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STUDY ON THE FUNCTION OF SYMBIOTIC BASIDIOMYCETES OF FUNGUS-GROWING TERMITES OF THAILAND

INTRODUCTION

Termites (also known as white ants) are any member of the order Isoptera , a small social insects living in a nest or colony system. Termites are abundant soil animals and play an important role in the process of litter decomposition in tropical terrestrial ecosystem due to their symbiosis with microorganisms. (Lee and Wood, 1971; Wood and Sands, 1978). Termites are generally divided into two groups; lower and higher termites. The Macrotermitinae (higher termites) have a great impact in tropical regions due to a highly efficient system for digesting plant litter due to their symbiotic relationship with basidiomycete fungi. The lower termites comprise of six families containing wood-feeders. Nevertheless, all known termites have a dense and diverse gut microbial community that aids in digestion (Ohkuma, 2003).

Termitidae (the higher termites) are the largest family with approximately 85% of all known genera. Members include the fungus-growing termites (subfamily Macrotermitinae) which cultivate a symbiotic fungus garden which is essential for the termite's survival (Slaytor, 1992; Abe *et al*., 2000). The fungus-growing termites feed on the wide range of dead and living plant material for example; grass, leaf litter and wood that is fully processed by their fungal symbiont (the basidiomycete, *Termitomyces*) on fungus combs in the nest. Fungus-growing termites construct fungus comb with dead plant material, and cultivate the symbiotic fungi on this fungus comb (or fungus garden). The symbiotic fungi are found as mycelia and white round structures, called fungus nodule in the termite nests. In a particular season, mushroom appear on the termite nests. These mushrooms are unique in nature, blooming only from the termite nests, and are commercially fascinating due to their prized edibility. These mushroom belonging to the genus *Termitomyces* (Order; Agaricales, family; Tricholomataceae). These termites have a great impact on plant

litter decomposition and carbon cycling in tropical ecosystems (Wood and Thomas, 1989; Yamada *et al*., 2005). For example, Burke and Cairney (2002) demonstrated that fungus-growing termites consumed 90% of the dry woody litter in an arid tropical area of Kenya. The fungus-growing termites genus *Macrotermes*, *Odontotermes*, *Microtermes* and *Hypotermes* are found in the Oriental region.

In most *Macrotermes* species, termite workers once ingest dead plant material and deposit undigested or partially digested feces on the top rim of the fungus comb. Thus, there is age gradient within a fungus comb. As the fungus comb aging, decolorization from dark to light brown is observed. After a certain period, the aged part of the fungus comb is eaten by the host termites (Wood and Thomas, 1989). Several roles of symbiotic fungi have been proposed, including the provision of glycosyl hydrolases (Martin and Martin, 1978), enrichment of nitrogen (Collins, 1983; Matsumoto, 1976) and lignin degradation (Hyodo *et al*., 2000; Rohrmann, 1978) with the significance of each role apparently varying in importance among host termite species (Hyodo *et al*., ,2003; Rouland-Lefevre, 2000). Jojima *et al.* (2003a) were isolated a novel hydrogen peroxidase-dependent phenol oxidase (TAP) from the basidiomycete *Termitomyces albuminosus*. This purified enzyme can oxidized various phenolic compounds. The deduced amino acid sequence of TAP showed 56% identity with dye-decolorizing heme peroxidase (DYP) from the ascomycete *Geotrichum candidum* Dec1. Johjima *et al*. (2003b) have investigated the mechanism of food processing by the fungus-growing termite *Macrotermes gilvus* and reported that the water-soluble phenolic compounds in fungus comb of *Macrotermes gilvus* were degraded during fungus comb aging. They concluded that the newer part of fungus comb, litter fiber was rigid structure and contains secondary metabolites such as phenolics. *Termitomyces* degrades the phenolics in fungus comb, resulting in reduction of toxicity. Laccase possibly involves in lignin degradation. Multiple laccases from *Termitomyces* could be responsible this process. Swift *et al.* (1979) reported that higher plants synthesize and accumulate a variety of phenolic compounds as secondary metabolites. Although physiological functions of plant phenolic compounds are not yet fully understood, it is thought that they contribute to plant defenses against pests and pathogens and therefore they also influence the

decomposition of plant litter by microorganisms in the detritus food chain. Consequently, the phenol degradation in fungus comb is considered to be important for improving palatability of termite food, especially containing high phenol content such as fallen leaves and bark.

In this study, the experiment was separated into 3 parts; first, the phylogenetic analysis of the *Termitomyces* strains isolated from various places and locations in Thailand. The fungus comb of various fungus-growing termites were collected from various locations in Thailand. *Termitomyces* strains were isolated from the fungal nodules and fruiting body and identified. Because there has been no report about developing the fruiting body from mycelia of the symbiotic fungi in a laboratory and mycelia give poor information about the taxonomy based on morphology, the *Termitomyces* cultures were identified using molecular technique. The phylogenetic relationship between the termites host and their location were discussed. Second, study on the phenol-oxidizing enzymes involved in the lignin degradation in the termite nests. The phenol-oxidizing enzymes in fungus combs of four species of fungus-growing termites and in 22 *Termitomyces* strains were investigated and laccase cDNA sequences were identified directly from the fungus combs and *Termitomyces* spp. to show the distribution and diversity of laccase genes in symbiotic fungi. The expression level of laccase genes were also investigated. Third, the EST analysis were performed in two cDNA libraries (NS and SB libraries) to investigate most abundant genes in the fungus combs and *Termitomyces* which let us more clearly understand the function of the symbiotic fungi in the termite nests.

OBJECTIVES

Overall objectives

To identified the symbiotic fungi in the fungus growing termites nest by using the molecular genetic method, and to investigate the enzymes involved in lignin degradation in the termite nests.

Specific objectives

1. To investigate the relationship between the symbiotic basidiomycetes of fungus-growing termites and their host.

2. To investigate the degradation enzymes and the involving genes of the symbiotic basidiomycetes (*Termitomyces* spp.) of fungus-growing termites.

3. To study the primary structures of laccase-like phenoloxidase in *Termitomyces* spp.

4. To investigate the distribution and diversity of laccase genes in these symbiotic fungi.

5. To study the expression level of laccase genes in the fungus combs

6. To investigate the most abundant genes in the fungus combs and *Termitomyces*.

LITERATURE REVIEW

1. General Introduction of Termites

1.1 Termites

Termites (also known as white ants) are any member of the order Isoptera, a small, pale and soft-bodied social insects living in a nest or colony system. Termites are common in all rain forests except in Queensland, Australia, where species diversity is low (Collins, 1983). Termites are abundant soil animals and play an important role in the process of litter decomposition in tropical terrestrial ecosystem due to their symbiosis with microorganisms. (Lee and Wood, 1971; Wood and Sands, 1978). Termites have chewing mouthparts. They feed chiefly on wood, from which they obtain cellulose. In primitive species cellulose is converted into various sugars by specialized gut protozoans and in the more highly evolved termites by specialized bacteria living symbiotically in the termite's digestive tract. The nearly 2,000 species are mostly tropical, and some build huge mounds to house their colonies.

1.2 Termite Colonies and Castes

Termite colonies are composed of three castes; the reproductives, known as alates (kings and queens, winged sexuals), the soldiers, and the workers. The kings and queens are sexually mature termites, with compound eyes and fully developed wings. The workers and soldiers lack wings and compound eyes.

When the surviving termites settle, the reproductive's wings become a weakened seam at the base. They then form pairs and establishes a new colony. A couple built a chamber in wood or soil, in which they mate; they remain permanently paired and the queen eventually produces as many as 30,000 eggs per day. Two or three weeks after mating, the young nymphs hatch and are fed on liquid secreted by

the parents and on fecal wastes, from which they obtain the protozoan or bacterial symbionts essential for life.

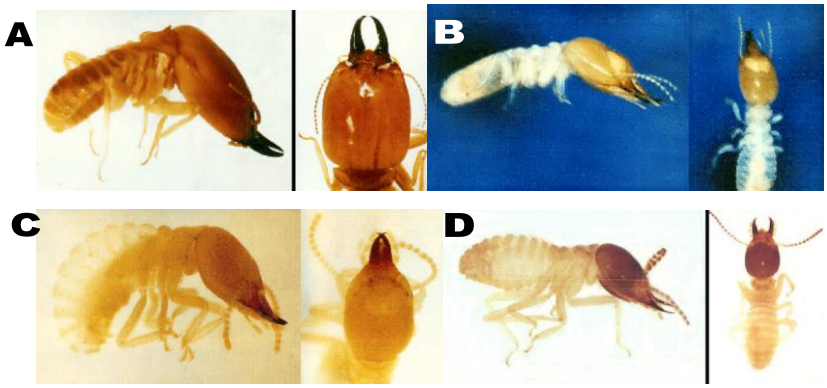

Figure 1 The soldiers of some termites in subfamily Macrotermitinae.
A) *Macrotermes bellicosus* soldier B) *Microtermes* sp. soldier
C) *Odontotermes formosanus* soldier D) *Hypotermes* sp. soldier
Source: Myles (2005)

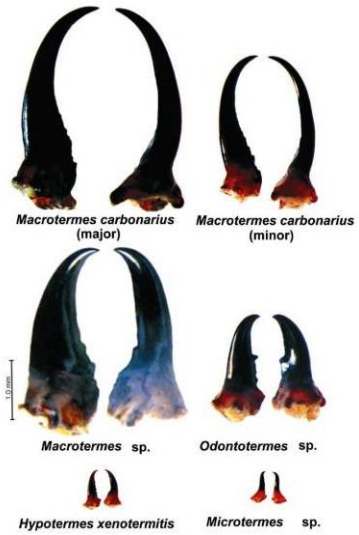

Figure 2 Soldier mandibles of Macrotermitinae
Source: Myles (2005)

The caste that the young termite, or nymph, will develops is depend on the amount of growth-inhibiting substance (a pheromone) that they get during feeding

and grooming. The pheromone is secreted by the reproductives, when the colony reaches a certain size, some of the nymphs begin to develop into reproductives. A large colony may have several pairs of reproductives. The soldiers and workers are added, since they do not produce the pheromone.

In some families of termites, no workers develop, and the nymphs perform worker functions, the soldiers, and the very young nymphs; caring for the eggs; grooming the queen; constructing and repairing the nest and foraging for food.

The soldier caste of termites is unique among social insects by morphology, development and behavior. The behavior of the soldier is specialized in defence. Two kinds of weapons are utilized, either alone or in combination; mandibles and defensive secretions.

1.3 Classification

In termites, three different feeding guilds are distinguished; soil-feeding, wood feeding and fungus-growing termites. These three major groups play different roles in decomposition processes. Termites can classified into seven families; Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae, Serritermitidae and Termitidae. Within these families, 14 subfamilies are recognized. Termitidae are the largest family with approximately 85% of all known genera, sometimes called the higher termites. Members include the fungus-growing termites (subfamily Macrotermitinae) which maintain a complex social order based on caste, age and feeding habits and which cultivate a symbiotic fungus garden which is essential for the termite's survival (Slaytor , 1992).

1.4 Symbiotic Organisms of Termites

The word "symbiosis" was coined by de Bary, who intended it to be an outcome-independent term describing the living together of two or more organisms which are not closely related in phylogeny, without the implication of beneficial

exchanges, but in modern usage it is normally assumed that symbiosis means mutual dependence as well as physical intimacy, i.e. mutualism (Saffo, 1993). In such relationships it is often the case that the partners are unequal in size, fundamentally different in biological organization and separated by large taxonomic distance. The term "termite symbiosis" is the mutualisms between higher animals (the host) and microorganisms (that can drawn from three domains: Archaea, Eubacteria, and Eucarya (protozoans and fungi)) where the microorganisms are housed internally (rarely on the external surface), and also 100% prevalent within the host (Bignell, 2000).

2. Subfamily Macrotermitinae (the fungus-growing termites)

The fungus-growing termites feed on the wide range of dead and living plant material for example; grass, leaf litter and wood that is fully processed by their fungal symbiont (the basidiomycete, *Termitomyces*) on fungus combs in the nest. The termite genera; *Sphaerotermes*, *Protermes*, *Megaprotermes*, *Pseudacanthotermes*, *Acanthotermes* and *Synacanthotermes* are found in the forests of tropical west and central Africa. *Allodontermes* is a southern African genus. *Ancistrotermes* is a savanna-inhabiting genus. Three genera are widely distributed in the Afrotropical and Oriental regions, that are; *Macrotermes*, *Odontotermes* and *Microtermes*. *Hypotermes* and *Euscaiotermes* are found in the Oriental region.

The fungus-growing termites (subfamily Macrotermitinae) have a great impact on the decomposition of dead plant material in the ecosystem due to their symbiotic fungi (Wood and Sands, 1978). Fungus-growing termites construct fungus comb with dead plant material, and cultivate the symbiotic fungi on this fungus comb (or fungus garden). These brain-like structures were identified for the first time by the German naturalist Koenig in 1799. These structures were later designated fungus combs or fungus gardens. More than half a century later, The English cryptogamist Gardner collected a large carpophore of the Agaricales order from a termite nest. Heim (1941) created a new genus for these fungi, *Termitomyces*, which contains all the "termitophilic Agaricales" (cited by Rouland-Lefevre, 2002).

Macrotermes spp. are the dominant soil animals and sometimes construct very large mounds up to 5 m in height in Africa and Asia. *M. gilvus* is a common mound-building termite which is widely distributed in south-eastern Asia. Soil modification by mounds-building termites has a marked effect on vegetation and also on the other animals (Wood and Sands, 1978). The mounds of *M. gilvus* in Thailand reach a height of 0.28-2.1 m and a basal diameter of 5-10 m. The mound surface was bare soil, but was overgrown with grass or herbs in the case of large mounds. Under a thick outer wall (13-48 cm thick), there was a living area referred to as the hive. The hive contained a royal chamber, a nursery zone, food store and fungus combs. Eggs and larvae of termites were found in the nursery zone and on fungus combs. Around the hive, fungus combs were distributed in thick-walled separate chambers in which termites stored food in the same way as in the hive (Inoue *et al*., 1997). The function of food store is not well understood. It is comprises small particles cut from plant litter and looks like moist sawdust. Lower concentrations of plant secondary metabolites have been detected in *Macrotermes gilvus* food stores than in the leaf litter around the mound (Johjima *et al*., 2003b). The food store may well help in detoxification or improve the palatability of the litter (Rouland-Lefevre , 2006).

2.1 The fungus comb in termite nests

The true nature of the fungus comb material was focus of attention for several years. The results clearly indicated the feces origin of the comb material. But Grasse' (cited by Rouland-Lefevre, 2000) noted that the structure of fecal balls used to build the fungus comb was clearly different from feces; he concluded that the fungus combs are built from plant material ingested after a brief mastication by the mandibles and passed rapidly through the gut without digestion. Materials accumulate in the rectum, which expels them in one piece forming the ball referred to as a mylosphere. The mylospheres are not really feces in the normal sense, but are commonly termed "primary feces" to distinguish them from "final feces" which are made from fully digested residues in the ordinary manner, old combs are consumed by the termites. At the macroscopic level, three categories of fungus combs can be distinguished;

- alveolar fungus combs ; whose dark upper part and clear lower part can be easily distinguished. These are typical of the genera *Macrotermes*, *Odontotermes* and *Protermes*.

- globular, oolitic and light-coloured fungus combs ; (with the exception of a darkish zone representing the oldest part of the comb). These combs are characteristic of the genera *Microtermes* and *Ancistrotermes*.

- fungus combs formed in vertical units ; arranged in an intricate or interlocking way in the manner of the parts of a jigsaw puzzle, their colour being evenly brown. They are found in the genus *Pseudacanthotermes*. The fungus comb of *Synacanthotermes* can also be classified in this group.

To construct fungus comb, different Macrotermitinae species feed on different plant materials; usually one of wood, leaf litter or grass. For example, *Macrotermes carbonarius* feeds on leaf litter while *Odontotermes* spp. are likely to be predominantly wood feeders. Fungus combs were subdivided into two parts: new and old (Hyodo *et al*., 2003). The two parts were defined differently depending on the termite genus (Rouland-Lefevre, 2000). In *Macrotermes* and *Odontotermes*, the top rim of the fungus comb constitutes the new part, and is constructed from fresh faeces. The old part is the bottom of the comb that has little recognizable mycelium and no fungal nodules; this part is largely consumed by the termites.

2.2 The symbiotic fungi

The symbiotic fungi are found as mycelia and white round structures, called fungus nodule in the termite nests. In a particular season, mushroom appear on the termite nests. These mushrooms are unique in nature, blooming only from the termite nests, and are commercially fascinating due to their prized edibility. These mushroom belonging to the genus *Termitomyces*. Seventeen species of *Termitomyces* have been reported in the tropics of Asia, Africa and South Pacific. Katoh *et al*.

(2002) demonstrated that the fungi comprising the fruiting bodies and fungus combs are identical.

Seven species of *Termitomyces* in South Africa were identified (Van Der Westhuizen and Eicker, 1990). Many researchers had investigated characteristic of the cultures and fruiting body of these fungi (Quimio, 1977; Van Der Westhuizen and Eicker, 1990; Botha and Eicker, 1991). Van Der Westhuizen and Eicker (1990) described the morphology of seven species of *Termitomyces* ; *T. clypeatus, T. microcarpus, T. sagittiformis, T. schimperi, T. striatus, T. umkowaani* and *T. reticulates* sp. nov. Botha and Eicker (1991) shown the cultures characteristic of five South African *Termitomyces*, namely *T. umkowaani, T. reticulates, T. sagittaeformis, T. clypeatus and T. microcarpus*. They found that *T. microcarpus* form significantly different culture with the other species and conclude that *T. microcarpus* should be transferred to the genus *Podabrella* Singer. Quimio (1997) was isolated *Termitomyces cartilaginous* into pure culture by tissue planting technique on malt extract agar. This fungus grew better at 30 °C than at 20 °C and did not grew at 10 and 40 °C, the optimum pH was pH 6-9 but can not induced this fungi to form fruiting body in laboratory.

Moreover, many reports about the phylogenetic relationship of these fungi and termites were published (Aanen *et al*., 2002; Katoh *et al*., 2002; Rouland-Lefevre, 2002; Froslev *et al*., 2003;). Rouland-Lefevre (2002) investigated the phylogenetic relationships of several African *Termitomyces* species and found that this group is clearly monophyletic and belong to the Tricholomataceae family. In congruence with the taxonomy based on morphology, *Termitomyces* appears to be monophyletic in members of the order Agaricales (Ohkuma, 2003). Froslev *et al*. (2003) shown that termitophilic fungi constitute a strongly supported monophyletic group within lyophylloid species.

Figure 3 Mushroom (*Termitomyces* sp.) blooming and fungus comb of fungus-growing termites. (Photos by Dr. Inoue)
Source: Ohkuma (2003)

2.3 The evolution of transmission of fungal symbionts in fungus-growing termites

Korb and Aanen (2003) concluded that in the fungus-growing termites (Macrotermitinae) horizontal transmission seems to be the rule as the termites normally acquire their cultivated fungus (*Termitomyces*) from the environment. As illustrated in figure 4a by the two separate life-cycle, the fungus produce fruiting bodies (basidiocarps) with sexual spores. These spores are carried into newly founded nests by the first workers of the new colony on their first foraging trips. In the laboratory experiments have shown that alates fail to establish a colony unless they are supplied with an external source of fungal spores. However, a few species of Macrotermitinae have developed vertical, uniparental symbiont transmission (for example; *Macrotermes bellicosus* and *Microtermes* spp.) as shown by a single life-cycle in figure 4b. In this case, the alates of one sex carry a bolus of conidia (asexual spores) in their foregut from the fungus combs of the parent colony to inoculate the first fungus combs in their newly founded colonies. This results in a lack of different mating types in the fungus so that sexual reproduction of the fungus is not possible. The difference in sex-specificity in fungus transmission indicates an independent origin of uniparental, vertical transmission in both groups (Korb and Aanen, 2003 and

reference there in). This was supported by molecular, phylogenetic investigations; termites with vertical transmission do not form a monophyletic group, but belong to two unrelated clades (Aanen *et al*., 2002). Katoh *et al*. (2002) also concluded that horizontal transmission of symbiotic fungi (*Termitomyces* spp.) among termite colonies (*Odontotermes formosanus*) in the Ryukyu Archipelago occurred during the evolutionary history of this symbiosis. They found that the termites display little genetic variation among the colonies, while the symbiotic fungi consist of two major genetic types. The molecular phylogenetic trees of the symbiotic fungi based on internal transcribed spacer and 18S rDNA suggested that these two types of fungi are different species. Aanen and Eggleton (2005) concluded that fungus-growing termites originated in African rain forest and that the main radiation leading to the extant genera occurred there (figure 5).

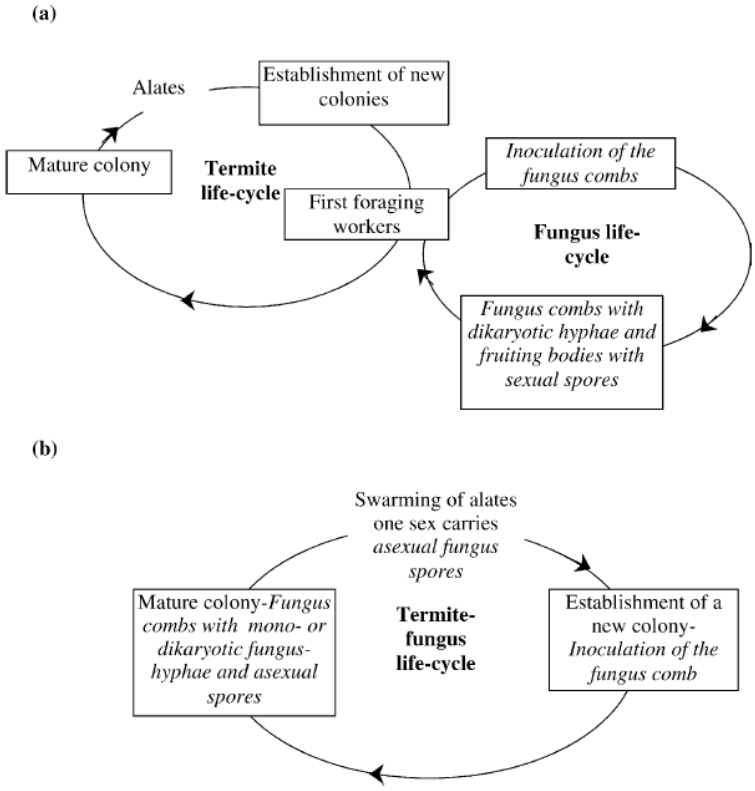

Figure 4 Transmission of fungus in Macrotermitinae during colony foundation. *Text in italics*: fungus life-cycle; regular text: termite life-cycle. a) Horizontal transmission b) Vertical, uniparental transmission

Source: Korb and Aanen (2003)

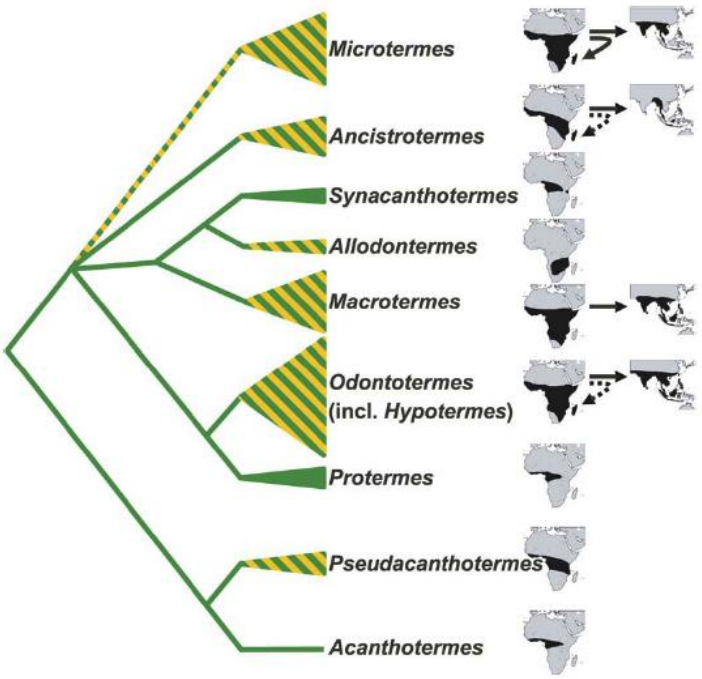

Figure 5 Simplified representation of the main biogeographical findings for fungus-growing termites. The phylogenetic relationships among the 10 termites genera. Colors of lines indicate estimated ancestral habitat (green, forest; striped green-yellow; ambiguous). Colors of triangles the habitat type (green, forest species; striped green-yellow, both forest species and savanna species). The African origin of the Malagasy *Odontotermes* and *Ancistrotermes* is hypothetical, as indicated by a broken arrow.

