavior of termites during establishment and maintenance of their fungal gardens exposes them to other contaminants like bacteria and other antagonistic fungi. These contaminats may be either introduced into the gardens or reside in the termite guts. Thus, they become the part of fungal diversity. Vigorous growth of antagonistic fungi was observed in the combs incubated in lab condition (**Figure.4.2**). But no significant changes were found in case of combs with termites.

(a) (b)



(c) (d)

Figure.4.2. Growth of contaminating fungi on combs from *Odontotermes* mounds. (a) five day old comb with *Xylaria* (b) Comb with *Trichoderma* growth (c), (d) Combs with *Xylaria*.

4.iv. Isolation, morphological and functional Studies of *Termitomyces* isolates.

Termitomyces culture in this study was started from nodules collected from fungal combs. For culturing *Termitomyces* three different types of media were used: (i) PDA: consists the following composition in g/liter. Infusion from potatoes 200; Dextrose 20; Agar 15. (ii) YMEA: This media composed of following compositions in g/liter. Peptone 5; Yeast extract 3; Malt extract 3; Dextrose 10; Ager 20. (iii) PDA+ YMEA: This media is PDA media modified by adding YMEA. Pure cultures of *Termitomyces* fungi were obtained after successive subculture of the first culture obtained from nodules on fresh combs inoculated on PDA + YMEA media. On PDA media

Termitomyces were not growing, but were greatly improved upon supplementation with YMEA. This is likely an indication that the fungal partner might get some more nutrients from the YMEA media. Optimum growth conditions for *Termitomyces* were 30˚C temperature and 65 Relative humidity in dark. Pure culture of *Termitomyces* obtained after two – three rounds of sub culturing.

(a) (b)

Figure.4.3.(a) (b) Cultures of *Termitomyces*

4.v. Isolation, morphological and functional studies of antagonistic fungi present in termite combs.

In the fungus combs morphologically different fungi were observed after few days of incubation. Termite associated *Xylaria* strains were not present in active combs. But in nests abandoned by termites they were frequently observed. This finding highlights the implications of fungus growing termites in regulation of fungi occurring within the combs.

(a) (b) (c)