Source: Aanen and Eggleton (2005)

2.4 The life cycle of *Termitomyces*

The life cycle of *Termitomyces* was usually assumed that these fungi have a heterothallic life cycle like most basidiomycetes. The figure 6 demonstrate the life cycle of *Termitomyces* with different transmission modes. In this life cycle, the general scheme, spores germinate and form a monokaryon, all cells have a single nucleus. Two monokaryons of the same species with different mating types can fuse and form stable dikaryon. A dikaryon can form fruiting bodies, where meiosis and spore formation take place. The symbiosis fungi of Macrotermitinae with a horizontal

transmission are likely to fit into this scheme. Alternatively, another scheme (vertical transmission), some of the *Termitomyces* fungi might have a homothallic mating system which a single fungal spore is sufficient to complete a fungal life cycle. In this case, the symbiotic fungi do not form fruit body.

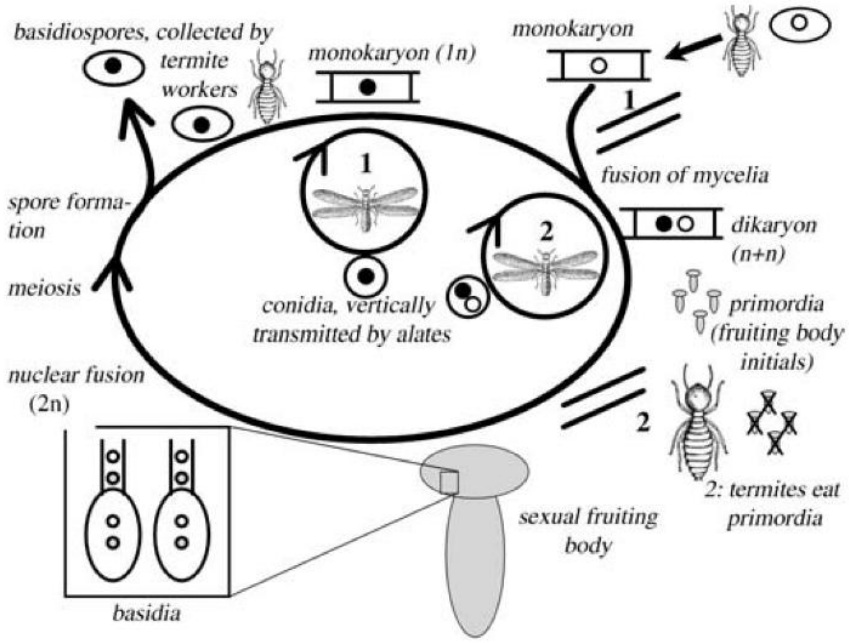

Figure 6 The life cycle of *Termitomyces* with different transmission modes. *Outside the main circle*, is the life cycle of a fungus that is horizontally transmitted as indicated. *Inside the main circle*, the potential cycles of a vertically transmitted fungus are drawn. For these fungi, the horizontal route is blocked (*parallel lines*) at either *1* (no fusion of monokaryons) or *2* (termites prevent fruiting of dikaryon by eating primordia). In 1, there is clonal propagation by alates of the monokaryotic stage (*small cycle 1*), whereas in 2 there is clonal propagation of the dikaryotic stage (*small cycle 2*).

Source: Korb and Aanen (2003)

Two type of life cycle are assume to be possible; possibility 1 (figure 6), the mycelium of these species is monokaryotic and this monokaryotic mycelium is clonally propagated by vertical transmission, keeping mycelia monokaryotic could be

a proximate explanation for the lack of fruiting bodies of some symbiotic fungi. Another possibility (possibility 2 in figure 6) is that the non-fruiting bodies fungi have a dikaryotic mycelium that is clonally transmitted by vertical transmission. In this case, the termites might actively prevent their fungal symbionts from fruiting, for example by eating the primordial. De Fine Licht *et al*. (2005) reported that *Termitomyces* sp. associated with the South Africa termite *Macrotermes natalensis* has a heterothallic mating system, with the fungus garden of the termite mound being in the heterokaryotic phase.

2.5 Role of the symbiotic fungi in termite nest

Several hypotheses have been proposed to explain the role of symbiotic fungi in termite nutrition (Bignell, 2000);

- Symbiotic fungi provide termites with cellulase and xylanases (Rouland *et al*., 1988a,b,c; Bignell *et al*., 1994).

- Fungi serve as nitrogen-rich food, which is advantageous because the dead plant material consumed by termites contains very little nitrogen.

- Fungi degrade plant lignin, which otherwise impedes the digestion of cellulose (Hyodo *et al*., 2000).

The activity of *Termitomyces* in fungus comb of termite nests have been proposed to play an important role in decomposition of plant litter, to degrade lignin and enhance the digestibility of cellulose for the termites (Rouland-Lefevre, 2000; Ohkuma, 2003). Matoub and Rouland (1995) were purified one endoxylanase (EC 3.2.1.8) and two endoxylanase (EC 3.2.1.37) from the termite *Macrotermes bellicosus* workers and its symbiotic fungus *Termitomyces* sp. Mora and Lattaud (1999) found laccase, an enzyme involved in phenol oxidation, in all fungus-growing termites tested, the symbiotic fungi and fungus combs. Hyodo *et al*. (2000) found the in vitro digestibility of cellulose in old fungus comb was approximately 3-fold higher than

that in the fresh part. Johjima *et al*. (2003b) shown that the water-soluble phenolics were degraded in the fungus comb.

Hyodo *et al*. (2003) had investigated the role of symbiotic fungi in lignin degradation and food provision differs among fungus-growing termites. They found that symbiotic fungi play different roles among fungus-growing termites. In *Macrotermes* spp., the main role of symbiotic fungi is to degrade lignin, so that the termites can utilize cellulose more efficiently, whereas in *Odontotermes* spp., *Hypotermes makhamensis, Ancistrotermes pakistanicus* and *Pseudacanthotermes militaris*, it is to serve as a food source.

Martin and Martin (1979) concluded that the fungal nodules in the termite nest (*Macrotermes natalensis*) are a source of C1-enzymes (active against crystalline cellulose) which acquired to digest the cellulosic materials. Slaytor (1992) was investigated the role of symbionts in cellulose digestion in termites *Macrotermes michaelseni* and found that the endo-β-1,4-glucanase and β-1,4-glucosidase activities were found in the fungal nodules associated with this termite and no evidance that bacteria are involved in cellulose digestion in termites. Various kinds of extracellular cell wall degradation enzymes were studied in *Termitomyces* spp.; cellulases (Martin and Martin, 1978; Rouland *et al*., 1988 a,b), α-amylase and endo-β(1,4)-glucanase (Ghosh and Sengupta, 1987; Sengupta and Sengupta, 1990), β-glucosidase (cellobiase) (Sengupta *et al*., 1991; Khowala and Sengupta, 1992; Rouland *et al*., 1992; Roy *et al*., 1994; Mukherjee and Khowala, 2002 a, b; Mukherjee *et al*., 2006), xylanases (Rouland *et al*., 1988c; Matoub and Rouland, 1995; Ghosh *et al*., 2002), hydrolytic enzymes (Mora and Rouland, 1994; Crosland *et al*., 1995), acetyl (xylan) esterase (Mukhopadhyay *et al*., 1997; Mukhopadhyay *et al*., 2003), amyloglucosidase (Ghosh *et al*., 1997), β-xylosidase (Bhattacharyya *et al*., 1997).

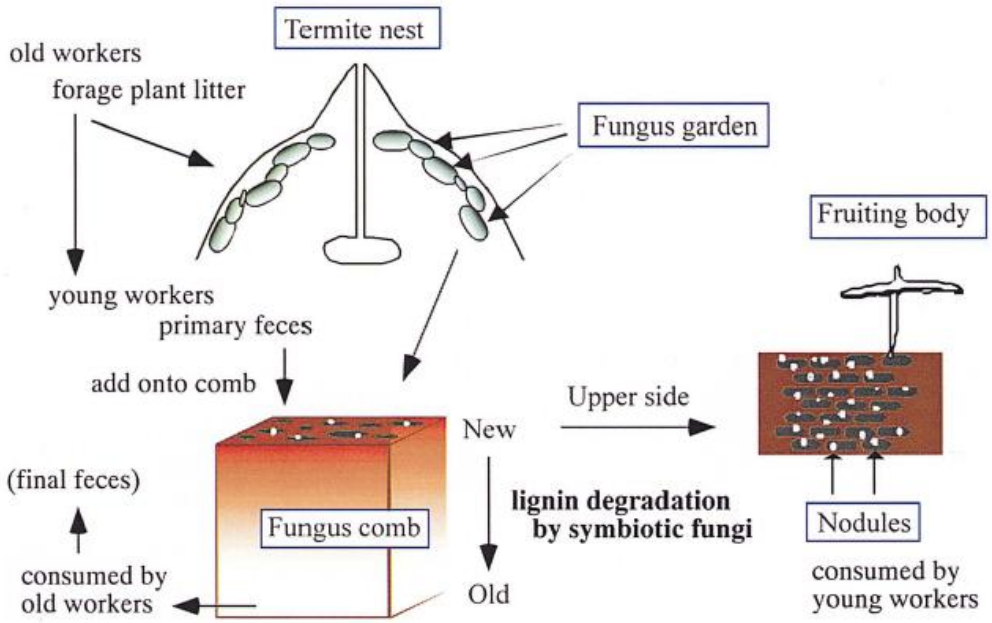

Figure 7 Symbiotic relationship between fungus-growing termites and *Termitomyces* fungi.

Source: Ohkuma *et al*., (2001)

3. Molecular genetics

Molecular genetics is the field of biology which studies the structure and function of genes at a molecular level. The field studies how the genes are transferred from generation to generation. Molecular genetics employs the methods of genetics and molecular biology. It is so-called to differentiate it from other sub fields of genetics such as ecological genetics and population genetics. An important area within molecular genetics is the use of molecular information to determine the patterns of descent, and therefore the correct scientific classification of organisms: this is called molecular systematics.

4. Molecular Approaches for Phylogenetic Study in Fungi

4.1 Ribosomal RNA (rRNA) is the central component of the ribosome which is the place for protein synthesis in the cell. The function of the rRNA is to provide a

mechanism for decoding mRNA into amino acids and to interact with the tRNAs during translation by providing peptidyl transferase activity. The ribosome in both prokaryotic and eukaryotic is composed of two subunits, large and small subunit. In prokaryote composed of 50S and 30S, whereas in eukaryote composed of 60S and 40S. The S units of the subunits represent measures of sedimentation rate rather than mass. The sedimentation rate of each subunit is affected by its shape, as well as by its mass. Eukaryotic ribosomes contain four different rRNA molecules; 18S, 5.8S, 28S and 5S rRNA. The 18S rRNA in most eukaryote is in the small ribosomal subunit and the large subunit contains three rRNA species (the 5S, 5.8S and 28S rRNAs). Eukaryotes generally have many copies of the rRNA genes organized in tandem repeats. rRNA is the most conserved (least variable) gene in all cells (Baennett and Lasure, 1985). Genes that encode the rRNA (rDNA) are sequenced to identify an organism's taxonomic group, calculate related groups and estimate rate of species divergence. For this reason many thousands of rRNA sequences are known and stored in specialized databases.

Table 1 Ribosome in Prokaryote and Eukaryote

Ribosome	Subunit	rRNA	Proteins
Bacterial ribosome 70S Mass: 2.5 x 10^6 D 66 % RNA	50S	23S = 2,904 bases 5S = 120 bases	31
	30S	16S = 1,542 bases	21
Mammalian ribosome 80S Mass: 4.2 x 10^6 D 60 % RNA	60S	28S = 4,718 bases 5.8S = 160 bases 5S = 120 bases	49
	40S	18S = 1,874 bases	33

Source: Modified from Lewin (1997)

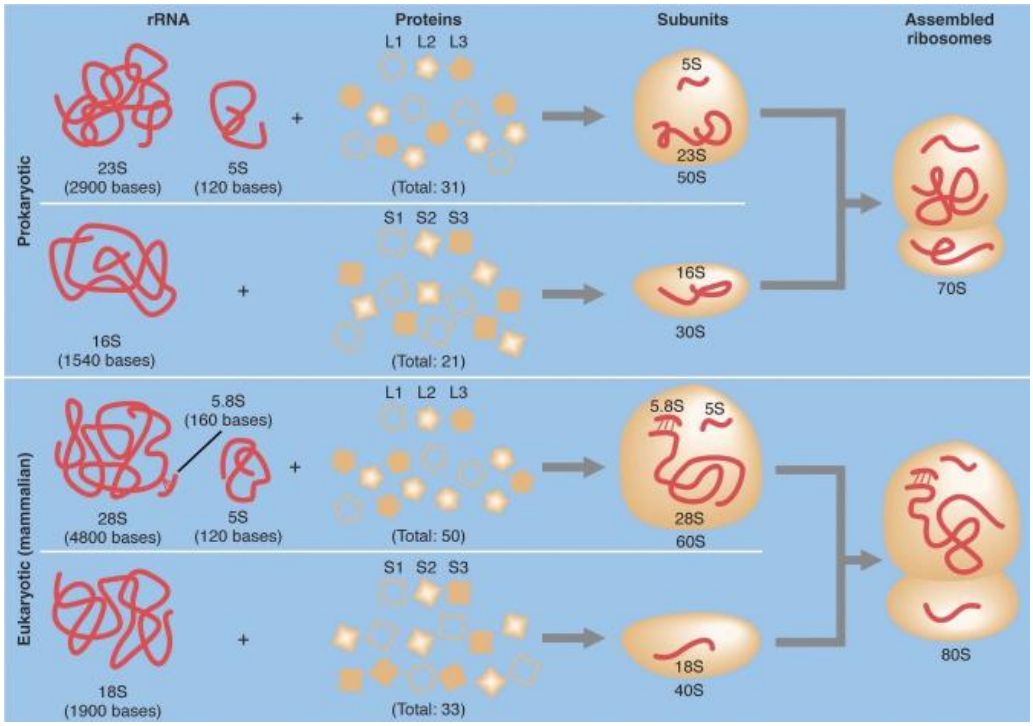

Figure 8 Structure of prokaryotic & eukaryotic ribosomes
Source: Carr (2005)

4.2 Spacer DNA is the part which separates one gene from the next in any gene cluster, or one gene cluster from the next. Spacer DNA can provide a source of DNA polymorphisms. Most use has been made of the internal transcribed spacers (ITS) within the transcription units of the DNA that correspond to the ribosomal RNAs (Moore and Frazer, 2002). The internal transcribed spacer (ITS) is a concept in molecular biology. The spacer is a sequence of RNA in a primary transcript that lies between precursor ribosomal subunits and is removed by splicing when the structural RNA precursor molecule is processed into a ribosome. These sequences are coded by ribosomal DNA. Eukaryotic organisms have two internal transcribed spacers; ITS-1 is located between the 18S gene and the 5.8S gene, and ITS-2 is located between the 5.8S and the 28S gene. Ribosomal genes and spacers occur in tandem repeats that are thousands of copies long, each separated by what is termed an intergenic spacer (IGS) or non-transcribed spacer (NTS). The ITS region is widely used in taxonomy and molecular phylogenetics. Use of PCR with

universal ITS primers produced amplified ITS products (http://www.biology.duke.edu/fungi/mycolab/primers.htm), which proved to have unique and easily identifiable fragment patterns for a majority of species of fungi (dermatophyte and wood-staining fungi) after digestion with the restriction endonuclease (Moore and Frazer, 2002).

The application of molecular techniques that exploit sequence variation within ribosomal RNA (rRNA) genes and associated spacer regions has enabled the field of fungal ecology to progress rapidly over the last decade (Anderson and Cairney, 2004). Within the rRNA gene cluster, the target regions most commonly used in ecological studies of fungal communities are the genes encoding 18S rRNA and 25/28S rRNA, and the internal transcribed spacer (ITS) region that incorporates the 5.8S rRNA gene. The highly variable nature of rapidly evolving rDNA spacer regions has made the ITS the most popular choice for species level identification of fungal taxa in environmental DNA pools (Anderson and Cairney, 2004). As a result, it is the region for which the largest amount of reference database sequence information is currently available for the molecular identification of fungi (Anderson and Parkin, 2006).

The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA (for small- and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. In addition to the standard ITS1+ITS4 primers used by most labs (figure 9), several taxon-specific primers have been described that allow selective amplification of fungal sequences.

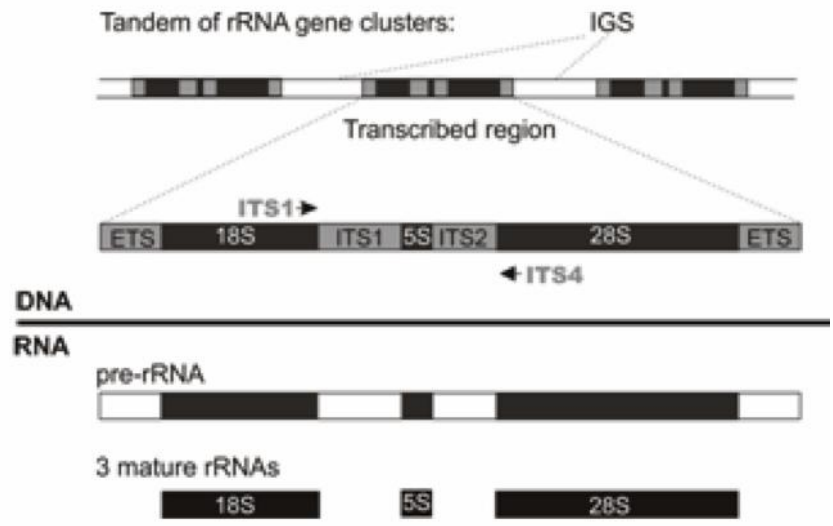

Figure 9 Schematic representation of rDNA region with primers ITS1 and ITS4 localization (arrows).ITS=internal transcribed spacers, ETS = external transcribed spacers, IGS=non-transcribed intergenic spacers. The transcription unit of the eukaryotic rDNA is typically hundreds of tandem repeats of 18S, ITS1, 5.8S, ITS2, and 28S.

Source: Korabecna (2007)

5. Phenol-oxidizing enzymes

Lignin is the most abundant aromatic polymer in nature. It serves to protect cellulose from most forms of microbial attack. Lignin biogenesis involves free radical polymerization of the precursors, *p*-coumaryl, coniferyl, and sinapyl alcohols, the relative proportions depending on the type of plant and tissue (Cullen and Kersten, 1996). Lignin composed of phenylpropanoid units linked through several major types of carbon-carbon and ether bonds so the mechanisms by which the fungal enzymes function must be oxidative rather than hydrolytic. In fungi, white-rot basidiomycetes have ability to degrade a variety of aromatic compounds, such as lignin (Kirk *et al*., 1987) and aromatic pollutants (Pointing, 2001). The fungi efficiently break down the lignin polymer into smaller, low molecular weight fragments that are available either

for conversion to fungal biomass or for further decomposition by bacteria (Lyons, 2002). It has been shown that extracellular phenol-oxidizing enzymes, lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13) and laccase (EC 1.10.3.2), are responsible for depolymerization of lignin (Kuwahara *et al*., 1984; Paszczynski *et al*., 1985; Gold *et al*., 1989; Kirk *et al*., 1987). Some white rot fungi produce all these enzymes while others produce only one or two of them (Hatakka, 1994).

Termitomyces spp. have ability to produce various kinds of phenol oxidizing enzymes, for example, Jojima *et al* (2003a) were isolated a novel hydrogen peroxidase-dependent phenol oxidase (TAP) from the basidiomycete *Termitomyces albuminosus*. This purified enzyme can oxidized various phenolic compounds. The deduced amino acid sequence of TAP showed 56% identity with dye-decolorizing heme peroxidase (DYP) from the ascomycete *Geotrichum candidum* Dec1.

5.1 Lignin peroxidase

Lignin peroxidase catalyzes the oxidation of various aromatic compounds to form aryl cation radicals (Kirk *et al*., 1987), the resulting aryl cation radicals degrade spontaneously via many reactions dependent on the structure of the substrate and on the presence of reactant. Veratryl alcohol (3,4-dimethoxybenzyl alcohol), a secondary metabolite of white rot fungi, acts as a cofactor for the enzyme. Veratryl alcohol is efficiently oxidized by LiP to form veratraldehyde at the expense of one equivalent of H_2O_2. Moreover, from the crystal structure of LiP, only veratryl alcohol can enter the active site. It is not possible for the lignin polymer to interact directly with the heme-group of the enzyme. Veratryl alcohol is used as an assay for enzyme activity due to the easily detectable absorbance of the product veratraldehyde at 310 nm. In the oxidative depolymerization process, phenolic compounds are formed. Manganese peroxidase (MnP) or laccases, will convert the phenolic LiP breakdown products to quinines form which are further metabolized (Schoemaker and Piontek, 1996).

5.2 Manganese peroxidase

Manganese peroxidases first discovered in *Phanerochaete chrysosporium*, are ubiquitous in lignin-degrading fungi. The enzyme has an absolute requirement for Mn^{2+} to complete the catalytic cycle. MnP oxidizes Mn^{2+} to Mn^{3+}, which diffuses from the enzyme and oxidizes various phenolic compounds. These enzymes require hydrogen peroxide for their activities (Cullen and Kersten, 1996). Catalysis involves initial oxidation of the ferric enzyme by H_2O_2, forming the two-electron-oxidized intermediate compound I. Compound I can oxidize phenols or Mn^{2+} by one-electron-producing phenoxyl radicals or Mn^{3+} and the one-electron-oxidized enzyme intermediate, compound II. The catalytic cycle is complete by the reduction of compound II; compound II has an absolute requirement for Mn^{2+} as the reductant. Mn^{3+} is a potent oxidant capable of oxidizing a large number of phenolic compounds. A distinguishing property of MnP and other fungal peroxidases is their high reactivity. Fungal peroxidases are capable of oxidizing substrates that other peroxidases are incapable of oxidizing (Ambert-Balay *et al*., 2002).

5.3 Laccase

Laccase also catalyzes the oxidation of various phenolic compounds and aromatic amines but this reaction is coupled with the reduction of molecular oxygen to water (Thurston, 1994). Although laccase catalyze the oxidation of phenolic substrates by O_2, but can also oxidize nonphenolic lignin model compounds in the presence of a suitable redox mediator (Cameron *et al*., 2000). Because of their broad substrate specificities, they can be also involved in degradation of a variety of plant phenols. Althought laccase was also called diphenol oxidase, monophenols like 2,6-dimethoxyphenol or guaiacol are often better substrates than diphenol, e.g. catechol or hydroquinone. Syringaldazine [N,N'-bis(3,5-dimethoxy-4-hydroxybenzylidene hydrazine)] is often considered to be a unique laccase substrate, and hydrogen peroxide is avoided in the reaction due to this compound is also oxidized by peroxidases (Leonowicz and Grzywnowicz, 1981; Baldrian, 2006).

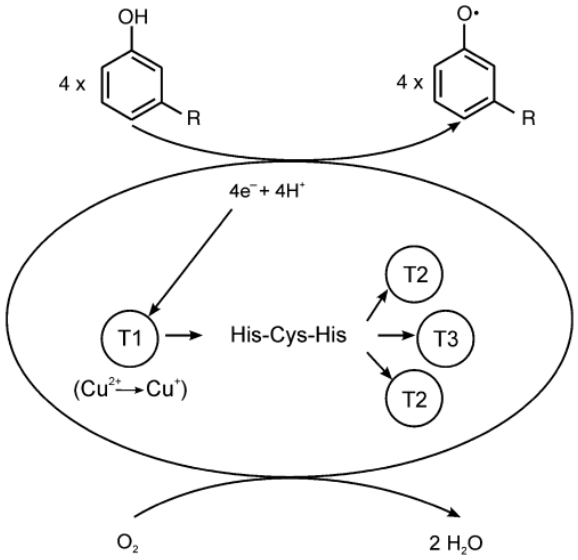

Figure 10 Catalytic cycle of laccase
Source : Baldrian (2006)

Laccases (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) belong to a group of polyphenol oxidases containing copper atoms in the catalytic centre and are in a large family of multicopper oxidases (MCOs) that also includes Fet3 ferroxidases, ascorbate oxidase (EC 1.10.3.3) and ceruloplasmin (EC 1.16.3.1) of plants (Larrondo *et al*., 2003; Baldrian, 2006). The typical substrates for laccase are phenolic systems. Their oxidation proceeds through an outer-sphere electron transfer process that generates a radical cation, which after fast proton abstraction generates a phenoxyl radical. Phenoxyl radicals are long-lived species with half-life times of hours (Crestini *et al*., 2003). Laccase is typically an extracellular enzyme with a molecular mass of ~65 kDa (Thurston, 1994). It was first demonstrated in the exudates of *Rhus vernicifera*, the Japanese lacquer tree and a few year later was also demonstrated in fungi. In many fungal species the presence of both constitutive and inducible laccases have been reported. Fungal laccases are extracellular soluble proteins. Usually the enzyme originates in the cytoplasm, but many instances of secretion of laccases have been reported. The outstanding white-rot fungi, *Phanerochaete chrysosporium* and *Phlebia radiata*, produce manganese peroxidase, lignin peroxidase and laccase when cultivated in the appropriate conditions.

Analogously to *P. chrysosporium*, lignin degradation by *P. radiata* requires a co-substrate and seem to be enhanced by an oxygen atmosphere and nitrogen limitation (Cabaleiro *et al*., 2002 and reference therein). Laccase activity has been detected in the bacterium *Azospirillum lipoferum*, indicated that laccase are not restricted to eukaryotes. Alexandre and Zhulin (2000) also reported that the laccase also widespread in bacteria. Current application for these enzymes in industrial processes include pulp delignification, textile dye bleaching, removal of phenolics from wines and transformation of antibiotics and steroids (Cherry and Fidantsef, 2003).

Basidiomycetes, which cause white-rot decay and thus are called white-rot fungus, are able to degrade lignin in wood efficiently. *Termitomyces* are also classified into white-rot fungi (Ohkuma, 2003). Mora and Lattaud (1999) reported the presence of the oxidation activity of 2,2'-azinobis (3-ethylbenzeothiazoline-6-sulfonic acid) (ABTS) and syringaldazine in the fungus combs of several fungus-growing termites in Africa and the authors considered that these reactions were catalyzed by laccase. Recent evidence, suggests that laccase, like lignin peroxidase, play a role in lignin degradation by fungi. The synergism between the laccase and manganese peroxidase of *Rigidopporus lignosus* is an example of the possible complex relationships that may be important in lignocellulose degradation (Cullen and Kersten, 1996). The involvement of laccase in ligninolysis is well established in *Pycnoporus cinnabarinus*, a fungus that lacks LiP and MnP (Eggert *et al*.,1997). However, some white rot fungi appear not to produce laccase, suggesting that this enzyme may not be essential for lignin decay (Hatakka, 1994). The most intensively studied white rot fungus, *Phanerochaete chrysosporium*, was thought to belong to this group. However, recently, laccase activity was detected in *P. chrysosporium* cultures grown under certain conditions (Gold and Alic, 1993; Srinivasan *et al*., 1995; Dittmer *et al*., 1997), but these results have not been widely accepted (Podgornik *et al*., 2001). Furthermore, Larrondo *et al.* (2003) report that the *P. chrysosporium* genome does not encode a typical laccase but rather encodes a unique extracellular multicopper oxidase with strong ferroxidase activity. The *P. chrysosporium* genome reveals an impressive array of genes encoding secreted oxidase, peroxidases and hydrolytic enzymes that cooperate in wood decay and revealed no conventional laccases but instead, four

multicopper oxidase (MCO) sequences are found and subsequent analysis has shown that it encodes a ferroxidase-like protein. (Martinez *et al*., 2004). Levin *et al*. (2002) demonstrated that the extracellular ligninolytic activities in the white-rot fungus *Trametes trogii* were induced by added 1 mM $CuSO_4 \cdot 5H_2O$ in the synthetic medium, higher copper concentrations inhibited growth. Chen *et al*. (2003) reported that the Basidiomycetes mushroom *Volvariella volvacea* strain V14 produced multiple forms of extracellular laccase when grown in submerged culture in a defined medium with glucose as sole carbon source and was regulated by copper (up to 200 µM $CuSO_4$) and by various aromatic compounds. Varela *et al*. (2003) have determined the liquid culture conditions of the brown-rot fungus *Gloeophyllum trabeum*, the results showed that the highest oxidoreductase activity was in the high carbon low nitrogen medium.

Laccases contain three types of copper that can be distinguished using UV/visible and EPR (electron paramagnetic resonance) spectroscopy. At least one type 1 (T1) copper (blue copper) should be presence with at least three additional copper ions; one type 2 (T2) and two type 3 (T3). Type 1 copper gives a blue colour to the protein from an absorbance at about 600 nm, owing to the charge transfer between Cu(II) and a cysteine residue and is EPR detectable. Type 2 confers no colour, shows a very weak absorption and functions as a one-electron acceptor, EPR is detectable. Type 3 copper is a pair of copper atoms that give a weak absorbance in the near UV (330 nm), functions as a two-electron acceptor and have no EPR signal (EPR-silent) (Solomon *et al*.,1996; Leontievsky *et al*., 1997). The substrates are oxidized by the T1 copper and the extracted electrons are transferred, probably through a strongly conserved His-Cys-His tripeptide motif, to the T2/T3 site, where molecular oxygen is reduced to water. Some enzymes lack the T1 copper so some authors use the term “yellow laccases” instance “true laccases” because these enzymes lack the characteristic absorption band around 600 nm (Leontievsky *et al*., 1997; Baldrian, 2006).

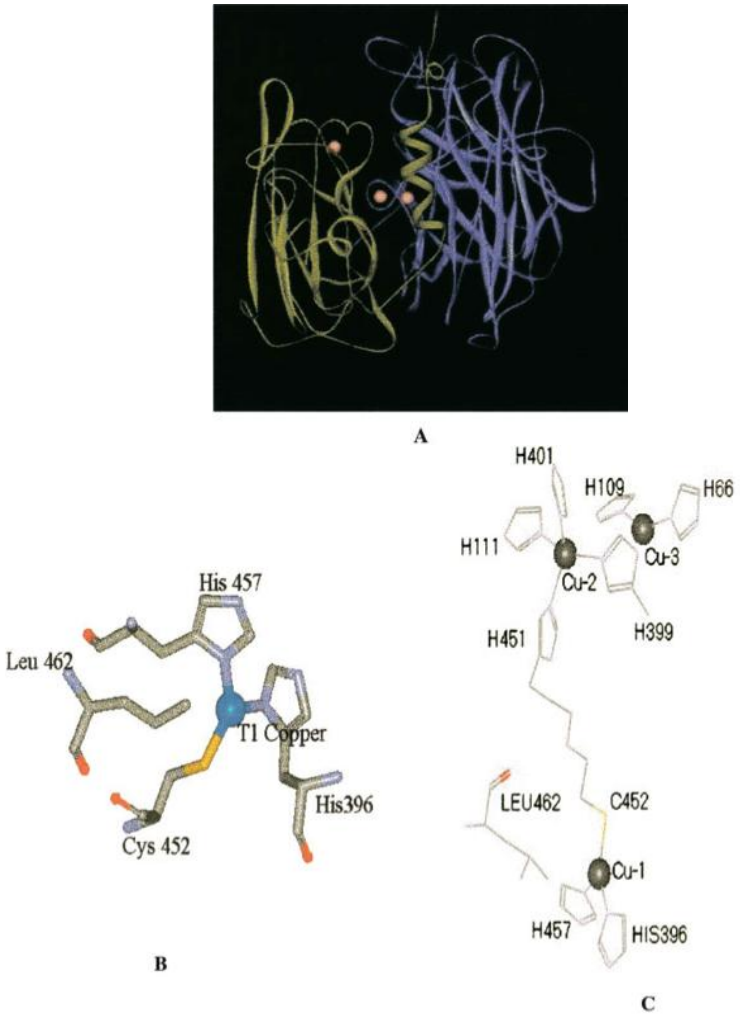

Figure 11 View of the ligands at T1 copper and trinuclear copper centers in *C. cinereus* laccase (a fungal laccase; PDB code 1A65) (adapted from Ducros *et al*., 1998; 2001). (A) Three-dimensional structure of backbone of entire domain of *C. cinereus* laccase showing all the copper ions at T1 and T3 copper centers. (B and C) Amino acid ligands in the vicinity of the T1 copper center and the trinuclear copper center with T2 and T3 copper. For T1 copper His396, His457, and Cys452 are equatorial ligands while Leu462 is an axial ligand. The His451–Cys452 electron bridge is highlighted.

Source: Kumar *et al*. (2003)

Because of fungal laccases are useful in biotechnological application, many research was emphasize on production, characterization and purification of fungal laccase (Petroski *et al*., 1980; Perry *et al*., 1993; Eggert *et al*., 1996; Leontievsky *et*

al., 1997; Munoz *et al*., 1997; Palmieri *et al*., 1997; Scherer and Fischer, 1998; Cambria *et al*., 2000; Dedeyan *et al*., 2000; Jung *et al*., 2002; Wang and Ng, 2006). Also the studied about laccase gene and/or cDNA sequences have been reported by several researchers; for example; in Basidiomycetes *Trametes* spp. by Collins and Dobson (1997), Galhaup *et al*. (2002), Hong *et al*. (2002), Gonzalez *et al*. (2003). Laccase mediator (low molecular weight compound) system (LMS) and nutritional condition also widely study to improve laccase production in various fungi. Arora and Gill (2001) found that the mineral salts malt extract broth was the best medium for laccase production and sugarcane bagasse was the best laccase inducer in white rot fungi; *Daedalea flavida*, *Phlebia brevispora*, *Phlebia radiata and Polyporus sanguineus*. Johannes and Majcherczyk (2000) found that the natural mediator such as, phenol, aniline, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol were as efficient as the systhetic compounds ABTS [2,2'-azinobis(3-ethylbenzeothiazoline-6-sulfonic acid)] and 1-hydroxybenzotriazole. Natural compounds such as methionine, cysteine and reduced glutathione, containing sulfhydryl groups were also active as mediator compounds. Carbajo *et al*. (2002) found that laccase activity was increase when *Coriolopsis gallica* was grown in the presence of tannic acid.

Ascomycetes *Podospora anserine* produces three laccase isoenzymes which differ in their substrate specificity, isoelectric point, molecular weight, amino acid and oligosaccharide composition and heat stability (Fernandez-Larrea and Stahl, 1996). Although the structure of the active site seems to be conserved in all the fungal laccases, there is great diversity in the rest of the protein structure (Mayer *et al*., 2002). The sequence features of multicopper oxidase have been report (Solomon *et al*., 1996) and the essential sequence features of fungal laccases was investigated based on multiple sequence alignments of more than 100 laccases. This has resulted in identification of a set of four ungapped sequence regions, L1–L4, as the overall signature sequences that can be used to identify the laccases, distinguishing them within the broader class of multi-copper oxidases (figure 12) (Kumar *et al*., 2003). Larrondo *et al*. (2004) have described a cluster of multicopper oxidase genes (*mco1*, *mco2*, *mco3*, *mco4*) from the lignin-degrading basidiomycete *Phanerochaete*

chrysosporium. This analysis, the *P. chrysosporium* genome revealed four putative MCO-encoding sequences (figure 13).

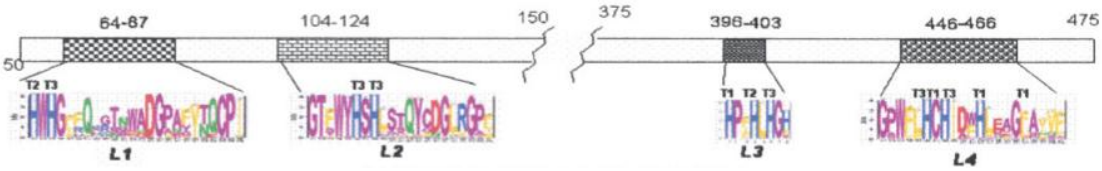

Figure 12 Overview of conserved regions in fungal laccases. Eighty percent consensus sequence obtained after multiple sequence alignment of 64 fungal laccase sequences.

Source: Kumar (2003)

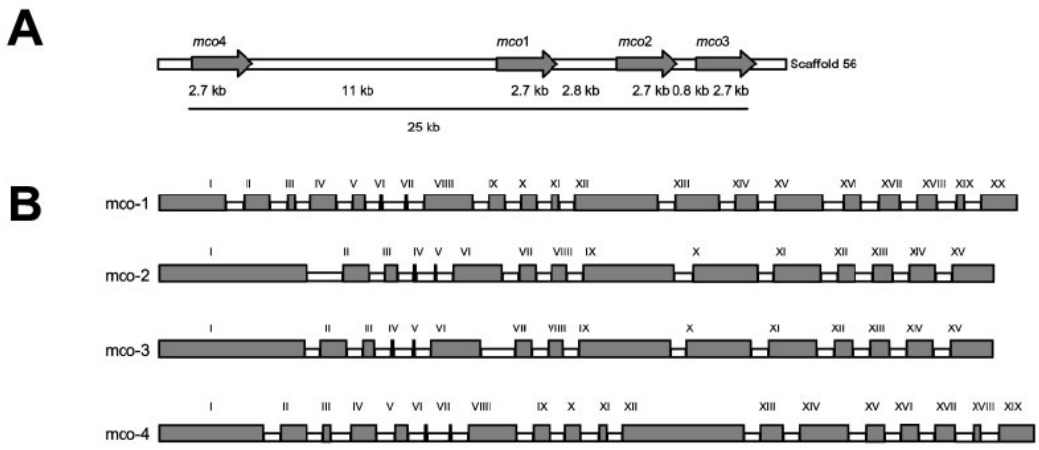

Figure 13 The *P. chrysosporium mco* gene cluster. A) Genomic organization of the *mco* genes present in scaffold 56. B) Intron/exon structure of the mco gene family members. Introns are presented as small white rectangles; exons correspond to the larger grey boxes. Exon numbers are indicated.

Source: Larrondo *et al*. (2004)

According to laccases consist of four regions which bind the copper atoms of the active centre. These copper binding regions are strongly conserved in all laccase (Figure 14) and were used for the design of generate primers to analyze and identify laccase genes of sample.

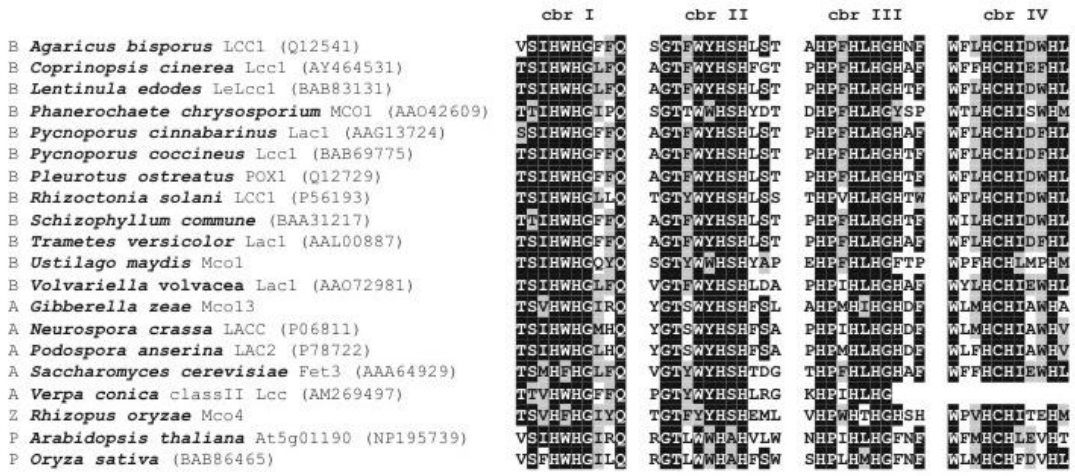

Figure 14 Amino acid alignment including the four well conserved copper binding region (cbr I-IV) of laccases used for the design of degenerate primers. B = Basidiomycetes, A= Ascomycetes, Z= Zygomycetes, P= Plants

Source: Kellner (2007)

6. Expressed sequence tag (EST) analysis

An expressed sequence tag or EST was the first method used for rapid identification of expressed genes and can be performed more easily than whole-genome sequencing. EST and complementary DNA (cDNA) sequences provide direct evidence for all the samples transcripts and are currently the most importance resources for transcriptome exploration. ESTs are short sub-sequence or a transcribed spliced nucleotide sequence (200-800 nucleotide bases in length), unedited, randomly selected single-pass sequence reads derived from cDNA libraries. At present, ESTs enable gene discovery, complement genome annotation, aid gene structure identification and facilitate proteome analysis (Nagaraj *et al*., 2007).

Messenger RNA (mRNA) sequences in the cell represent copies from expressed genes. As RNA can not be cloned directly, they are reverse transcribed to double-stranded cDNA using a specialized enzyme, the reverse transcriptase. The resultant cDNA is cloned to make libraries representing a set of transcribed genes of the original cell, tissue or organism. Subsequently, these cDNA clones are sequenced

randomly from both direction in a single-pass run with no validation or full-length sequencing to obtain 5′ and 3′ ESTs.

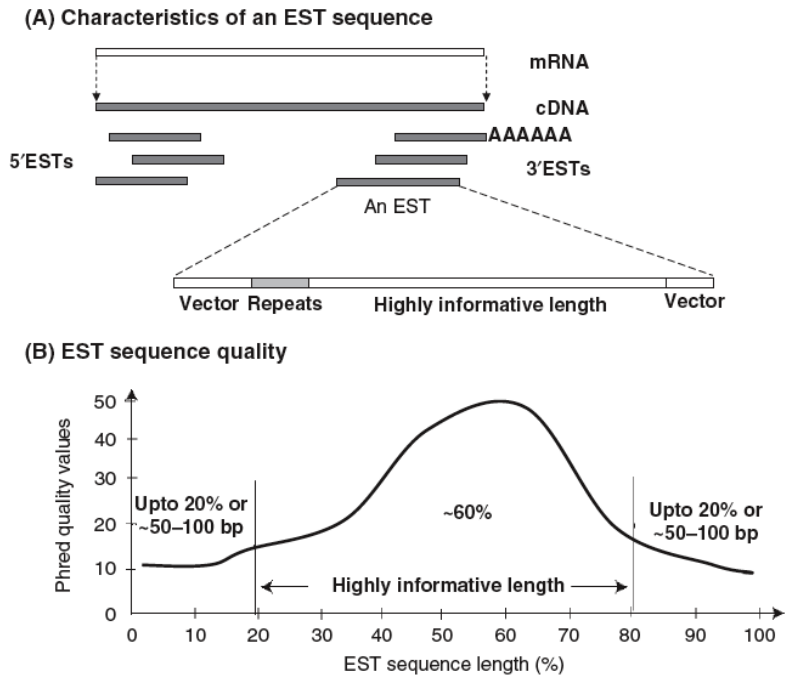

Figure 15 Characteristics of EST sequences. A) An EST sequence usually starts and ends with vector-contaminated bases, interspersed with possible repeats or low-complexity regions. B) Phred quality scores are plotted as a function of sequence length for a hypothetical EST sequence shown in A).

Source: Nagaraj *et al*. (2007)

A typical EST sequence (figure 15A) is only a very short copy of the mRNA itself and is highly error prone, especially at the ends. The overall sequence quality is usually significantly better in the middle (figure 15B). Vector and repeat sequences either in the end or rarely in the middle are excised during EST pre-processing. As ESTs are sequenced only once, they are susceptible to errors. Generally, the quality of base reads in individual EST sequences is initially poor (upto 20% or ~50-100 bp), gradually improves and then diminishes once again towards the end. The overall sequence quality is usually significantly better in the middle (highly informative length). Phred scores provide a measure of sequence quality with higher values

corresponding to better sequence quality. Phred examines the peaks around each base call to assign a quality linked to error probabilities (Nagaraj *et al*., 2007).