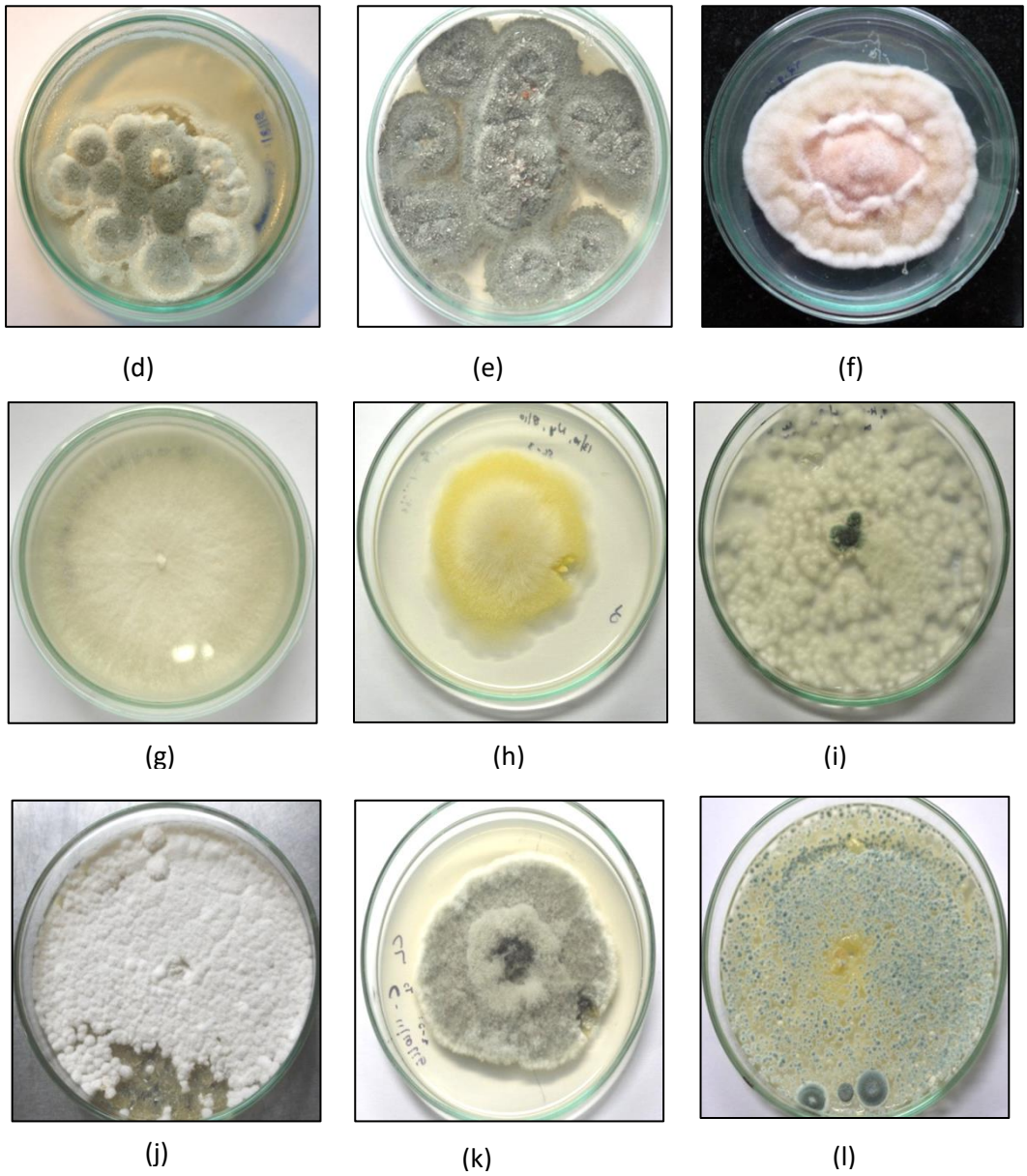


Figure.4.4. Cultures of different fungus from combs of *Odontotermes*. (a) *Trichoderma* sp. (b) *Xylaria* sp. (c) *Aspergillus* sp. (d) *Penicillium* sp. (e) *Talaromyces* sp. (f) *Fusarium* sp. (g) *Trichoderma* sp. (h) *Rhizomucor* sp. (i) *Penicillium* sp. (j) *Cochliobolus* sp. (k) Unidentified (l) *Penicillium* sp.

Twelve morphologically different fungal species were identified from *Odontotermes* combs. We also got some samples which were unidentified using NCBI. All of these antagonistic fungi that we got from incubated combs belong to phylum Ascomycota.

4.vi. Identification of contaminating fungus

We have estimated phylogenies of fungus-growing termites and their associated mutualistic fungi of the genus *Termitomyces* using Bayesian analysis of DNA sequences.