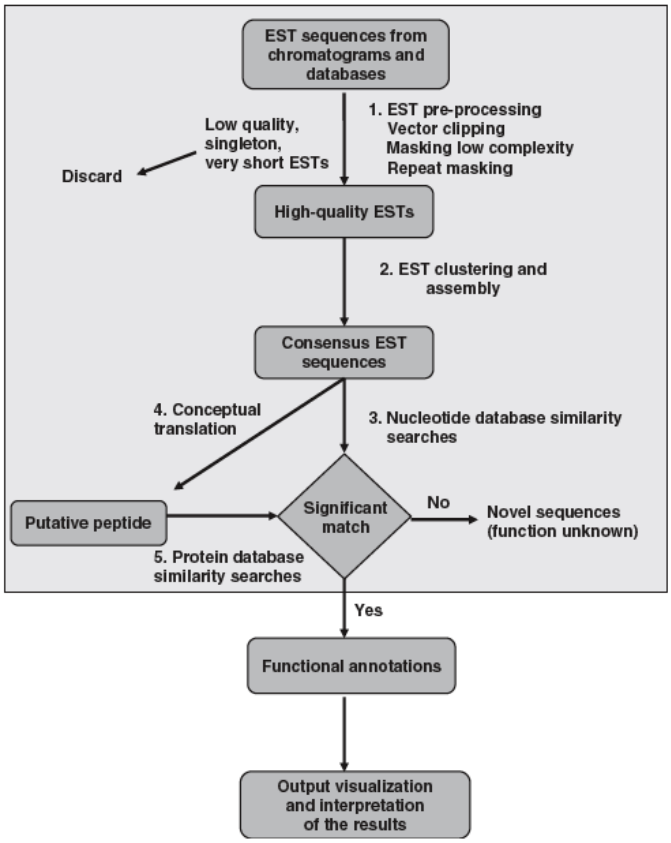

Figure 16 Generic steps involved in EST analysis. 1. Raw EST sequences are checked for vector contamination, low complexity and repeat regions, which are excised or masked. Low quality, singleton and very short sequences are also removed. 2. ESTs are then clustered and assembled to generate consensus sequences (putative transcripts). 3. DNA database similarity searches are carried out to assign, identify homologues and sign possible function. 4. Putative peptides are obtained by conceptual translation of consensus sequences. 5. Protein database similarity searches are performed to assign putative function(s). The analysis is extended to functional annotation followed by visualization and interpretation of results. The steps enclosed by the grey box alone are implemented in the current available pipelines.

Source: Nagaraj *et al*. (2007)

The different steps in the analysis of EST data sets is shown in figure 16. Chromatograms or EST sequences extracted from database are pre-processed (step 1, figure 16) into high-quality ESTs, then high-quality ESTs are grouped into cluster (step 2, figure 16) based on sequence similarity. This step serves to elongate the sequence length by culling information from several short EST sequences simultaneously. Subsequently, database similarity searches are performed against relevant DNA databases (step 3, figure 16). Additionally, a consensus sequence can be conceptually translated to a putative peptide (step 4, figure 16) and compared with protein sequence databases (step 5, figure 16) (Nagaraj *et al*., 2007).

There are two main problems associated with EST sequences; 1) the overall representation of host genes within a library. The mRNA population within a single cell, tissue or organism represents the collection of genes that are being actively transcribed to maintain genes that are expressed to achieve growth, pathogen defence or any other response from a wide assortment of available effects. A standard cDNA library is a faithful representation of the ratio of mRNAs present within a specific tissue under exact conditions at the time of sampling. Poorly expressed genes will be poorly represented within libraries, and genes that are not expressed will be absent. This limitation is difficult to resolve. We can only conclude that there was no measurable transcript under the conditions from which the particular tissue was sampled. 2) The overall quality of any individual sequence within a collection. Sequence quality describes the faithfulness with which an EST sequence represents the gene sequence from which it was reverse-transcribed and cloned. A low quality EST sequence is a poor representation of its cognate host gene. ESTs are not the complete representation of the parental cDNA. An ESTs length is limited to a few hundred nucleotides of reliable sequence. Even if both ends of the cDNA have been sequenced (producing two ESTs), the ESTs will not overlap in most cases, although the clones can be physically joined into a single logical pseudomolecule. The only viable alternatives to EST sequencing that address the attributes of incomplete sequence coverage and nucleotide quality are the full-length cDNA sequences. Full-length cDNA sequences are obtained by shotgun sequencing cDNA clones that have been selected for both 5′ and 3′ ends. Such a strategy yields many individual ESTs

that can assembled into a single contig. Each individual residue will be sequenced many times and the consensus will be of the highest quality (Rudd, 2003).

Expressed sequence tag data from fungi are also showing to be useful to specific and diverse aims, such as mapping previously characterized genes, investigation of patterns of fungal genome evolution, prediction of novel genes, prediction of pathogenicity determinants, identification of disease-related sequences, improvement of functional assignments and identification of alternatively spliced mRNA species (Ribichich *et al*., 2006)

MATERIALS AND METHODS

I. The phylogenetic analysis experiment

A. Samples collection

Fungus nodules and fruiting body from fungus comb of fungus-growing termites were collected from four sampling areas of Thailand, that are; Prachinburi, Saraburi, Chanthaburi and Nakronratchasima. For Nakronratchasima, only the fruiting body of mushroom (*Termitomyces*) was collected. The termite hosts and location used in this study was shown in Appendix Table A1.

The sampling sites were located at distances of at least 70 km to one another as shown in figure 17. For the samplings, a single fungus comb in a nest in each case was collected except for *Macrotermes annandalei* from Chanthaburi province, two strains from different nests were cultivated.

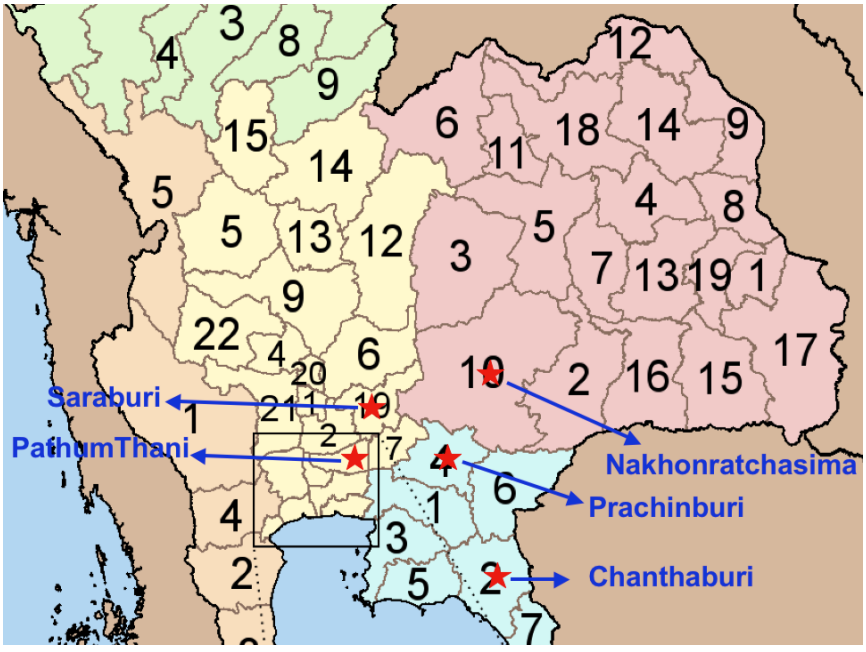

Figure 17 Geographic area show the distances between each sampling site

B. Fungus combs and *Termitomyces* strains isolation

Termitomyces strains were isolated from the fungus nodules on the fungus combs of each termite hosts. The fungus nodules were carefully picked up using sterile forceps, rinsed with 0.6% sodium hypochlorite solution, washed with steriled water and cultivated on Potato Dextrose Agar (PDA) in darkness at room temperature. One strain was cultivated from the fruiting body of *Termitomyces* sp. (inoculated with the tissue of the internal part of the stipe) blooming from the nest of *Odontotermes* sp. in Nakronratchasima.

C. DNA extraction from fungal cultures

DNA were extracted from the three weeks-old cultures using an ISOPLANT kit (Nippon Gene) according to the manufacturer's instruction. The fungal mycelium plugs were transferred to 1.5 ml tube and 300 µl of solution I with 2% mercaptoethanol was added and mixed for 1-2 sec and then 150 µl of solution II was added. Following the incubation at 50 °C for 15 min, then 150 µl solution III was added. The mixture was incubated on ice for 15 min. After centrifugation at 14,000 rpm at 4 °C for 15 min, the mycelium and agar were removed and supernatant containing the DNA was collected. 1/20 volume of 5M NaCl or 3M sodium acetate, 1 µl of ethachinmate and 2.5 volumes of absolute ethanol were added and incubated at -20 °C for 10 min followed by centrifugation at 14,000 rpm at 4 °C for 10 min, the supernatant was discarded and 1 ml of 70% ethanol was added subsequently by centrifugation at 12,000 rpm at 4 °C for 10 min. The precipitate was collected and dried using vacuum dryer for 2 min. Finally, 100 µl of TE buffer pH 8.0 was added to dissolved DNA. The DNA solution was treated with 1% v/v of 100 mg/ml of ribonuclease A (RNase A) and incubated at room temperature for at least 10 min. 100 µl of phenol/chloroform solution was added and centrifuged at 10,000 rpm for 5 min and take the upper part into new 1.5 ml tube. Five µl of 5M NaCl and 2.5 volumes of absolute ethanol were added and incubated at -20 °C for 10 min followed by centrifugation at 15,000 rpm at 4 °C for 15 min, the supernatant was discarded and 1 ml of 70% ethanol was added subsequently by centrifugation at 15,000 rpm at 4 °C

for 5 min. The precipitate was collected and dried using vacuum dryer for 2 min. Finally, 20 µl of TE buffer pH 8.0 was added to dissolved DNA. The DNA solution was kept at -20 °C.

D. Polymerase Chain Reaction (PCR)

The DNA region consisting of ITS1, 5.8S rDNA, ITS2 and partial large subunit (LSU) rDNA was amplified by PCR using ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White *et al.*, 1990) and LR7 (5′-TACTACCACCAAGATCT-3′) primers. Reaction mixture (total volume 30 µl) containing:

rTaq DNA polymerase (Toyobo)	0.15 µl
10x Buffer	3 µl
2.5 mM dNTP mixture	3 µl
25 mM $MgCl_2$	2.4 µl
Each of 5 pmol/µl ITS5 and LR7 primers	2 µl
Sterile deionized water	17.45 µl
DNA template	2 or 0.5 µl (4 ng or 1 ng)

PCR was carried out using a PTC-200 thermalcycler (MJ Research). Thermal cycling consisted of initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 45 sec, and extension at 72 °C for 2 min.

The amplified product from PCR was checked by electrophoresis on 1% agarose gel. The 1% agarose gel in 1x TAE (Tris-Acetate EDTA) buffer was prepared by adding 1 g of powder agarose to 100 ml of 1x TAE buffer. The slurry was heated in a microwave oven until the agarose completely dissolved. The warm agarose solution was cooled to 45-55 °C and then poured into the mold which had a comb placed above the mold. The gel should be between 3-5 mm thick and air bubbles were not allowed to be under or between the teeth of the comb. The gel was allowed to set at room temperature for 30 min. After that the comb was carefully removed and

ready for loading the sample. The gel was then placed in the electrophoresis tank and 1x TAE buffer was added to cover the gel to a depth of about 1 mm before closing the lid of the gel chamber. The 5 μl of PCR products were mixed with 1 μl of 6x loading buffer (Toyobo). Five μl (total 50 ng loading) of the DNA marker, λ/*Hind*III digest-ϕX174/*Hae*III digest (Toyobo), was used for estimating the DNA size. The PCR product solution or size-standard marker, was pipetted into the slots of the submerged gel. After that the electrical leads were attached to the electrophoresis tank so that the DNA will migrate toward the anode. The voltage was 100 volts for 30 min. The gel was run until the gel loading buffer have migrated the appropriate distance through the gel. The gel was then stained with ethidium bromide in 1xTAE buffer (0.5 μg/ml) for 15-20 min and examined under ultraviolet light of an UV-transilluminator (UVP Inc.) to visualize DNA bands and to print the photograph. A band size was estimated by bands of DNA marker. The expected size of amplified product was approximately 2 kbp.

E. Direct Purification of PCR product

The PCR product were purified using a Wizard PCRpreps DNA purification system (Promega) as followed;

1. The PCR product was transferred into 1.5 ml tube. 100 μl of direct purification buffer and 1 ml of resin were added. Vortex for 20 sec to mix.

2. For each PCR product prepared one Wizard Minicolumn. Attached the syringe barrel to the luer-lock extension of each minicolumn. Inserted the tip of the minicolumn/syringe barrel assembly into the vacuum manifold.

3. Transferred the solution from 1. into syringe barrel. Applied a vacuum to draw the solution into the minicolumn.

4. Added 2 ml of 80% isopropanol to the syringe barrel, and reapplied a vacuum to draw the solution through the minicolumn.

5. Dried the resin by continuing to draw a vacuum for 30 sec after the solution had been pulled through the column. Removed the syringe barrel and transferred the minicolumn to a 1.5 ml microcentrifuge tube. Centrifuged the minicolumn at 10,000 x g in a microcentrifuge for 2 min to removed any residual isopropanol.

6. Transferred the minicolumn to a new 1.5 ml tube. Applied 50 µl of water or TE buffer to the minicolumn and waited 1 min. Centrifuged the minicolumn for 20 sec at 10,000 x g to eluted the DNA fragment. The purified PCR products was kept at -20 °C.

The purified PCR products were used for either direct DNA sequencing as templates or cloning into a pGEM-T vector (Promega).

F. Cloning and Transformation

The purified PCR products were cloned into the pGEM-T vector (Promega). Reaction mixture (total volume 20 µl) containing;

The purified PCR product	9 µl
pGEM-T vector (10ng/ml)	1 µl
Ligation enzyme	10 µl

The mixture was mixed gently and incubated at 14 °C overnight and followed by transformation step; the tube of 100 µl of competent cells (*E. coli* JM109) were removed from -80 °C and thawed on ice for 10 min following pipetted into the ligation mixture and placed on ice for 30 min, then rapid transferred to 42 °C for 30 sec and placed on ice for 1 min. 900 µl of SOC medium was added and incubated at 37 °C for 1 hour subsequently centrifuged at 5,000 rpm for 10 min and discarded some supernatant. The remain supernatant and the pellet were mixed together. 50 µl of the mixture was spreaded on Luria-Bertani (LB) agar plates containing 50 µg/ml of ampicillin, 2% X-gal and 0.1 M IPTG. Following incubation at 37 °C overnight.

Eight white colonies per one sample were selected and picked up for insertion determination prior to sequencing.

G. Insertion determination

Inserted fragment was analyzed by colony-PCR amplification. The white colony on replica plate was picked up and mixed in the premixture. Premixture (total volume 20 µl) containing;

r*Taq* DNA polymerase (Toyobo)	0.15	µl
10x Buffer	3	µl
2.5 mM dNTP mixture	3	µl
25 mM $MgCl_2$	2.4	µl
Each of 5 pmol/µl M13F and M13R primers	1	µl
Sterile deionized water	10.45	µl

PCR was carried out using a PTC-200 thermalcycler (MJ Research). The inserted fragment was amplified by temperature profile, 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 1 min and final extension at 72 °C for 1 min.

The primers M13F (5'- GTAAAACGACGGCCAG- 3') and M13R (5'- CAGGAAACAGCTATGAC- 3') were used. The amplified product (5 µl) was size-fractionated by electrophoresis on 1% agarose gel in 1x TAE (Tris-Acetate EDTA) buffer. The amplified fragments approximately 2.4 kbp were used for sequencing.

H. Isolation and Purification of PCR products prior to sequencing

Eight clones per sample were picked up and cultivated in 2 ml of LB broth containing 100 µg/ml of ampicillin. The plasmid-containing bacterial cells were incubated in shaker at 37 °C overnight (12-16 hours). The plasmid DNA were extracted and purified. The cultures were centrifuged at 10,000 rpm for 2 min at

25 °C, the supernatant was removed. Added 200 µl cell resuspension solution with 6 µl of RNaseA (10 mg/ml) and resuspended the cells completely by vortexing. Added 200 µl of cell lysis solution and gently mix (invert the tube 4-6 times) to lyse the cells until the lysate becomes clear. Added 350 µl of Neutralization solution, then immediately and gently mixed the solution. Centrifuged for 10 minutes, meanwhile attached the syringe barrel to the luer-lock extension of each minicolumn. Inserted the tip of the minicolumn/syringe barrel assembly into the vacuum manifold. Pipetted the supernatant into syringe barrel. Applied a vacuum to draw the solution into the minicolumn. Added 2 ml of column wash solution and reapplied a vacuum to draw the solution through the minicolumn. Transfered the minicolumn to a new 1.5 ml tube and centrifuged at 10,000x g in a microcentrifuge for 2 min to removed any residual solution. Transfered the minicolumn to a new 1.5 ml tube. Applied 50 µl of water or TE buffer to the minicolumn and waited 1 minute. Centrifuged the minicolumn for 20 sec at 10,000 rpm to eluted the plasmid DNA. The purified plasmid DNA was precipitated using alcohol precipitation step and the pellet was dissolved in 20 µl steriled deionized water.

DNA yield was determined by measuring the concentration from its absorbance at 260 nm (A_{260}). Fifty µl of the eluated was measured using a spectrophotometer. The value at A_{260} allows calculation of total nucleic acid whereas the value at A_{280} determines the amount of proteins in the DNA solution. The A_{260} of 1 (with a 1-cm detection path) corresponded to 50 µg double-strand DNA per milliliter. Water should be used as diluent when measuring DNA concentration since the relationship between absorbance and concentration is based on extinction coefficients calculated for nucleic acids in water. The ratio of A_{260}/A_{280} of 1.8 to 1.9 revealed the high purity of DNA.

I. Restriction fragment length polymorphism (RFLP)

Plasmid DNA of the clones was analyzed by restriction fragment length polymorphism (RFLP) with *Hae*III and *Hha*I. Reaction mixture (total volume 20 µl) containing;

Plasmid DNA	1 µl
10x buffer	2 µl
*Hae*III or *Hha*I	0.2 µl
Sterile deionized water	16.8 µl

The mixture was incubated at 37 °C over night and checked the RFLP pattern by electrophoresis on 2% agarose gel in TAE buffer. The representative clone from each strain was analyzed by automated sequence analyzers.

J. Sequencing analysis

The representative clone from RFLP pattern of each strain was analyzed by automated sequence analyzers (ABI model 377) using the sequencing primers ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′), ITS3 (5′-GCATCGATGAAGAACGCAGC-3′), ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) for the ITS1-5.8S rDNA-ITS2 region determination and primers LROR (5′-ACCCGCTGAACTTAAGC-3′), LR3 (5′-CCGTGTTTCAAGACGGG-3′), LR3R (5′-GTCTTGAAACACGGACC-3′), LR5 (5′-TCCTGAGGGAAACTTCG-3′) and LR16 (5′-TTCCACCCAAACACTCG-3′) for the LSU rDNA region determination.

Sequencing reaction was performed with Bigdye terminator sequencing kit (Perkin Elmer). This kit included ddNTPs labeled with four different kinds of fluorescent dyes. Each extended fragment that reacted either with ddATP, ddTTP, ddCTP or ddGTP was simultaneously directed. The reaction mixture (total volume 20 µl) containing:

5x sequencing buffer	4	µl
Ready reaction premix	4	µl
1 pmol/µl of each primer	3.2	µl
Sterile deionized water		µl
DNA template solution	1	µg

Temperature profile was 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60°C for 4 min.

1. Purification of extension product

The entire content of each extension product was transferred into 1.5 ml-microcentrifuge tube and then 64 µl of 95% ethanol and 16 µl of deionized water was added. After vortex briefly, the tube was left at room temperature for 15 min and centrifuged at 15,000 rpm at room temperature for 20 min. Then, supernatant was carefully discarded by pipetting because DNA pellet may not be visible. Following 250 µl of 70% ethanol was added, the mixture was centrifuged at 15,000 rpm at room temperature for 10 min. Then the supernatant was discarded and centrifuged at 15,000 rpm at room temperature for 1 min. After that, the supernatant was discarded again. Finally, the DNA pellet was dried by vacuum centrifugation for 2 min and stored at –20 °C.

2. Sequencing

The purified extension product was used to long-read sequencing by an automatic sequence analyzer model 377 (ABI PRISM). The sequencing gel was prepared using mixture (total volume 50 ml) containing:

Urea (Research Organics)	18	g
PAGE-plus concentrate (Amresco)	5	ml
10x TTE (Tris-TAPS-EDTA.Na_2)	5	ml
Adjusted volume with sterile deionized water to	50	ml

The mixture (total volume 50 ml) was mixed by low speed of stirrer. Then 250 µl of 10% ammonium persulfate (Amresco) and 25 µl of TEMED (N,N,N’,N’-Tetramethyl ethylenediamine) (nacali tesque) were added and mixed gently. The gel mixture was casted on glass plates.

For DNA sample preparation, the dried DNA sample from 10.1 was dissolved with 6 μl of loading buffer containing 5 μl of deionized formamide and 1 μl blue dextran (ABI PRISM), then the mixture was denatured at 95 °C for 2 min and immediately chill on ice. The mixture 2 μl was loaded on the sequencing gel.

K. Phylogenetic Analysis

GENETYX-MAC V10.0 (Software Development) was used for basic analysis of sequences except a multiple alignment of gene sequences, which was performed by DNASIS-MAC V3.7 (Hitachi), using Higgins's method (Higgins, 1988). At first, all the ITS1-5.8S rDNA-ITS2 gene sequences region (530-645 bp) were analyzed for taxonomically assignment by the BLASTn program and grouped. The sequences showing at least 99% of the identities were classified to the same group. Then, one representative of each group from the ITS1-5.8S rDNA-ITS2 gene sequences were analyzed the LSU rDNA sequences (1.2-1.4 kbp) using BLASTn program and compared to the representative basidiomycetes' DNA sequences of Agaricales available in the databases. The closely related sequences from database were retrieved and added to the alignment. The sequences of internal transcribed spacer (ITS) region were used to compared and grouped the sequences of all samples. The sequences of large subunit RNA (LSU rDNA) of one representative from all groups were used for tree construction.

The DNA sequence data were aligned using the CLUSTAL W package and check manually. The programs implemented in PHYLIP 3.5c (distributed by Felsenstein, J., Department of Genetics, University of Washington, Seattle) were used to infer the neighbor-joining and the parsimony trees and to obtain bootstrap confident estimates. The program PUZZLE 4.0 was used with 10,000 puzzling steps to infer the quartet-puzzling maximum likelihood tree.

II. The laccase experiment

A. Fungus combs and *Termitomyces* strains isolation

The fungus combs of six fungus-growing termites (Macrotermitinae) were collected. The termite hosts and sampling locations in this study were *Macrotermes gilvus, Microtermes* sp. and *Odontotermes* sp. from Prachinburi province, *Odontotermes longignathus* and *Hypotermes* sp. from Saraburi province and *Macrotermes gilvus* from PathumThani province. The sampling location were separated from each other by at least 50 km as shown in figure 17.

The fungus combs were collected by 2 procedures; as the fresh samples and immediately put into liquid nitrogen and stored at -80 °C until used.

Twenty-two *Termitomyces* isolated strains from the fungus nodules from the previous experiment were used. One new strain (NS/Mg strain) was isolated from the fungus comb of *M. gilvus* from PathumThani provience by the method discussed previously.