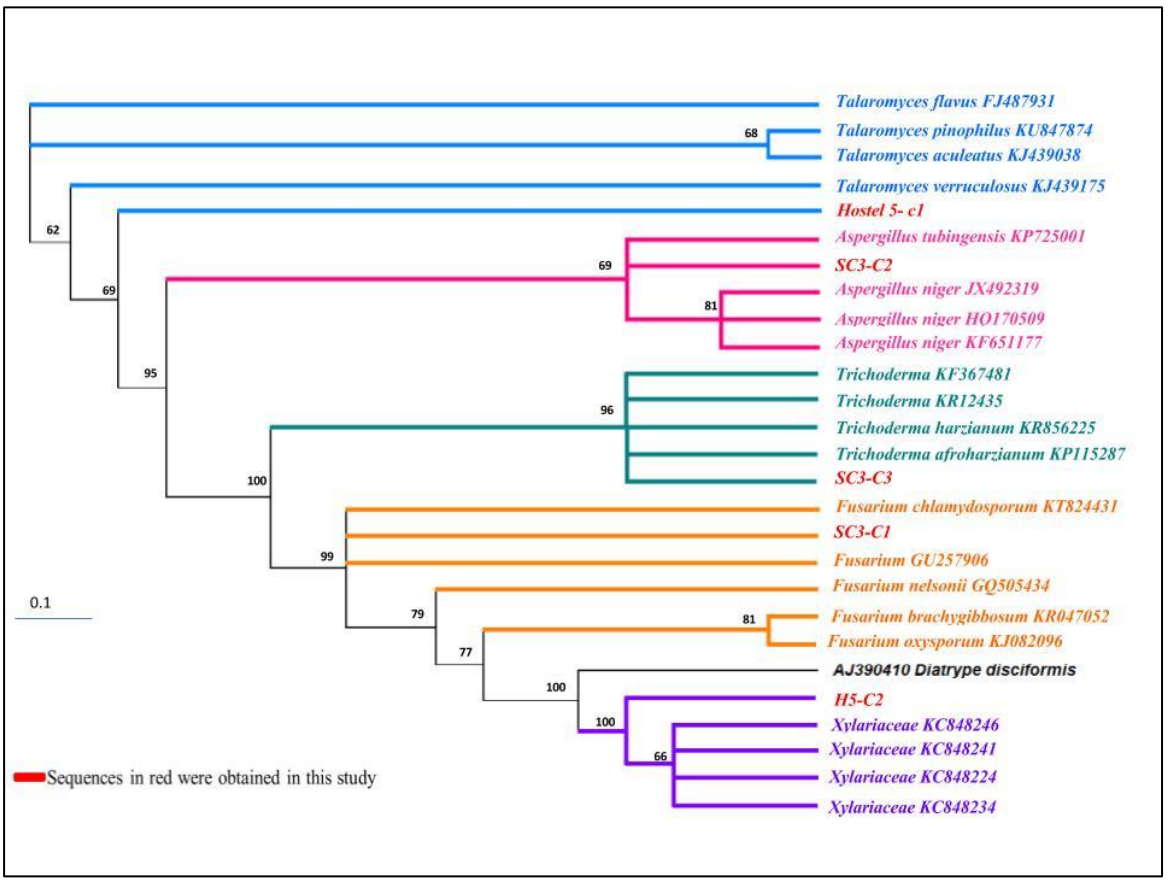


Figure.4.5. Phylogenetic tree of antagonistic fungi inferred from *ITS* region.

Different fungi associated with combs were difficult to identify on the basis of their morphology. Therefore, molecular information was used by amplifying the ITS region of cultivated fungi. Using Bayesian phylogenetic analysis we found that *Talaromyces* sp., *Aspergillus* sp., *Trichoderma* sp., *Fusarium* sp., *Xylaria* sp. are the main antagonistic fungi appeared on the combs after few days of incubation.

4.vii. Staining of fungi samples

Staining of collected fungus samples were done to confirm their identity. Identification of stained samples was also done on the basis of morphology. All the contaminating fungi belong to phylum Ascomycota, while Termitomyces belongs to phylum Basidiomycota. Members of Ascomycota are known as spore shooters. They produce microscopic spores inside special elongated cells or sacs, known as asci. Presence of asci were clearly visible in all the stained fungi.