B. Screening for peroxidative fungi

Twenty-two *Termitomyces* spp. strains (as described in Appendix Table A2, except for the *Termitomyces* strain from *M. gilvus* comb from PathumThani province) were recultivated on Kirk's agar media containing 0.01% guaiacol including 1.2 or 12 mM ammonium tartrate (low or high nitrogen source) and incubated in dark condition at room temperature and checked for growth and brown zone formation once a week for 4 weeks. If the fungi produced phenol-oxidizing enzyme(s), a brownish pigment will be observed.

C. Screening for ligninolytic enzyme producing *Termitomyces* spp. strains

The *Termitomyces* spp. strains (except for the *Termitomyces* strain from *M. gilvus* comb from PathumThani province) were recultivated on 120 ml of Kirk's liquid media containing 120 µl of 5% Remazol Brilliant Blue R (RBBR) dye including 1.2 or 12 mM ammonium tartrate (low or high nitrogen source) and cultured with agitation (120 rpm) at 28 °C in dark condition and measured the decolorization of RBBR dye every 2 day for 45 days. The decolorization of RBBR dye was measured as decrease in absorbance 592/500.

D. Measurement of phenol-oxidizing enzyme activities

1. *Termitomyces* spp. cultures preparation

The activities of phenol-oxidizing enzymes in liquid cultures of 22 *Termitomyces* spp. strains (except for the *Termitomyces* strain from *M. gilvus* comb from PathumThani province) were examined by inoculated 4 mycerial plugs (4 mm in diameters) of each *Termitomyces* strain grown on PDA to 500 ml flask containing 120 ml of Kirk's liquid media with 1.2 or 12 mM ammonium tartrate (low or high nitrogen source) and 120 µl of 5% Remazol Brilliant Blue R (RBBR) dye and cultured with agitation (120 rpm) at 28 °C in dark condition. The enzyme activities were determined from the culture media every 2 day for 45 days.

2. Enzyme extract from the fungus comb

The activities of phenol-oxidizing enzymes in the fungus combs of *M. gilvus* (PathumThani), *M. gilvus* (Prachinburi), *Odontotermes* sp. (Prachinburi) and *Microtermes* sp. (Prachinburi) were examined. The fungus comb was ground using mortar and pestle. Approximately 0.1 g of the ground comb was added to a 1.5 ml polypropylene tube containing 1 ml MiliQ water and subsequently mixed. The supernatant was recovered by centrifugation at 10,000x g for 5 min at 4 °C, and enzyme activities were determined.

3. Phenol-oxidizing enzyme activities determination

Three kinds of phenol-oxidizing enzymes activities were investigated; Lignin peroxidase (LiP), Manganese peroxidase (MnP) and Laccase (Lac).

a) Lignin peroxidase (LiP) activity was measured follow the method of Tien and Kirk (1988), using 3,4-dimethoxybenzyl alcohol (veratryl alcohol, VA) as substrate. LiP can oxidize VA to 3,4-dimethoxybenzardehyde (Vad) in the presence of hydrogen peroxide. Reaction mixture (total volume 1 ml) containing;

20 mM Na-tartrate, pH 3.0-3.5
0.5 mM VA
0.1 mM H_2O_2
100 µl culture medium or comb extracted

The rate of veratraldehyde formation was determined from the increase in absorbance at 310 nm.

b) Manganese peroxidase (MnP) activity was measured follow the method of Wariishi *et al*. (1992) using Mn^{II} as substrate. MnP can oxidize Mn^{II} to Mn^{III} in the presence of hydrogen peroxide and some organic acids. Reaction mixture (total volume 1 ml) containing;

50 mM Na-malonate, pH 4.5
0.5 mM $MnCl_2$
0.1 mM H_2O_2
100 µl culture medium or comb extracted

The formation of Mn^{III}-malonate complex was determined from the increase in absorbance at 270 nm.

c) Laccase (Lac) activity was measured using 2,6-dimethoxyphenol (DMP) or syringaldazine [N,N´-bis-(3,5-dimethoxy-4-hydroxybenzylidene) hyfrazine,

(ABTS)] as substrate. Laccase can oxidizes phenolic in the presence of oxygen. Reaction mixture (total volume 1 ml) containing;

50 mM Sodium citrate, pH 5 or 4.5
0.5 mM DMP or ABTS
100 µl culture medium or comb extracted

The formation of quinone dimmer was determined from the increase in absorbance at 469 nm or 415 nm for DMP or ABTS, respectively.

E. DNA extraction from the *Termitomyces* spp.

Termitomyces spp. (strains KU418, KU446, KU430, KU432, KU428, KU426 and NS/Mg) were culture in Potato Dextrose Broth (PDB), the three weeks old cultures were extracted DNA according to the method of Maeda (2001). The mycelia were recovered by filtration using a 32 µm nylon mesh filter and then frozen quickly in liquid nitrogen. The frozen mycelia were lyophilized and ground into a powder, the powder was then added to an extraction buffer (100 mM Tris/HCl pH 8, 100 mM EDTA, 250 mM NaCl, 100 µg proteinase K ml^{-1}, 1% sodium *N*-lauryl sarcosine), and the mixture was incubated for 2 h at 55 °C. Debris was removed from the solution by centrifugation. Total DNA was precipitated in 2-propanol.

F. RNA extraction

1. RNA extraction from the fungus combs

Poly(A)$^+$RNA was extracted from the fungus combs of *M. gilvus* (PathumThani), *M. gilvus* (Prachinburi), *Odontotermes* sp. (Prachinburi), *Microtermes* sp. (Prachinburi), *O. longignathus* (Saraburi) and *Hypotermes* sp. (Saraburi). The fungus comb was ground to be a fine powder with a mortar and pestle under liquid nitrogen. Approximately 0.1 g of this powder was then transferred to a polypropylene tube containing 0.2 ml of extraction buffer (4M guanidine thiocyanate, 0.1M Tris-

HCl, pH 7.5, 1% 1,4-dithiothreitol, 0.5% lauroylsarcosine). After mixing thoroughly, 0.6 ml of the dilution buffer (0.1M Tris-HCl, pH 7.5, 0.4M LiCl and 20 mM EDTA) was added. The supernatant containing RNA was separated from the debris by centrifugation at 17,000 x *g* 15 min at 25 °C. Poly(A)$^+$ RNA was isolated from the supernatant using an Oligotex mRNA kit according to the manufacturer's protocol (Takara). Ten µl of Oligotex suspension was added to the supernatant containing RNA and mixed thoroughly and placed at 37 °C for 10 min follow by centrifuged at 15,000 rpm 25 °C for 10 min and carefully removed the supernatant, then 200 µl of Oligotex Wash buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) was added and mixed thoroughly, follow by centrifuged at 15,000 rpm 25 °C for 10 min and carefully removed the supernatant. 100 µl of RNase-free water was added and mixed by pipetted, then placed at 65 °C for 5 min follow by centrifuged at 15,000 rpm 25 °C for 10 min and carefully transferred the supernatant to new RNase-free tube. Ten µl of 3M sodium acetate (Wako, Nippon Gene) and 1 µl of Ethachinmate (Wako, Nippon Gene) were added. To precipitate RNA, on ice, 2.5 times volume of chilled absolute ethanol was added and placed on ice for 10 min, then the mixture was centrifuged at 15,000 rpm 4 °C for 15 min. The supernatant was discarded. The RNA pellet was added with 200 µl of chilled 70% ethanol and precipitated using same centrifugation for 5 min. The RNA in tube was left at room temperature to dry up for 3 min. Finally, the RNA was dissolved in 10 µl of RNase-free water.

DNA contaminated in RNA solution was digested using DNase I (Takara) according to manufacturer's instruction. On ice, the digestion mixture was prepared. The components of 20 µl mixture were shown at below. The mixture was gently mixed and incubated at 37 °C for 30 min using alumi bath (ALB-301, Iwaki, Japan).

RNA solution	9.0	µl
10x DNase I buffer (Takara)	4.0	µl
RNase free DNase I (5U/µl, Takara)	1.0	µl
RNase free water	6.0	µl

After DNA digestion, the extracted RNA was purified using RNeasy Mini kit for RNA Cleanup (QIAGEN) according to manufacturer's instruction. After purification, the purified RNA was dissolved in 50 μl of RNase-free water.

First-strand cDNA was synthesized from 0.1 μg of poly(A)$^+$ RNA using Superscript II (Invitrogen) and poly-T primer [5'-TTTACCTCTTCAGC$(T)_{19}$-3'] at 42 °C for 50 min and subjected to PCR.

2. RNA extraction from the *Termitomyces* spp. cultures

Total RNA was extracted from a 23-day-old *Termitomyces* culture (strain KU418 and NS/Mg). *Termitomyces* spp. were culture in Potato Dextrose Broth (PDB), the three weeks old cultures were recovered the mycelia by filtration using a 32 μm nylon mesh filter. The frozen mycelia were suspended in LETS buffer [100 mM LiCl, 10 mM Na_2EDTA, 10 mM Tris-HCl pH 7.4, 0.2% Sodium Lauryl Sulfate (SDS), 0.1% Diethyl pyrocarbonate (DEPC)]. Then, the resulting solution was mixed well and incubated at 65 °C for 30 min. After that, the solution was centrifuged at 9,000 rpm 4 °C for 20 min. Supernatant was collected into a new tube. Equal volume of the phenol-chloroform was added and mixed well, then the solution was centrifuged at 9,000 rpm 4 °C for 10 min then the supernatant was carefully collected into a new tube, this step was performed for 2 times or until the RNA solution is clear (no white precipitant of protein). 1/20 volume of 5M NaCl was added. To precipitate RNA, on ice, 2.5 time volume of chilled absolute ethanol was added and kept at -20 °C for 10 min, then the mixture was centrifuged at 12,000 rpm, 4 °C, for 20 min. The supernatant was discarded. The RNA pellet was added with 1-2 ml of chilled 70% ethanol and precipitated using same centrifugation for 5 min. The RNA in tube was left at room temperature to dry up for 5 min. Finally, the RNA was dissolved in 500 μl of RNase-free water. To easy dissolve, the solution was incubated at 65°C for 15 min and gently mixed. RNA solution was purified using phenol-chloroform and follow by ethanol precipitation, finally, the purified RNA was dissolved in 150 μl of RNase-free water. mRNA was isolated from total RNA by means of Oligotex TM-dT30 Super (TAKARA) as described in 1. First-strand cDNA was synthesized from

1 µg of poly(A)$^+$ RNA using Superscript II (Invitrogen) and poly-T primer [5'-TTTACCTCTTCAGC(T)$_{19}$-3'] at 42 °C for 50 min and subjected to PCR.

G. Isolation of the laccase genes

Degenerate primers, primer 1 (5′-GGMACSTTCTGGTAYCAY-3′) and primer 2 (5′-CCRTGCARRTGGAAKGGRTG-3′, where Y represents C or T, S represents G or C, R represents A or G, M represent A or C, and K represents G or T) targeting a copper binding domain of basidiomycete laccases were designed to amplify laccase-like sequences from the fungus combs and the *Termitomyces* sp. KU418 strain. PCR was carried out using a PTC-200 thermalcycler (MJ Research) and *Ex-Taq* (Takara). Premixture (total volume 50 µl) containing;

Template (diluted 10x)	2	µl
5 U/ µl Ex-*Taq* DNA polymerase (Takara)	0.25	µl
Ex-*Taq* Buffer	5	µl
2.5 mM dNTP mixture	4	µl
primer 1 (10 pmol/µl)	1	µl
primer 2 (10 pmol/µl)	1	µl
Sterile deionized water	36.75	µl

Thermal cycling consisted of 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, and extension at 72 °C for 1 min. The amplified product from PCR was checked by electrophoresis on 1% agarose gel in 1x TAE (Tris-Acetate EDTA) buffer. A band size was estimated by bands of DNA marker [λ/*Hind*III digest-ϕX174/*Hae*III digest (Toyobo)].

PCR products (ca. 900 bp) were purified from 1% agarose gel using a Minelute gel extraction kit (Qiagen) according to the manufacturer's instruction. Gel slice was determined by weight. Three volumes of Buffer QG (3 times the volume of gel slice) were added to the agarose gel piece that contain DNA. The tube was incubated in a 50 °C water bath incubator and mixed by vortexing the tube every 2-3

min during the incubation for 10 min or until the agarose gel was completely dissolved. 1 gel volume of isopropanol was added and mixed by inverting the tube several times and then applied the sample to the MinElute column and centrifuged for 1 min at 13,000 rpm. 500 µl of Buffer QG was added to the column and centrifuged for 1 min. 750 µl of Buffer PE was added to the column and centrifuged for 1 min and then centrifuged the column for an additional 1 min to completely removed ethanol residue. The obtained DNA was eluted from the column by added 10 µl of Buffer EB (10 mM Tris-HCl pH 8.5) to the center of the membrane, waited for 1 min and then centrifuged for 1 min at 13,000 rpm and followed by ethanol precipitation of DNA. Finally, the purified product was used for further cloning.

H. Cloning and Transformation

The purified product was cloned into the pGEM-T vector (Promega) to construct laccase cDNA libraries for all fungus combs and *Termitomyces* KU418. The ligation reaction mixture (total volume 10 µl) containing;

The purified PCR product	4.5	µl
pGEM-T vector	0.5	µl
Ligation enzyme	5	µl

The mixture was mixed gently and incubated at 14 °C overnight and follow by transformation step; the tube of 100 µl of competent cells (*E. coli* JM109) were removed from -80 °C and thawed on ice for 10 min following added into the ligation mixture and placed on ice for 30 minutes, then rapid transferred to 42 °C for 30 seconds and placed on ice for 1 minute. 900 µl of SOC medium was added and incubated at 37 °C for 1 hour subsequently centrifuged at 5,000 rpm for 10 minutes and discarded some supernatant. The remain supernatant and the pellet were mixed together. 50 µl of the mixture was spreaded on Luria-Bertani (LB) agar plates containing 50 µg/ml of ampicillin, 2% X-gal and 0.1 M IPTG. Following incubation at 37 °C overnight. Approximately 30 white colonies per one sample were selected and picked up for insertion determination prior to sequencing.

I. Insertion determination

Approximately 30 clones of each library were randomly selected and their insert DNA sequences were amplified by PCR using universal primer set of the vector. Premixture (total volume 30 µl) containing;

r*Taq* DNA polymerase (Toyobo)	0.15	µl
10x Buffer	3	µl
2.5 mM dNTP mixture	3	µl
25 mM $MgCl_2$	2.4	µl
Each of 5 pmol/µl M13F and M13R primers	1	µl
Sterile deionized water	20.45	µl

The universal primers M13F (5′- GTAAAACGACGGCCAG- 3′) and M13R (5′- CAGGAAACAGCTATGAC- 3′) were used. PCR was carried out using a PTC-200 thermalcycler (MJ Research). The inserted fragment was amplified by temperature profile, 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 30 second, annealing at 60 °C for 30 second, extension at 72 °C for 1 minute and final extension at 72 °C for 1 minute.

The amplified product (5 µl) was size-fractionated by electrophoresis on 1% agarose gel in 1x TAE (Tris-Acetate EDTA) buffer and classified according to the result of restriction fragment length polymorphism using either *Hae* III or *Hha* I.

J. Restriction fragment length polymorphism

The PCR product from I. was analyzed by restriction fragment length polymorphism (RFLP) with *Hae*III and *Hha*I. Reaction mixture (total volume 20 µl) containing;

Plasmid DNA	1	µl
10x buffer	2	µl

*Hae*III or *Hha*I	0.2 µl
Sterile deionized water	16.8 µl

The mixture was incubated at 37 °C for 1 hour and checked the RFLP pattern by electrophoresis on 2% agarose gel in TAE buffer. The representative clone from each strain was analyzed by automated sequence analyzers.

K. Purification of PCR products prior to sequencing

The PCR product of the representative clone from each strain was purified using ExoSAP-IT (usb). This method, the primers were hydrolyzed by exonuclease I and phosphoric acids (Pi) of dNTPs were removed by alkaline phosphatase. Five µl of PCR product was mixed with 1 µl of diluted ExoSAP-IT (Exonuclease I and Shrimp Alkaline Phosphatase in specially formulated buffer) and incubated at 37 °C for 15 min, follow by incubated at 80 °C for 15 min and kept at -20 °C until used.

L. Sequence and phylogenetic analysis

1. Sequencing; The DNA sequences of the representative clones from each library were determined using M13 forward and reverse primers on a ABI PRISM 377^{TM} DNA sequencer (PE Applied Biosystems, Perkin-Elmer) and a Big-Dye Terminator Sequencing Kit (Perkin Elmer). This kit included ddNTPs labeled with four different kinds of fluorescent dyes. Each extended fragment that reacted either with ddATP, ddTTP, ddCTP or ddGTP was simultaneously directed. Approximately 200 ng of DNA template (estimated by comparing with 50 ng loading DNA marker) or 0.5-2.0 µl (used here) was used as template in sequencing reaction. The reaction mixture (total volume 20 µl) containing;

Sterile milliQ water	12.0- 13.5	µl
5x sequencing buffer	3.0	µl
Ready reaction premix	2.0	µl
1.6 pmol/µl primer solution	1.0	µl

DNA template	0.5- 2.0	μl

The reaction was performed using the thermal cycle profile: 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min by PCR machine. The Ready reaction premix and the sequencing reaction were avoided light exposure that decrease a dye signal intensity. The sample was purified and sequenced as discussed previously.

2. Phylogenetic Analysis; GENETYX-MAC V10.0 (Software Development) was used for basic analysis of sequences except a multiple alignment of gene sequences, which was performed by DNASIS-MAC V3.7 (Hitachi), using Higgins's method (Higgins *et al*., 1988). All the DNA gene sequences were first analyzed for taxonomically assignment by the BLASTn program. The sequences were compared to sequences from databases and closely related sequences from database were retrieved and added to the aliment. The sequences that appeared to contain introns based on sequence alignment and the "GT-AG" rule were manually removed and used for further analyses. The protein sequence were aligned using ClustalX ver1.8 (Thompson *et al*., 1997). A neighbor-joining tree was constructed using the MEGA package with a PAM matrix (Kumar *et al*., 2001). GENETYX ver10.1 (Software Development) was used to calculate sequence identity. The representative isolate (NS/Mg stain) was selected for determination of full length laccase cDNA sequence.

M. Full-length cDNA sequences of laccase gene

Full-length cDNA sequences of laccase gene of two representative clones (*lcc1-2* and *lcc2-5*) were isolated from the fungus comb RNA from PathumThani using rapid amplification of cDNA ends (RACE)-PCR. Primer 3 (5′-GGTGGCAAAGCAGCCTCAAGATCT-3′) and primer 4 (5′-TGGAACATCAATAATGTTTCGTAC-3′) were used for amplification of the 3′-end sequences of *lcc1-2* and *lcc2-5*, respectively. The PCR condition for *lcc1-2* were 35 cycles of 94 °C for 30 sec followed by 65 °C for 1.5 min. The PCR condition for *lcc2-5* were 35 cycles of 94 °C for 30 sec, 58 °C for 1 min and 72 °C for 1 min. For the

amplification of the 5′ end, reverse transcription reactions for *lcc1-2* and *lcc2-5* were preformed with primer 5 (5′-TACCAGTCGGCAAGAG-3′) and primer 6 (5′-ATGCTTGAGTTGTGCC-3′), respectively using a 5′ RACE-PCR kit version 2 (Gibco BRL). The PCR condition were 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min. PCR products from the 3′ and 5′ RACE-PCR were cloned and sequenced using ABI PRISM 377^{TM} DNA sequencer (PE Applied Biosystems, Perkin-Elmer) and a Big-Dye Terminator Sequencing Kit (Perkin Elmer) as described previously.

N. Amplification of coding region of the *lcc1-2* and *lcc2-5* genes

The coding regions of the *lcc1-2* and *lcc2-5* genes were amplified from genomic DNA extracted from the *Termitomyces* sp. strain NS/Mg using an Isoplant kit (Nippon Gene) as described previously. The 5′- and 3′- flanking regions of the *lcc1-2* and *lcc2-5* genes were obtained using inverse PCR with primer 7 (5′-CCTCAAGATCTCCTTCCTTCT-3′) and primer 8 (5′-AGATCCTTCAGAGGATCGTTC-3′) for *lcc1-2* and primer 9 (5′-TCTGTCATTGAGGTCGACTTC-3′) and primer 10 (5′-TAAATGAGCCGAAGGATCTTCG-3′) for *lcc2-5*. Genomic DNA (0.5 µg) from *Termitomyces* sp. strain NS/Mg was digested with either *Pst*I or *Eco*RI for the *lcc1-2* and *lcc2-5* amplifications, respectively. PCR conditions were 30 cycles of 94 °C for 20 s and 67 °C for 8 min or 5 min for *lcc1-2* and *lcc2-5*, respectively. PCR products were purified using a PCR purification kit (QIAGEN) and analyzed by direct sequencing.

O. Semi-quantitative analysis of gene expression

1. Setting up PCR condition

In order to estimate expression level of laccase genes from cDNA of the fungus combs of *M. gilvus* in Prachinburi and PathumThani and *Hypotermes* sp. from Saraburi semi-quantitatively, the PCR conditions were optimized by changing

parameters related to the PCR cycle, annealing temperature and extension time (Hongoh *et al*., 2003). The reaction mixture (total volume 50 µl) containing;

Template (undiluted)	0.5	µl
5 U/ µl Ex-*Taq* DNA polymerase (Takara)	0.5	µl
Ex-*Taq* Buffer	5	µl
2.5 mM dNTP mixture	4	µl
primer 1 (10 pmol/µl)	1	µl
primer 2 (10 pmol/µl)	1	µl
Sterile deionized water	38	µl

Thermal cycling consisted of initial denaturation at 95 °C for 2 min followed by 16, 18 or 20 cycles of 95 °C for 30 sec, 50 °C for 1 min and 72 °C for 3 min and subsequently final extension at 72 °C for 10 min.

2. Estimate concentrations of PCR products

Concentrations of PCR products were estimated by electrophoresis on an agarose gel stained with SYBR Green I using Molecular Imager FX (Bio-Rad). The agarose gel thickness not more than 8 mm was put on the tray and inserted into the scanner. After the scan was completed, the image was analyzed by compared with a calibration curve of known quantity of standard marker.

3. Purification of PCR products

PCR products were purified using a Minelute PCR purification kit (Qiagen) as follow;

a) Five volumes of Buffer PB was added to 1 volume of the PCR product and mixed.

b) Applied the sample to the MinElute column and centrifuged for 1 min.

c) Added 750 µl Buffer PE to the MinElute column and centrifuged for 1 min.

d) Centrifuged the column for an additional 1 min at maximum speed.

e) Placed the MinElute column in a clean 1.5 ml centrifuge tube.

f) Eluted DNA with 10 µl Buffer EB (10 mM Tris-Cl, pH 8.5) or H_2O, stand the column for 1 min and then centrifuged for 1 min. The purified PCR products were used for cloning.

4. Cloning and Transformation

The purified PCR products were cloned into a pCR®2.1 vector using a TOPO TA-cloning kit (Invitrogen). The cloning reaction mixture (total volume 6 µl) containing;

The purified PCR product	4	µl
pCR®2.1-TOPO	1	µl
Salt solution (1.2M NaCl, 0.06 M $MgCl_2$)	1	µl

The reaction mixture was mixed gently and incubated at room temperature for 30 min and follow by transformation step as discussed previously.

5. Insertion determination

Ninety-six clones of each library were randomly selected and their insert DNA sequences were amplified by PCR using universal primer set of the vector. Premixture (total volume 20 µl) containing;

Ex-*Taq* DNA polymerase (1.5 U/100µl PCR mixture)		µl
Ex-*Taq* Buffer	2	µl
2.5 mM dNTP mixture	1.6	µl
Each of 5 pmol/µl M13F and M13R primers	0.7	µl
Sterile deionized water		µl

PCR was carried out using a PTC-200 thermalcycler (MJ Research). The inserted fragment was amplified by temperature profile, 94 °C for 5 min followed by 25 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 1 min and final extension at 72 °C for 10 min.

The amplified product (2 µl) was size-fractionated by electrophoresis on 1% agarose gel in 1x TAE (Tris-Acetate EDTA) buffer and classified by comparing RFLP results against results obtained from reference sequences. Expression levels were estimated from the abundance (%) of each clone.

6. Restriction fragment length polymorphism

The PCR product from 5 was analyzed by restriction fragment length polymorphism (RFLP) with *Hha*I. Reaction mixture (total volume 10 µl) containing;

PCR product	3 µl
10x buffer	1 µl
*Hha*I	0.2 µl
Sterile deionized water	5.8 µl

The mixture was incubated at 37 °C for 6 h and checked the RFLP pattern by electrophoresis on 3% NuSieve 3:1 agarose in TBE buffer. Expression level were expressed as the clone abundance (percent) of each RFLP group. The representative clone from each group was analyzed by automated sequence analyzers.

7. Purification of PCR products prior to sequencing

The PCR product of the representative clone from each group was purified using ExoSAP-IT (usb). Five µl of PCR product was mixed with 1 µl of diluted ExoSAP-IT (Exonuclease I and Shrimp Alkaline Phosphatase in specially formulated buffer) and incubated at 37 °C for 15 min, and then at 80 °C for 15 min and kept at -20 °C until used.

8. Nucleotide sequencing and sequence analysis

DNA sequencing was performed using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer). Approximately 200 ng of DNA template (estimated by comparing with 50 ng loading DNA marker) or 0.5-1.0 μl (used here) was used as template in sequencing reaction. Twenty-μl total volume of reaction mixture shown at below was performed on ice. The reaction was performed using the thermal cycle profile: 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min by PCR machine. The Ready reaction premix and the sequencing reaction were avoided light exposure that decrease a dye signal intensity.

Sterile milliQ water	13.0-13.5	μl
5x sequencing buffer	3.0	μl
Ready reaction premix	2.0	μl
3.2 pmol/μl M13F primer	1.0	μl
DNA template	0.5-1.0	μl

9. Purification of elongation product

The sequencing reaction was purified to exclude contaminating components as salts, primers, dye terminators, etc. that may disturb the sequencing and cause low quality of sequence result. Preparation of purification mini-column, powder of Sephadex G-50 Superfine was loaded into the well of 96-well loader plate (45 μl column loader) and then the loaded powder was further transfered into MultiScreen HV plate (Millipore). Using multi-channel pipetter, 300 μl of sterile milliQ water was loaded into each well. The plate was covered with a lid and warped with plastic wrap to avoid a water evaporation and then incubated at room temperature for at least 3 hours. After incubation, the sephadex resin was washed. The MultiScreen HV plate was placed on a 96-well microplate (ABI PRISM) using a connection frame. The plates was centrifuge at speed 900 g for 5 min to exclude water from mini-column of MultiScreen HV plate to the 96-well microplate. The excluded water was discarded and the plate was reused. The mini-column was added with 150 μl of sterile milliQ

water and the centrifugation at the same speed was performed again. After that the MultiScreen HV plate was placed on MicroAmp Optical 96-well reaction plate using the connection frame. To purified the sequencing reaction, whole reaction solution was carefully loaded into the each well of mini-column and the MultiScreen HV plate was covered with lid and sealed with plastic sticker to avoid plate moving. The sequencing reaction was filtrated using centrifugation at the same speed. The MicroAmp Optical 96-well reaction plate contains purified product was sealed with aluminum sticker and used for sequencing.

10. Sequencing and analysis of laccase genes

The sequencing was performed by ABI PRISM^{TM} 3700 Genetic Analyzer and the 3700 sequencing data collection software version 3.6 (Applied Biosystems) according to the manufacturer's instructions. The partial sequences of laccase gene of all isolated strains were mannually checked and corrected. The software DNASIS-MAC version 3.7 (Hitachi) was used to correct the sequence. To determine the identities among sequences, the software DNASIS-MAC version 3.7 and GENETYX-MAC version 10.0 were used. The sequences were identified and comparing with public laccase sequences in the databases by BLASTn searching.

III. Expressed sequence tag (EST) analysis

A. Fungus comb, fungal strain and culture media

Fungus comb of *M. gilvus* from PathumThani (same orchard with the samples of laccase experiment) were collected. Termites were completely removed from the fungus combs, which were immediately frozen with liquid nitrogen in the field. The fungus combs were stored at -80 °C until used. *Termitomyces* sp. strain NS/Mg (JCM13351) which isolated from a fungus comb in the same termite nest and also used in laccase experiment, was maintained on potato dextrose agar (PDA; 0.4% potato extract, 2% glucose; Nissui, Tokyo, Japan) at 30 °C.

B. Construction of cDNA libraries

The construction of cDNA libraries from the fungus comb and 21-day-old mycelium of *Termitomyces* sp. strain NS/Mg grown on a PDA plate were preformed by a modification of the single-strand linker ligation method (Shibata *et al*, 2001). This method used DNA ligase to add a double-strand linker to first-strand cDNA. The linkers have random 6-bp (dN_6 or dGN_5) 3′ overhangs that can ligate to any cDNA sequence. The synthesized cDNAs have tags sequences at the 5′- and 3′-ends that originated from the linker and oligo(dT) primer for reverse transcription, respectively. This means that the cDNA sequence between these tags can be amplified by PCR. Whole fungus comb was subjected to poly $(A)^+$RNA by using oligo(dT) latex (Takara, Otsu, Japan) as described previously. First-strand cDNA was synthesized by using superscript II reverse transcriptase (Invitrogen) and primer 1 (5′-TTTAAGCAGTGGTATCAACGCAGAGAAGCT_{16}VN-3′). The double-strand linkers GN5 and N6 were prepared by mixing oligonucleotides A (5′-AAGCAGTGGTATCAACGCAGAGAATTC$\underline{GN_5}$-3′) and B (5′-GAATTCTCTGCGTTGATACCACTGCTT-3′, 5′-end phosphorylated), and oligonucleotides B and C (5′-AAGCAGTGGTATCAACGCAGAGAATTC$\underline{N_6}$-3′), respectively. The double-strand linker was ligated with the first-strand cDNA over night then the first-strand cDNA with the linker was dissolved in TE (pH 8.0) and used to construct nonsubtractive (NS) and substractive (SB) cDNA libraries. Subtractive hybridization was carried out with a super subtraction kit (Sawady Technology, Tokyo, Japan). With this method, driver RNA was immobilized on the surface of a carbodiimide-coated microplate. Driver RNA/cDNA hybrid was formed on the surface of the microplate and unbound cDNAs were recovered from the aqueous phase. Driver RNA was prepared from 21-day-old mycelium of *Termitomyces* sp. strain NS/Mg grown on a PDA plate. Poly$(A)^+$RNA was extracted with the same method used for the fungus comb sample. Approximately 7 µg of poly$(A)^+$RNA was immobilized, and one third of the first-strand cDNA with the double-strand linker was used for the hybridization. Subtractive hybridization was carried out twice at 65 °C for 24 hr according to the manufacturer's instructions. Unbound cDNAs were purified with a MicroSpin S400HR (Amersham, Buckingghamshire, UK) followed by ethanol precipitation and was subsequently

dissolved in 30 µl of 10 mM Tris-HCl, pH 8.0. The cDNAs were amplified with primer 1 (5´-AAGCAGTGGTATCAACGCAGAGAATTC-3´) and 2 (5´-TTTAAGCAGTGGTATCAACGCAGAGAAGCT-3´). PCR was performed using a PTC-200 thermal cycler (MJ Research, Watertown, USA) and Ex-*Taq* (Takara). Thermal cycling consisted of 94 °C for 2 min followed by 22 cycles of 95 °C for 20 s and 68 °C for 6 min. The PCR reaction mixture contained 10 µl of the subtractive cDNA or 2,000 times diluted first-strand cDNA solution (nonsubtractive) and 0.2 mM deoxyribonucleotide triphosphate containing 5-methyl deoxycytidine triphosphate. The PCR product was size-fractionated (>400 bp) with a SizeSep400 spun column (Amersham) and directionally cloned into pBlueScript (KS+) vector (Stratagene) at the EcoRI/HindIII sites (Meissner *et al*. 1987), before being transferred into XL10-gold cells (Stratagene, La Jolla, USA). The NS and SB libraries which contained $3.5x10^4$ and $2.5x10^4$ individual clones, respectively.

C. DNA sequencing, data processing and annotation

Sequencing templates were prepared using PCR with universal primers for the vector from *Escherichia coli* colonies [pBlueScript (KS+) vector (Stratagene)]. The PCR products were purified with an ExoSAP-IT kit (USB, Cleveland, USA) as mentioned previously. Plasmids were also used for sequencing when the amplification of insert cDNA was difficult. The DNA sequences of the 5´-end of the cDNA were determined using T7HT primer (Toyobo, Osaka, Japan) with DNA sequencers (ABI models 3700 and 377; Applied Biosystems Japan, Tokyo). The resultant sequences were processed with the Staden package version 1.4.1 (https://sourceforge.net/projects/staden/). Sequence chromatograms were trimmed to eliminate low-quality regions, and vector and linker sequences were removed using the pregap4 program in the Staden package. Cleaned sequences were assembled using a pregap4 shotgun assembler with default parameters. The annotation of ESTs and bioinformatics works were performed by the Environmental Molecular Biology Laboratory in RIKEN.

RESULTS AND DISCUSSION

I. The phylogenetic analysis of *Termitomyces* spp. from fungus growing termites.

A. The feature of the fungus combs and fungal nodules

To investigate the symbiotic fungi associated with the termites, eleven species of fungus-growing termites (Order Isoptera, Family Termitidae, Subfamily Macrotermitinae); *Macrotermes gilvus* (Prachinburi), *Ma. carbonarius* (Saraburi), *Ma. annandalei* (Chanthaburi), *Microtermes* spp. (Prachinburi and Chanthaburi), *Odontotermes* spp. (Prachinburi, Saraburi, Chanthaburi and Nakronratchasima), *Odontotermes longignathus* (Saraburi) and *Hypotermes* sp. (Saraburi) were collected their combs and fruiting body. The picture of mound, fungus combs, soldiers and workers of *Ma. gilvus* in PathumThani were showed in Figure 18. The fungus nodules and the internal part of the stipe were cultivated on artificial medium (PDA).

Figure 18 The mound, fungus combs, soldiers and workers of *Ma. gilvus* in PathumThani province.

B. Isolation and identification of *Termitomyces* strains

Termitomyces strains were isolated from the fungal nodules and fruiting body. The fungus nodules were carefully picked up using sterile forceps, rinsed with 0.6%

sodium hypochlorite solution, washed with steriled water and cultivated on Potato Dextrose Agar (PDA) in darkness at room temperature. One strain was cultivated from the fruiting body of *Termitomyces* sp. (inoculated with the tissue of the internal part of the stipe) blooming from the nest of *Odontotermes* sp. in Nakronratchasima. The representative cultures grown on Potato Dextrose Agar (PDA) were shown in Figure 19. A total of 39 strains were successfully cultivated. The strains cultured in this study have been deposited in the Japan Collection of Microorganisms under the accession numbers; JCM11082-JCM11106, JCM11110, JCM11115, and JCM11153-11164.

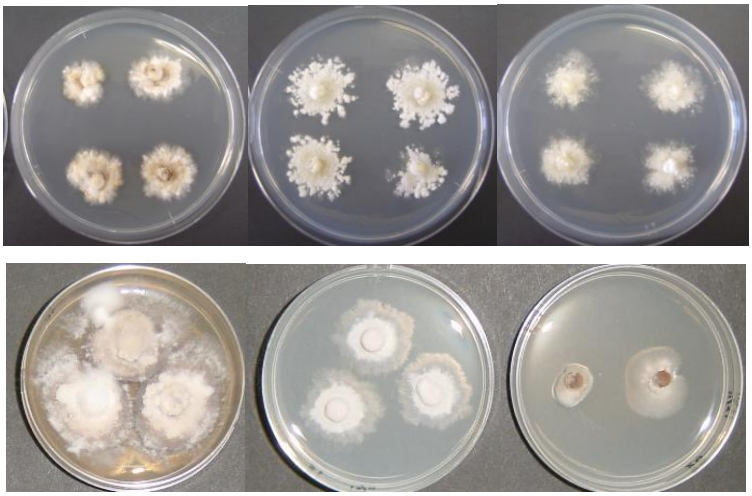

Figure 19 *Termitomyces* cultures grown on Potato Dextrose Agar (PDA)

There has been no report about developing the fruiting body from mycelia of the symbiotic fungi in a laboratory. Since mycelia give poor information about the taxonomy based on morphology, molecular sequences are expected to be useful to identify the symbiotic fungi grown on the fungus comb. Also, a reliable phylogeny of the symbiotic fungi is important in order to understand the symbiotic relationship with termites. In this study, the fungal symbionts were analyzed their nuclear ribosomal DNA sequences and compared the phylogeny of symbiotic fungi in relation to the termite hosts and their locality.

Mycelia of *Termitomyces* strains were grown for three weeks and their DNA was extracted using ISOPLANT kit (Nippon Gene) and amplified ITS1, 5.8S rDNA, ITS2 and partial large subunit (LSU) rDNA region using primers ITS5 and LR7, then the amplified product was purified.

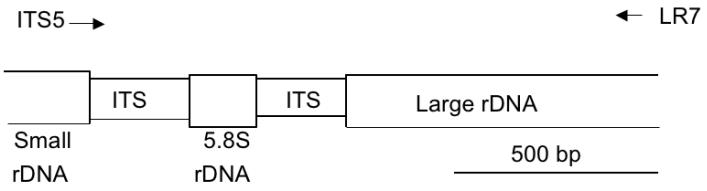

Figure 20 The rDNA region that was analyzed in this study.

The amplified products from PCR (2.1 kbp) were purified using a Wizard PCRpreps DNA purification system (Promega) and determined its quality and quantity using gel electrophoresis by comparison with standard on an agarose gel by using the image analysis (Image Gauge version 3.1, Fuji Image Gauge, MAC^{TM}) as shown in Figure 21.

The purified PCR products were used for either direct DNA sequencing as templates or cloning into a pGEM-T vector (Promega), then inserted fragment was analyzed by colony-PCR amplification using M13F and M13R primers. The amplified product (5 μl) was size-fractionated by electrophoresis on 1% agarose gel in 1x TAE (Tris-Acetate EDTA) buffer (Figure 22).

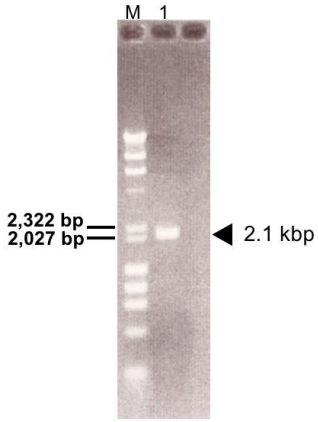

Figure 21 The purified PCR products amplified ITS1, 5.8S rDNA, ITS2 and partial large subunit (LSU) rDNA region using primers ITS5 and LR7 with the temperature profile; 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, at 55 °C for 45 sec, and 72 °C for 2 min and a final extension at 72 °C for 1 min. The expected size was specified by an arrow head. Lane M is molecular size standards (λ/ *Hin*dIII digest plus ϕX174/*Hae*III digest [Toyobo]). The size of each band was, from top to bottom, 23130, 9416, 6557, 4361, 2322, 2027, 1353, 1078, 872, 603 and 310 bps. Lane 1 is PCR amplified products.

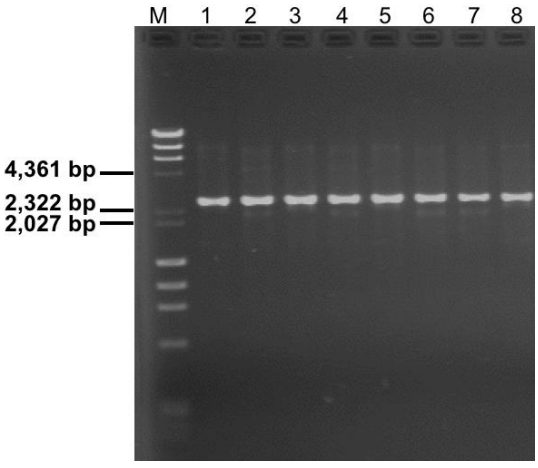

Figure 22 The colony-PCR amplification of the purified PCR products.
Lane M: size standard marker (λ/*Hind*III digest-ϕX174/*Hae*III digest)
Lane 1-8: PCR amplified products

The amplified fragments approximately 2.4 kbp were used for sequencing. Plasmid DNA of the clones was firstly analyzed by restriction fragment length polymorphism (RFLP) with *Hae*III and *Hha*I, to confirm that the clones from a single strain were equivalent to one another.

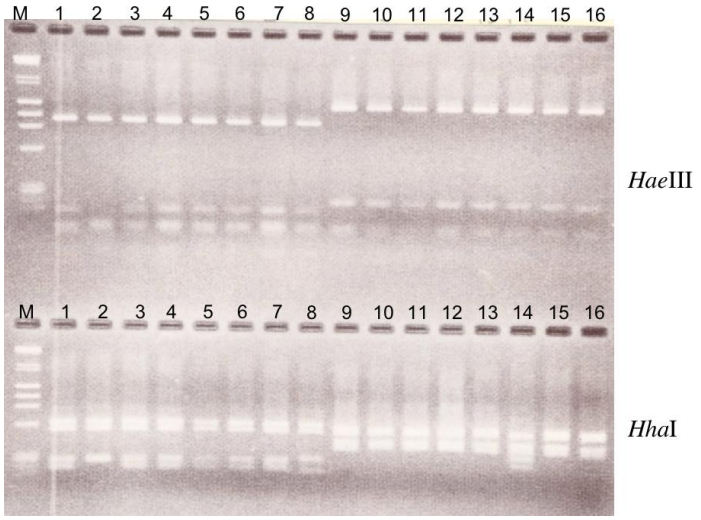

Figure 23 The result of RFLP analysis using *Hae*III (upper) and *Hha*I (lower)
Lane M: size standard marker (λ/*Hind*III digest-ϕX174/*Hae*III digest)
Lane 1-8: an example from *Termitomyces* strain KU 454
Lane 9-16: an example from *Termitomyces* strain KU 455

C. Sequence analysis of *Termitomyces* clones

The representative clone from RFLP pattern of each strain was analyzed by automated sequence analyzers (ABI model 377) using the sequencing primers ITS2, ITS3, ITS4 and ITS5 for the ITS1-5.8S rDNA-ITS2 region determination and primers LROR, LR3, LR3R, LR5 and LR16 for the LSU rDNA region determination. The primers direction used in this study were showed in figure 24.

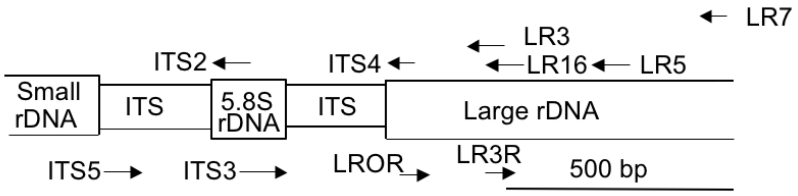

Figure 24 The primers direction used in this study.

The DNA sequence data were aligned using the CLUSTAL W package and check manually. The programs implemented in PHYLIP 3.5c (distributed by Felsenstein, J., Department of Genetics, University of Washington, Seattle) were used to infer the neighbor-joining and the parsimony trees and to obtain bootstrap confident estimates. The program PUZZLE 4.0 was used with 10,000 puzzling steps to infer the quartet-puzzling maximum likelihood tree.

Comparison of the sequence similarity showed that 38 strains from the fungus-nodules and from the fruiting body of *Termitomyces* sp. were significantly related, except for one strain (KU416) from the fungus-nodule of *Odontotermes* sp. at Kho Kitchagoot which form distinct group to another. Among the 38 strains, the DNA sequence of the ITS1-5.8S rDNA-ITS2 (530-645 bp) showed more than 72% nucleotide identity to one another. Based on the comparison of the ITS1-5.8S rDNA-ITS2 region, the 38 strains were classified into eight groups (as shown in Table 2 and figure 25).

Table 2 Number of strains among the 38 *Termitomyces*-related basidiomycetes in each of the eight phylogenetic groups

		Phylogenetic group							
Location	Host termite	1	2	3	4	5	6	7	8
Prachinburi	*Macrotermes gilvus*					5			
Prachinburi	*Microtermes* sp.		6			1			
Prachinburi	*Odontotermes* sp.					5			
Saraburi	*Ma. carbonarius*	5							
Saraburi	*Hypotermes* sp.			3					
Saraburi	*Od. longignathus*				6				
Saraburi	*Odontotermes* sp.				2				
Chanthaburi	*Ma. Annandalei**					1			1
Chanthaburi	*Microtermes* sp.							1	
Chanthaburi	*Odontotermes* sp.		1						
Nakronratchasima	*Odontotermes* sp.						1		

* The two strains were from different nests of this termite species. In the other cases, strains of each termite host were from only one fungus comb.

Group 1: This group compose of all isolated strains (5 strains) that isolated from nodule fungi from the comb of *Macrotermes carbonarius* at Saraburi province, bootstrap probability of this grouping is 99 and 100 from ML and NJ method respectively (figure 25).

Group 2: This group compose of one isolated strain from the comb of *Odontotermes* sp. at Chanthaburi province and 6 isolated strains from the comb of *Microtermes* sp. at Prachinburi province, bootstrap probability of this grouping is 99 and 100 from ML and NJ method respectively.

Group 3: This group compose of 3 isolated strains from the comb of *Hypotermes* sp. at Saraburi province, the bootstrap probability of this grouping is 99 and 100 from ML and NJ method respectively.

Group 4: This group compose of 2 isolated strains from the comb of *Odontotermes* sp. and 6 isolated strains from the comb of *Odontotermes longignathus*

at Saraburi province, the bootstrap probability is 100 and 100 from ML and NJ method respectively.

Group 5: This group compose of the isolated strains from various places and termite host; 5 strains from the comb of *Macrotermes gilvus* at Prachinburi province, 5 strains from the comb of *Odontotermes* at Prachinburi province, one strain from the comb of *Microtermes* sp. at Prachinburi province and one strain from the comb of *Macrotermes annandalei* at Chanthaburi province. The bootstrap probability of this grouping is 98 and 93 from ML and NJ method respectively.

Group 6: This group compose of one isolated strain from fruiting body of *Termitomyces* sp. from the nest of *Odontotermes* sp. at Nakronratchasima province.