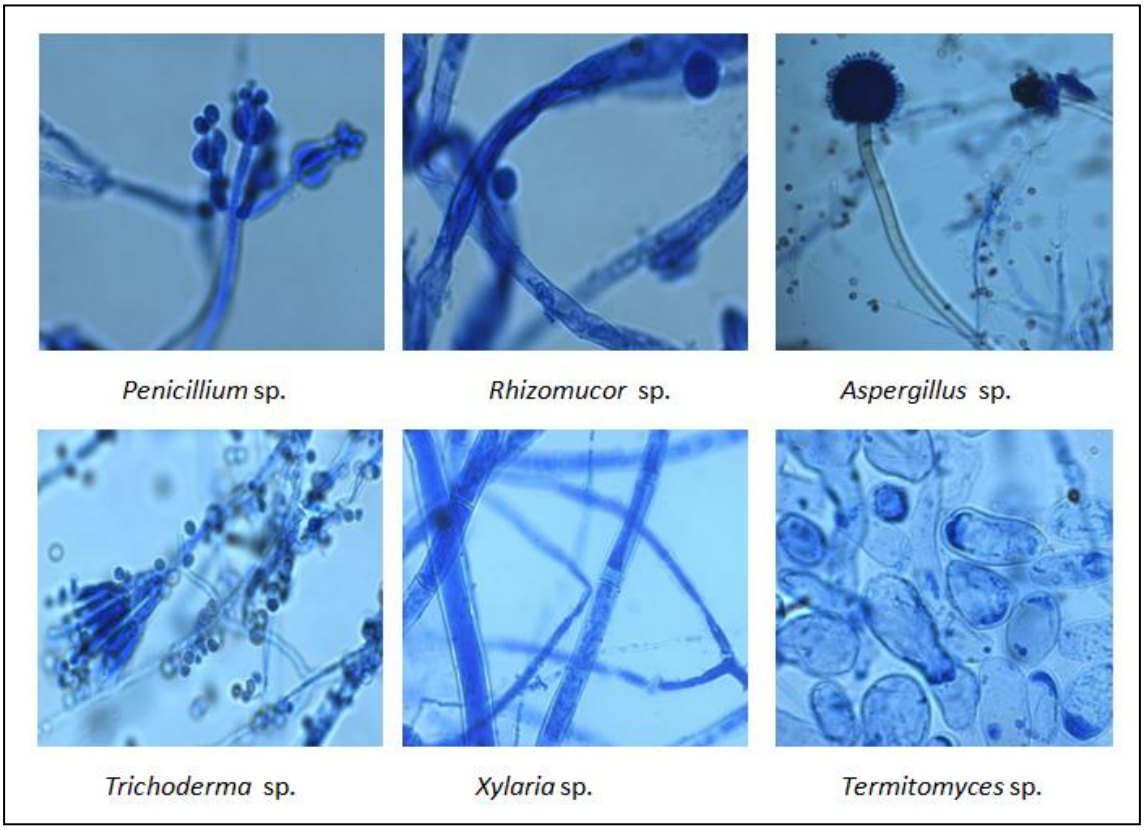


Figure.4.6. Staining images of different fungi collected from *Odontotermes* combs

The fungi in the phylum Basidiomycota are characterised by sexualy reproducing club-shaped fruiting bodies called basidia which are the swollen terminal cell of a hyphae. Basidia is most likely derived from the ascus found in Ascomycota, with which it shares several characteristics. The main difference is that basidium bears its spores outside while ascus retain them inside the structure.