Group 7: This group compose of one isolated strain from the comb of *Microtermes* sp. at Chanthaburi province.

Group 8: This group compose of one isolated strain from the comb of *Macrotermes annandalei* at Chanthaburi province.

Within each group, the DNA sequences showed more than 99% nucleotide identity to one another. Strains between the groups 4 and 5 showed approximately 97% nucleotide identity to each other, whereas those between the other groups showed less than 90% nucleotide identity to one another. The grouping was supported by phylogenetic analyses of this DNA region (as showed in Figure 25), in which the branching order of the groups was not strongly supported except for the clustering of groups 1 and 2, and that of groups 4 and 5.

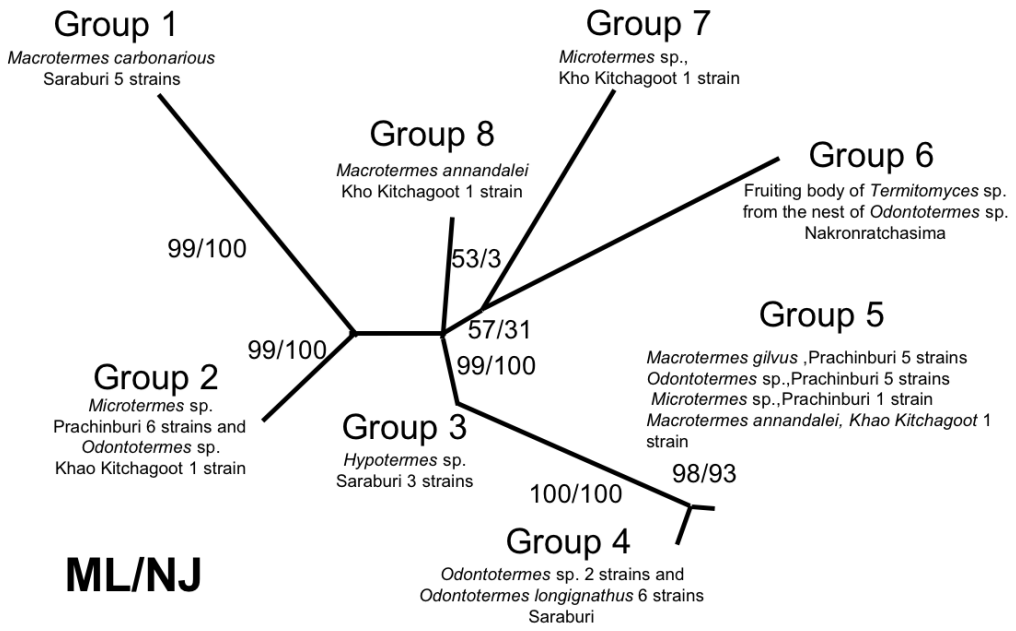

Figure 25 Grouping of the isolated fungi on the basis of sequence similarity and phylogenetic analysis (using sequence of ITS region)

At first, the DNA sequence of at least 330 bp of the LSU rDNA region was analyzed in all the 38 strains and showed more than 93% nucleotide identity to one another. The comparison of the LSU rDNA region indicated that the strains within each group showed more than 99% nucleotide identity to one another, being consistent with the grouping based on the ITS regions. However, this DNA region was too similar to distinguish the grouping in some cases. The strain between groups 1 and 2, and those between groups 4 and 5, showed more than 99% nucleotide identity to each other, respectively.

The longer LSU rDNA region (1.2-1.4 kbp) of at least one representative of each of the eight groups was analyzed for their DNA sequences. As the result, all the representatives of the eight groups were closely related to one another, showing more than 92% nucleotide identity. Then, the LSU rDNA sequences were evaluated the phylogenetic relationship comparing with 24 database sequence of the fungi in Order Agaricales including 4 database sequence of the fungi in Ascomycota and constructed tree by Neighbor joining method (NJ) (as showed in Figure 26). The nucleotide

sequence data found in this study will appear in the databases under accession numbers AB073496-AB073545 and AB073739.

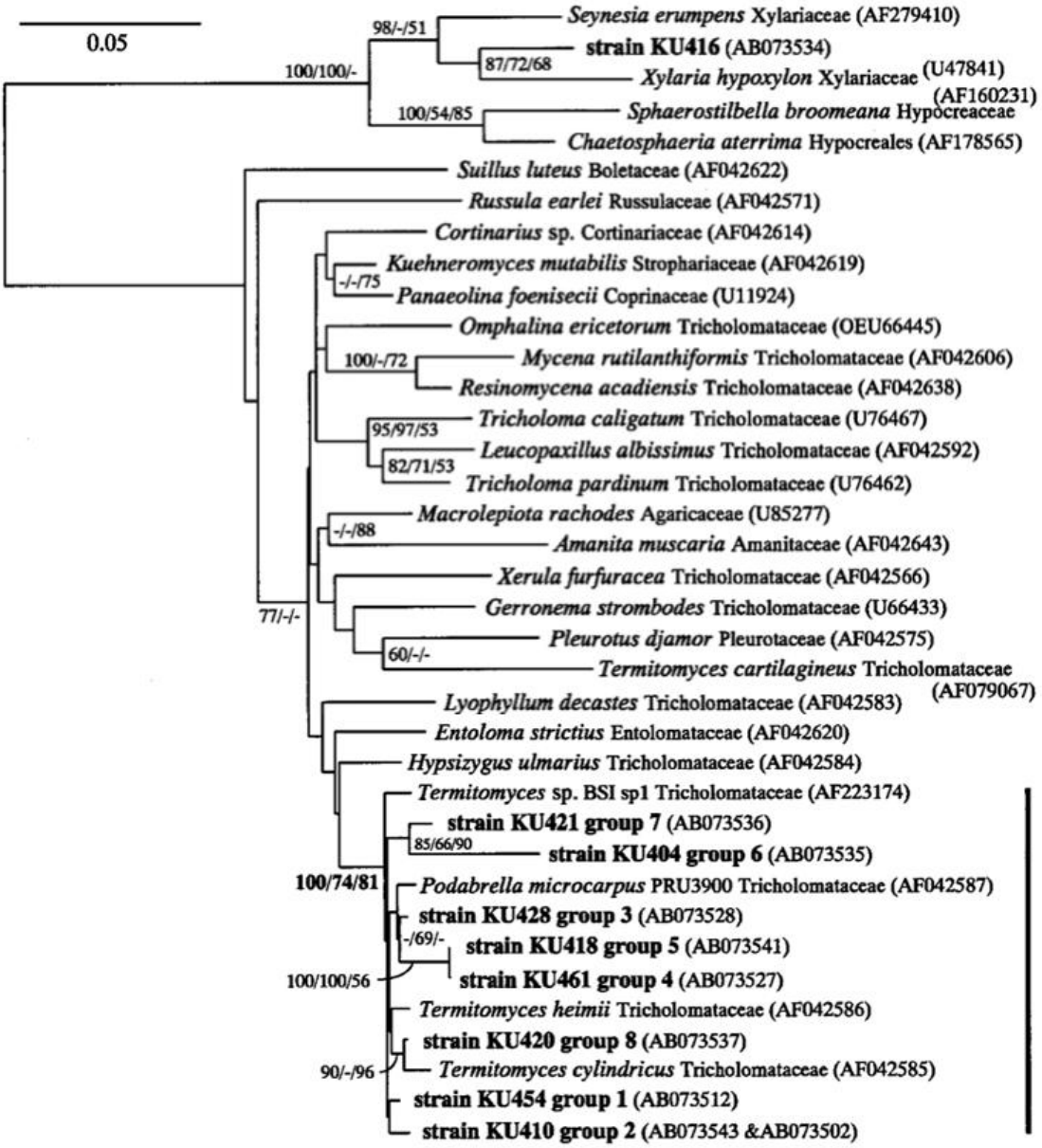

Figure 26 Phylogenetic positions of the strains symbiotic with fungus-growing termites.

The phylogenetic analyses of the LSU rDNA indicated that all the eight groups identified from the termite nests were clustered together with *Termitomyces heimii*, *Termitomyces cylindricus* and *Termitomyces* sp. strain BSI sp1. This cluster also

contained *Podabrella microcarpus* strain PRU3900 (Tricholomataceae). The close relationship between *Termitomyces* and *Podabrella* has already been established by molecular phylogenetic analysis, (Moncalvo *et al*., 2000) and in fact, the same strain has recently appeared as *Termitomyces microcarpus* in the database (AF357023). The clustering was fully supported by the statistical analyses, showing 100% and 74% bootstrap values for the neighbor-joining and the parsimony methods, respectively, and 81% occurrence in the quartet-puzzling steps. The results suggest that all the 38 strains are *Termitomyces*-related basidiomycetes and confirm that *Termitomyces* are true symbionts which grow on the fungus comb as fungus nodules. The eight groups probably represent species or subspecies of this genus. Close relationships were found in the case between group 4 and 5, between group 6 and 7, and between group 8 and *T. cylindricus*, which were supported by the statistical analyses, respectively.

Among the 39 strains analyzed in this study, 38 strains were related to *Termitomyces*. The only exception was strain KU416, cultivated from *Odontotermes* sp. in Khao Kitchagoot. The LSU rDNA analysis clearly indicated that this strain was affiliated to Ascomycota of the family Xylariaceae that includes the genus *Xylaria*. The LSU rDNA of some members of this family showed the highest sequence identity, around 92%, with this strain. It has been reported that *Xylaria*-like fungi inhabit the fungus comb as mycelia. (Thomas, 1987) However, they never produce spores or stroma until the fungus comb is removed from the nest or termites abandon the nest. The presence of termites probably prevents *Xylaria*-like fungi from proliferating on the fungus comb.

Relationships of the *Termitomyces*-related fungi cultivated from the fungus comb with their host termites were not simple. The groups 2 and 5 of the symbiotic fungi were identified from the termites of different genera. Also, the group 4 was identified from the different species of *Odontotermes*. Some species of *Termitomyces*, such as *T. eurhizus* and *T. medius*, are reported as symbionts of several species of more than two genera of termites, respectively (Wood and Thomas, 1989). The symbionts are thought to be generalists rather than specialists with respect to the relationship with their hosts. From a single genus of the termite, more than two groups

of the symbiotic fungi were identified when the termites of multiple species or multiple locations were examined. From the genus *Microtermes*, for example, the symbionts of groups 2, 5 and 7 were identified. Also, from the genus *Odontotermes*, those of group 2, 4, 5 and 6 were identified. The choice of the symbionts by the hosts, at least at the genus level of the termites, is not fixed to a single group of the symbionts. Furthermore, it is noted that a single fungus comb harbored two different groups of the symbiotic fungi in the case of *Microtermes* sp. in Prachinburi. In the case of *Macrotermes annandalei* in Chanthaburi, two groups were obtained from the different nests.

The locality of the host termites seemed to affect the choice of the symbionts because the groups of the symbiotic fungi obtained in a single sampling site were limited, at least in Prachinburi and Saraburi. In Prachinburi, only the groups 2 and 5 were found. In the case of two *Odontotermes* species in Saraburi, only the group 4 was found. However, more than two groups were identified from a single location in each of the three locations where the multiple termite genera were investigated. In the case of groups 2 and 5 of the symbionts, they were identified from both Prachinburi and Chanthaburi. Thus, the locality alone is not crucial for the selection of the symbionts, and some *Termitomyces* lineages of the symbionts and dispersed across the locations.

Probably, these complex phylogenetic relationships between the host termites and their symbiotic fungi are reflected by the methods to acquire the symbionts during the establishment of a new colony of the termites. Two methods are discussed to have evolved within fungus-growing termites. (Johnson *et al*., 1981; Sieber, 1983) One is that the symbiont is carried over by reproductive alates of the termites, and the other is the collection of the symbiont by foraging workers of the termites in the early stage of colony foundation. The apparently complex relationships cannot be explained if the only one of the two methods is used by the termites. More extensive study is necessary to clearify what kinds of methods are used by each termite species, taking differences of their behavior prominently into account. The DNA sequences reported in this study can be the basis for such further study.

II. Phenol- oxidizing enzyme activities from symbiotic fungi *Termitomyces* strains and fungus combs

A. Screening for phenoloxidases/peroxidase fungi from *Termitomyces* strains

The ability of the symbiotic fungi to produce phenol-oxidizing enzymes was examined in 22 *Termitomyces* strains from fungus combs of various termite species in previous experiment using a plate assay method. The screening was based on the polymerization of guaiacol on agar plate caused by extracellular phenoloxidases and/or peroxidases excreted by the fungi. Figure 27 show the brown-coloured zone produced by the representative fungus when grown on PDA with 0.01% guaiacol compare with the control (the medium without 0.01% guaiacol). The result of growth and brown-coloured zone of all samples were shown in Figure 28 and 29.

Figure 27 The brown-coloured zone produced by the representative fungus compare with the control (the medium without 0.01% guaiacol).

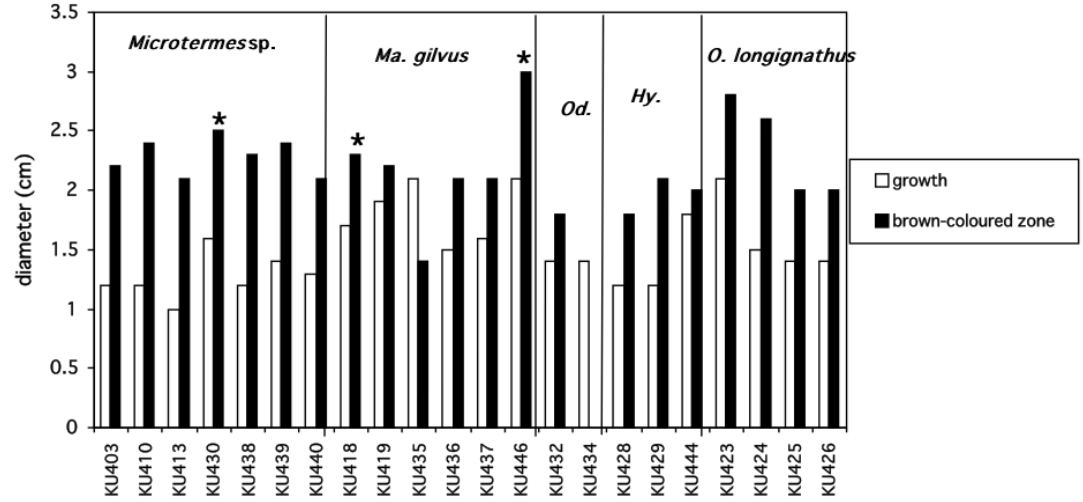

Figure 28 Growth and brown-coloured zone produced by nodule fungi grown on Kirk medium +1.2mM Ammonium tartrate (LN) for 4 weeks. The fungal strains used for further study were marked by asterisks.

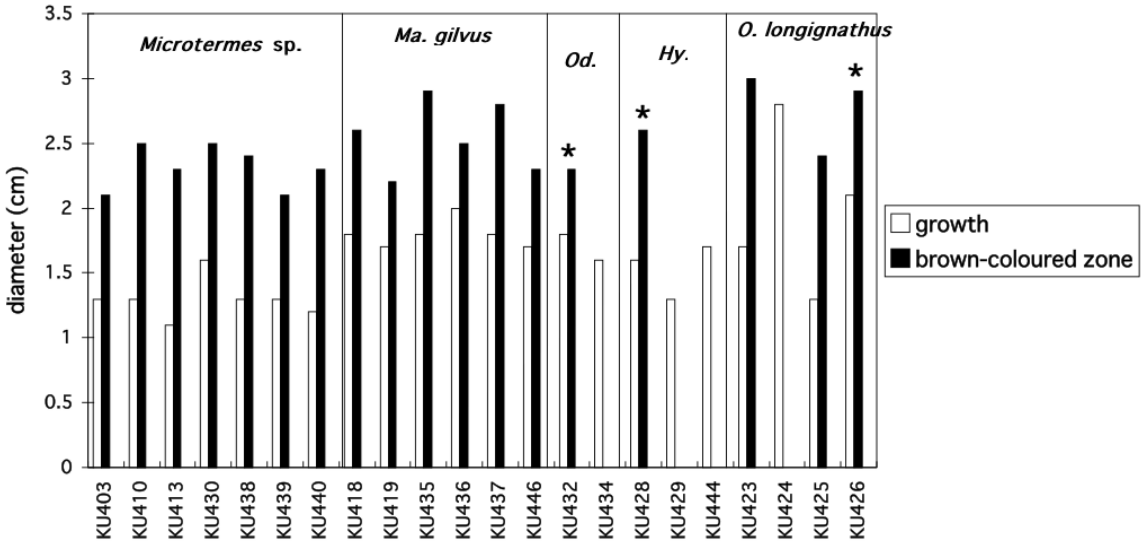

Figure 29 Growth and brown-coloured zone produced by nodule fungi grown on Kirk medium +12mM Ammonium tartrate (HN) for 4 weeks. The fungal strains used for further study were marked by asterisks.

Phenol-oxidizing enzyme was observed under both low nitrogen (LN) and high nitrogen (HN) condition in most of the strains assayed, except for two strains from *Hypotermes* sp. (KU429 and KU 444) and one strain from *O. longignathus* (KU424) that only exhibited the activity under LN conditions. No activity was

observed from one *Odontotermes* sp. strain (KU434) under both condition. The strains that exhibited higher activity from each host termite (marked by asterisks in Figure 28 and 29) were used for further study.

B. Screening for ligninolytic enzyme producing *Termitomyces* strains

The representative *Termitomyces* spp. strains from A. (KU418, KU446, KU430, KU432, KU426, KU428) were recultivated on 120 ml of Kirk's liquid media containing 120 µl of 5% Remazol Brilliant Blue R (RBBR) dye including 1.2 or 12 mM ammonium tartrate (low or high nitrogen source) and cultured with agitation (120 rpm) at 28 °C in dark condition and measured the decolorization of RBBR dye by the ligninolytic enzymes every 2 day for 45 days. The results were showed in figure 30 and 31.

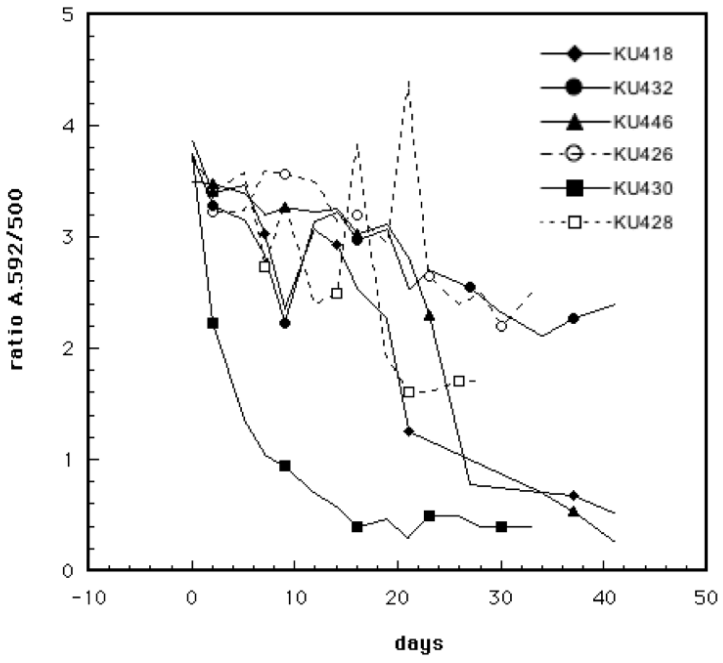

Figure 30 Decolorization of RBBR by the ligninolytic enzyme from *Termitomyces* strains grown on Kirk medium under low nitrogen condition.

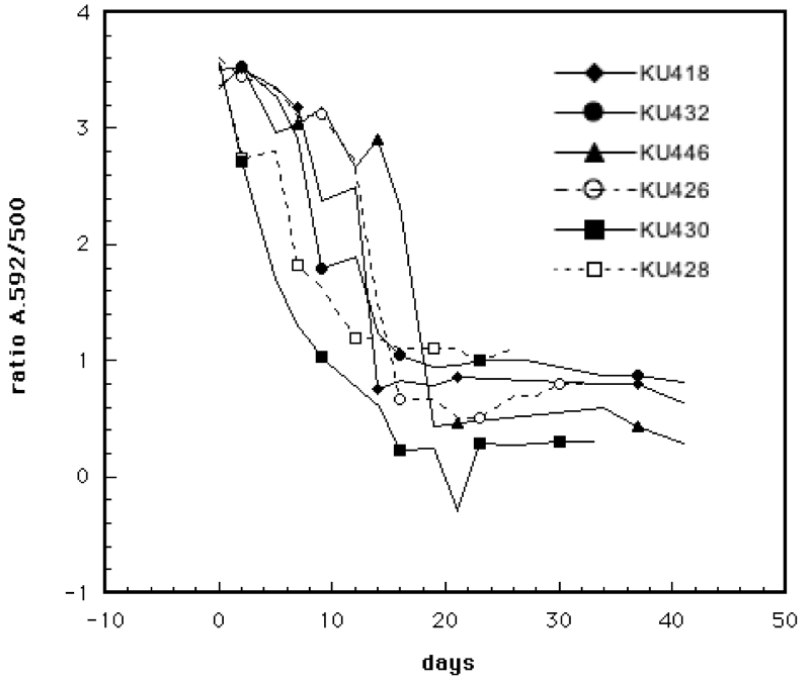

Figure 31 Decolorization of RBBR by the ligninolytic enzyme from *Termitomyces* strains grown on Kirk medium under high nitrogen condition.

The decolorization of RBBR dye by the extracellular enzymes was measured as decrease in absorbance 592/500. In low nitrogen medium (figure 30), all samples except for the *Termitomyces* strains isolated from *O. longignathus* (KU426) and *Hypotermes* sp. (KU428) showed decolorization of RBBR dye. In high nitrogen source medium (figure 31), all samples showed decolorization of RBBR dye. All *Termitimyces* spp. strains used in this study were used for examined the series of phenol-oxidizing enzymes activities.

C. The activities of phenol-oxidizing enzymes from *Termitomyces* strains

As known that basidiomycetes produce three kinds of extracellular phenol-oxidizing enzymes, that are lignin peroxidase (LiP), manganese peroxidase (MnP) and Laccase. Lignin peroxidase catalyzes the oxidation of various aromatic compounds to form aryl cation radicals while manganese peroxidase oxidizes Mn(II) to Mn(III), which diffuses from the enzyme and oxidizes various phenolic compounds. These enzymes require hydrogen peroxide for their activities. Laccase also catalyzes the

oxidation of various phenolic compounds and aromatic amines, but this reaction is coupled with the reduction of molecular oxygen to water. Because of their broad substrate specificities, they can be also involved in the degradation of a variety of plant phenols. In this study, three kinds of these enzymes activities were investigated.

The activities of phenol-oxidizing enzymes of 6 isolated strains from A (KU418, KU446, KU430, KU432, KU426, KU428) were examined by the cultures grown on Kirk's liquid media with low or high nitrogen source, the results of phenol-oxidizing enzyme activities from each culture were showed in figure 32, 33, 34, 35, 36 and 37 respectively.