4.ix. Fungus interaction assay

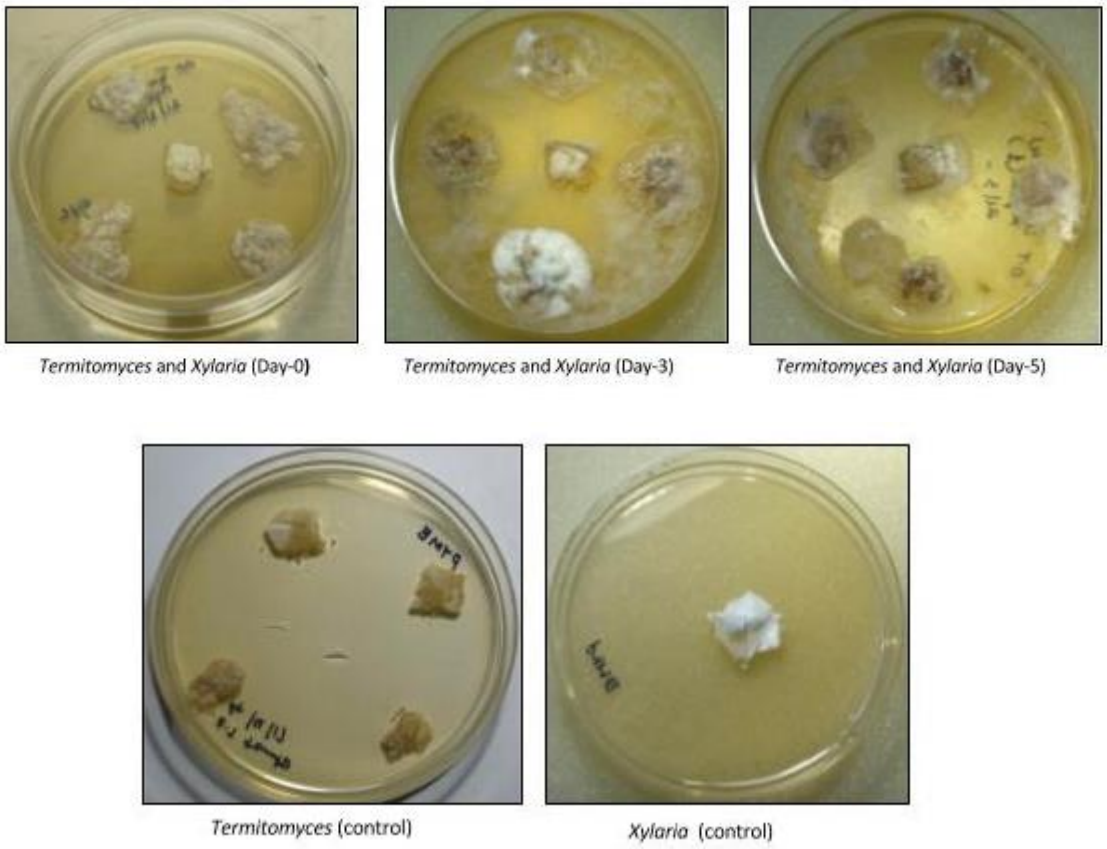


Figure.4.7 Representative example of *Termitomyces*- *Xylaria* interaction assay.

This study was carried out to assess the interaction of comb associated 'weedy' fungus with the cultivar fungus *Termitomyces*. From the plate assays, it was observed that *Termitomyces* is unable to inhibit the growth of *Xylaria*, where as *Xylaria* mycelia covered the *Termitomyces* plugs within few days of culture. The mycelia of Xyalri appeared to be healthy after interacting with *Termitomyces*. Growth of both *Termitomyces* and *Xylaria* were less in experimental plates as compared to control plates.

Discussion and Conclusion

Through this study we tried to explore the fungal diversity in the combs of *Odontotermes*. We tried to study the interaction between symbiotic fungus *Termitomyces* and other contaminating fungi. From our study, we could conclude that our study area (IISER Mohali campus) is dominated by *Odontotermes* which are higher termites. These *Odontotermes* show symbiotic relationship with a specific fungus *Termitomyces* (Phylum: Basidiomycota). It is well known fact that in the absence of termites different varieties of antagonistic fungi cover the termite combs. But there are some mechanisms through which termites suppress the growth of these contaminating fungi. A fungus interaction assay was performed between *Xylaria* and *Termitomyces* to monitor the growth rate of *Termitomyces* in the presence of *Xylaria*. We observed a reduction in the growth rate of cultivar fungus. This indicates that, there exists a competition between *Termitomyces* and *Xylaria*. In this study different types of fungi were collected from the combs of *Odontotermes*. Identification of these fungi was done using molecular methods and also by staining.

Further studies have to be done to see how termites are suppressing the growth of antagonistic fungi from combs. In fungus growing ants, it is well known that a particular species of bacteria is involved in removal of contaminating fungi from their nests. Like this, in termites also different types of bacteria can be present. To confirm the presence of bacterial diversity, their role and probability of functioning as a mutualist in the removal of antagonistic fungi from termite combs, analysis of microbial community structure in the gut and fungus combs of *Odontotermes* termites using both culture dependent and culture independed approaches have to be done.

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