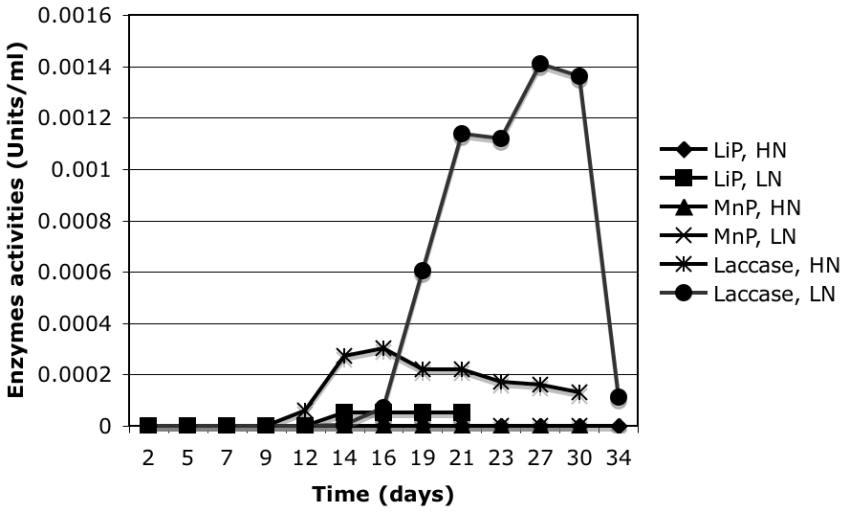

Figure 32 The enzyme activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU418 strain isolated from *M. gilvus* in Prachinburi under low (LN) or high (HN) nitrogen conditions.

From figure 32, strain KU418 that was isolated from the fungus comb of *Macrotermes gilvus*, when compared with lignin and manganese peroxidase, laccase showed highest activity, significant laccase activity was detected under LN conditions.

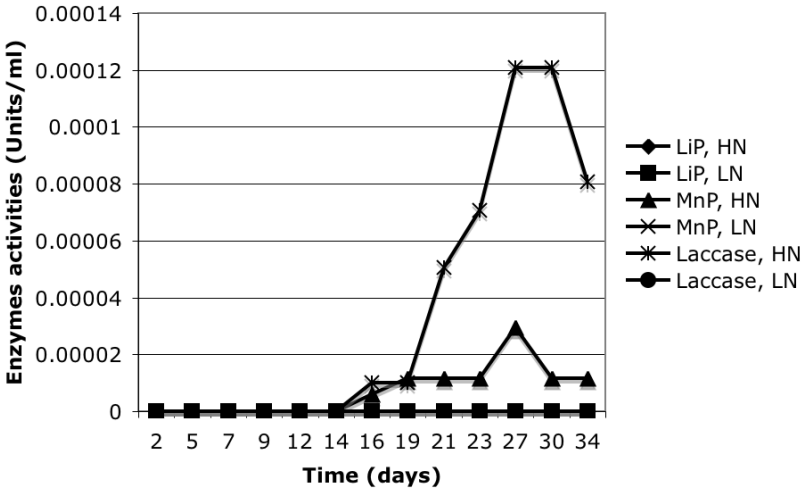

Figure 33 The enzyme activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU446 strain isolated from *M. gilvus* in Prachinburi under low (LN) or high (HN) nitrogen conditions.

From figure 33, strain KU446 that was isolated from the fungus comb of *Macrotermes gilvus*, when compared with lignin and manganese peroxidase, laccase showed highest activity under HN conditions.

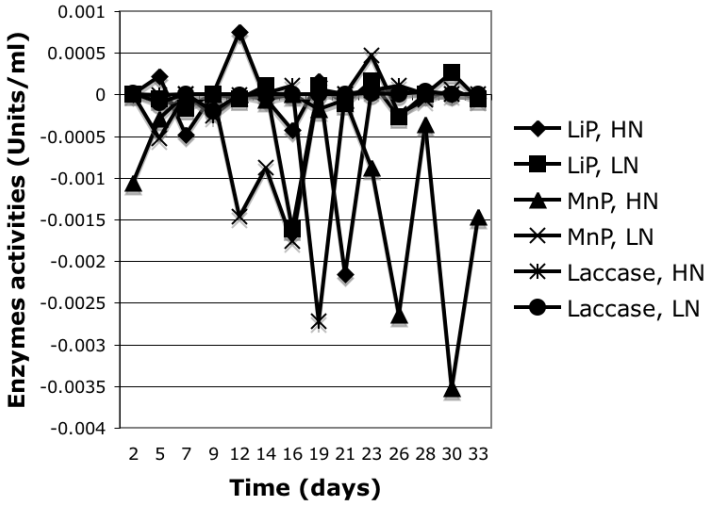

Figure 34 The enzyme activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU430 strain isolated from *Microtermes* sp. in Prachinburi under low (LN) or high (HN) nitrogen conditions.

From figure 34, strain KU430 that was isolated from the fungus comb of *Microtermes* sp., weak lignin peroxidase activity was detected under HN conditions, no laccase activity was detected in this strain.

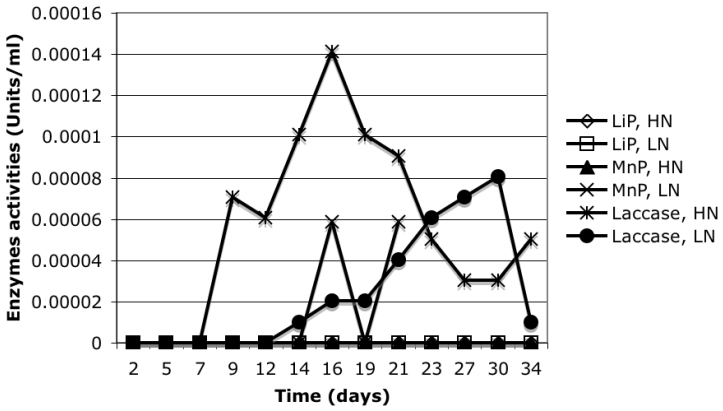

Figure 35 The enzyme activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU432 strain isolated from *Odontotermes* sp. in Prachinburi under low (LN) or high (HN) nitrogen conditions.

From figure 35, strain KU432 that was isolated from the fungus comb of *Odontotermes* sp., weak laccase activity can detected under both conditions and weak manganese peroxidase activity can detected under LN conditions, no lignin peroxidase activity was detected in this strain.

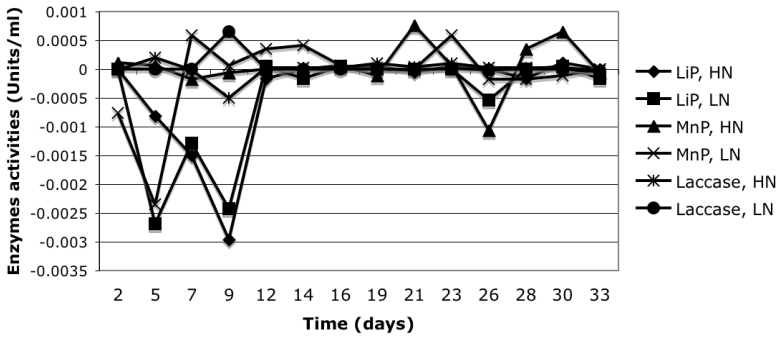

Figure 36 The enzyme activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU426 strain isolated from *Odontotermes longignathus* in Saraburi under low (LN) or high (HN) nitrogen conditions.

From figure 36, strain KU426 that was isolated from the fungus comb of *O. longignathus* can detected weak activities in all enzymes studied under both conditions.

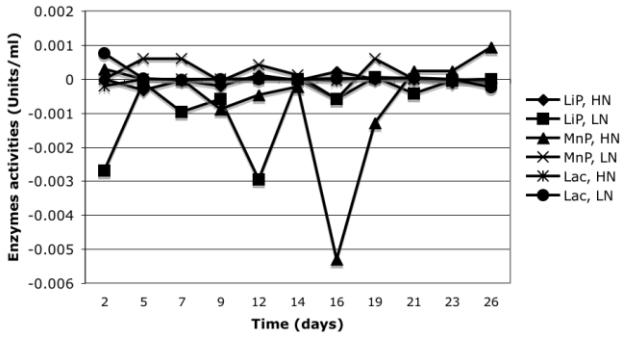

Figure 37 The enzyme activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU428 strain isolated from *Hypotermes* sp. in Saraburi under low (LN) or high (HN) nitrogen conditions.

From figure 37, strain KU428 that was isolated from the fungus comb of *Hypotermes* can detected weak activities in all enzymes studied under both conditions, except for lignin peroxidase which no activity under LN condition.

D. The phenol-oxidizing enzyme activities in the fungus comb

A series of phenol-oxidizing enzymes, laccase, lignin and manganese peroxidases in the fungus combs of *Microtermes* sp., *Odontotermes* sp. and *M. gilvus* were examined. Of these enzymes, only laccase activity was detected in all of the fungus combs examined (Table 3).

Table 3 Laccase activity in fungus combs

Termite species (location)	Activity (µkat/g of comb)[a]
Ma. gilvus (Prachinburi)	32.6
Ma. gilvus (PathumThani)	33.3
Odontotermes sp. (Prachinburi)	9.58
Microtermes sp. (Prachinburi)	5.15

[a] The data shown are mean values of duplicate experiments.

III. Cloning and characterization of putative laccase genes of *Ma. gilvus* from PathumThani

In order to identify laccase genes expressed in the *Termitomyces* spp. and the fungus combs, *Termitomyces* spp. from A. (strains KU418, KU446, KU430, KU432, KU428, KU426) and NS/Mg were extracted their DNA and laccase genes were amplified with degenerate primers targeting the highly conserved copper binding domains II and III (figure 38). RNA were also extracted from two *Termitomyces* strains (KU418 isolated from *M. gilvus* fungus comb in Prachinburi and NS/Mg isolated from *M. gilvus* fungus comb in PathumThani) and from the fungus comb of *M. gilvus* (PathumThani), *M. gilvus* (Prachinburi), *Odontotermes* sp. (Prachinburi), *Microtermes* sp. (Prachinburi), *O. longignathus* (Saraburi) and *Hypotermes* sp. (Saraburi). Reverse transcription (RT)-PCR was carried out on RNA extracted from the fungus combs and *Termitomyces* spp. (strain KU418 and NS/Mg) with degenerate primers targeting the highly conserved copper binding domains II and III (figure 38). PCR and RT-PCR products of an appropriate size (ca. 900 bp; Lyons *et al*, 2002) were obtained (figure 39) and cloned. The clones were checked for RFLP pattern using restriction enzymes *Hae*III and *Hha*I (figure 40) and the representative clone from each strains was analyzed by automated sequence analyzers.

A total of 71 sequences were identified from the fungus combs of five termite species and from strain KU 418 grown in KB liquid culture (figure 32). Of these, 69 sequences showed significant similarities to basidiomycete laccase (BLAST scores and E values ranged from 441 to 234 and 1e-122 to 2e-60, respectively). Termination codons were found in putative open reading frames of two sequences from the fungus comb of *Odontotermes* sp. and one sequence from strain KU418, and these were not analyzed further.

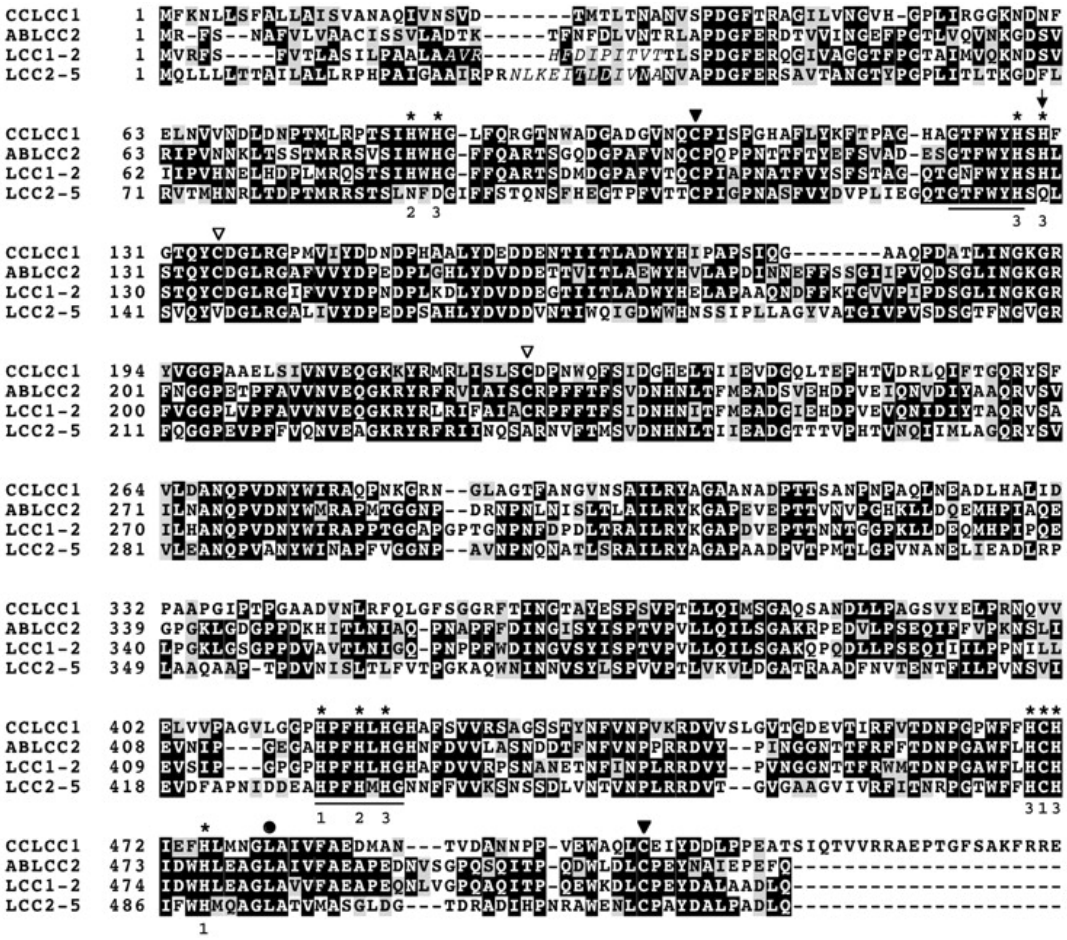

Figure 38 Sequence comparison among *C. cinerea* laccase 1 (CCLCC1) (DDBJ accession no. AAD30964), *A. bisporus* laccase 2 (ABLCC2) (accession no. Q12542), LCC1-2, and LCC2-5. Identical and similar amino acids are shaded. The conserved amino acid residues potentially involved in copper ion binding are marked by asterisks, and position 129 of *A. bisporus* laccase 2 is also marked with an arrow. Numbers under the His and Cys residues indicate types of copper ions that bind to each residue. The amino acid sequences that are used to design the degenerate primers are underlined. The Cys residues that form disulfide bonds in *C. cinerea* laccase 1 are marked with closed inverted triangles (Cys 103 and Cys 505) and open inverted triangles (Cys 135 and Cys 222). A closed circle indicates the Leu residue that influences a laccase redox potential.

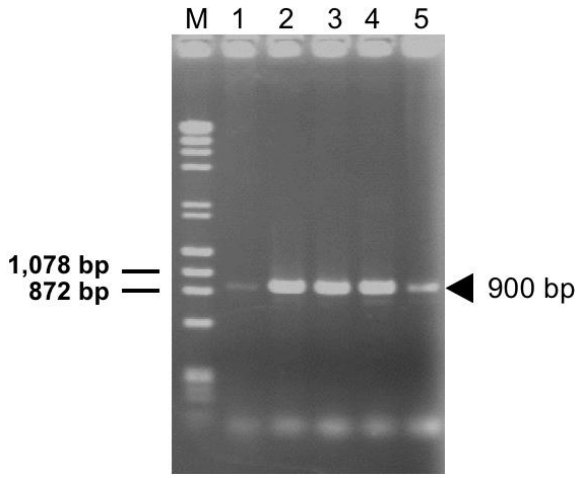

Figure 39 RT-PCR products amplified from cDNA with temperature profile as 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min. Lane M is molecular size standards (λ/ *Hin*dIII digest plus ϕX174/*Hae*III digest [Toyobo]). The size of each band was, from top to bottom, 23130, 9416, 6557, 4361, 2322, 2027, 1353, 1078, 872, 603 and 310 bps.

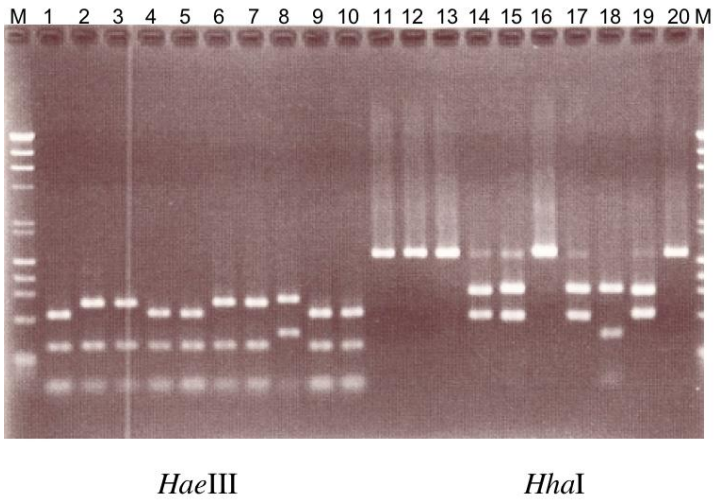

Figure 40 The example of RFLP pattern cut by restriction enzymes *Hae*III (Lane 1-10) and *Hha*I (Lane 11-20). Lane M is molecular size standards (λ/ *Hin*dIII digest plus ϕX174/*Hae*III digest [Toyobo]).

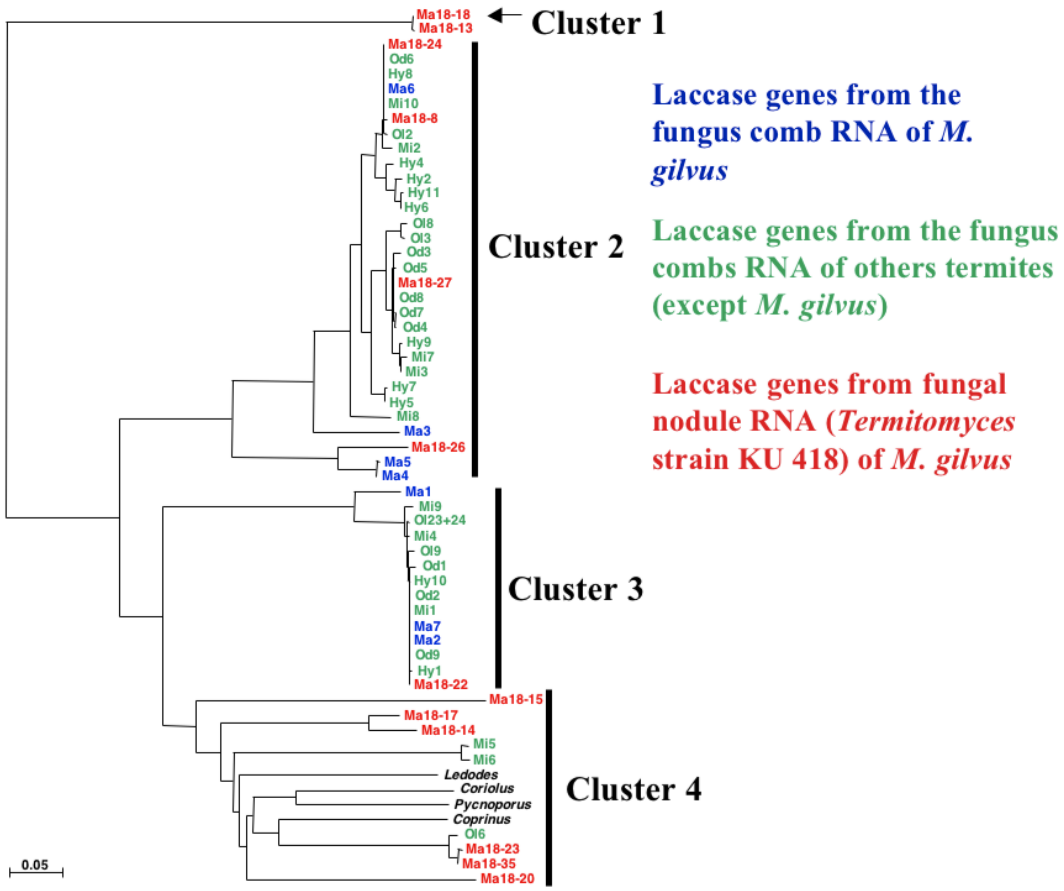

Figure 41 Phylogenetic analysis of the laccase genes expressed from fungus combs RNA and *Termitomyces* RNA (strain KU 418) compare with some laccase genes from database. Red and blue are from same comb whereas green is from another different comb.

Laccase genes obtained from fungus combs and isolated *Termitomyces* sp. strain KU418 were formed into 4 clusters (figure 41). Typical laccase from basidiomycetes were affiliated with cluster 4. Most clones from fungus combs (indicated as green and blue color) were found in cluster 2 and 3, while clones from cultured *Termitomyces* indicated as red color were found in all clusters, suggesting that *Termitomyces* possesses multiple laccase genes that expression seems to be controlled depending on the culture conditions.

A. Full-length cDNA sequences of laccase gene

The full-length cDNAs of two representative clones (*lcc1-2* and *lcc2-5*) were isolated from the fungus comb RNA from PathumThani province. The corresponding gene sequences were also identified from genomic DNA of the symbiotic fungal strain NS/Mg isolated from the same fungus comb. Comparison between the genomic and cDNA sequences revealed that the coding regions of genomic *lcc1-2* and *lcc2-5* were interrupted by 23 and 21 introns and encoded 524 and 534 amino acids, respectively. The LCC1-2 amino acid sequence contained all of the amino acid residues that are essential for copper ion binding in laccase, whereas the copper binding domains in LCC2-5 were incomplete (figure 38). The LCC2-5 amino acid sequence lacked 3 of 11 amino acid residues involved in copper ion binding. Two disulfide bonds, Cys 103-Cys 505 and Cys 135-Cys 222, were found in the crystal structure of laccase 1 from *Coprinus cinerea* (Ducros *et al*., 1998). The equivalent four Cys residues were also conserved in the LCC 1-2 sequence, while one set of Cys residues was present in the LCC2-5 sequence. A BLAST search showed that best hits for LCC1-2 and LCC2-5 amino acid sequences were laccase 1 (Q12541) and laccase 2 (Q12542), respectively, from the basidiomycete *Agaricus bisporus*. Amino acid identities and similarities between LCC1-2 and laccase 1 were 66 and 76%, while those between LCC2-5 and laccase 2 were 47 and 62%, respectively.

In addition to the *lcc1-2* and *lcc2-5* genes, the numbers of distinct cDNA sequences eventually identified were six from *M. gilvus*, three from *Odontotermes* sp. and six from *Microtermes* sp. in Prachinburi; seven from *Hypotermes* sp. and five from *O. longignathus* in Saraburi; one from *M. gilvus* in PathumThani; and nine from strain KU418.

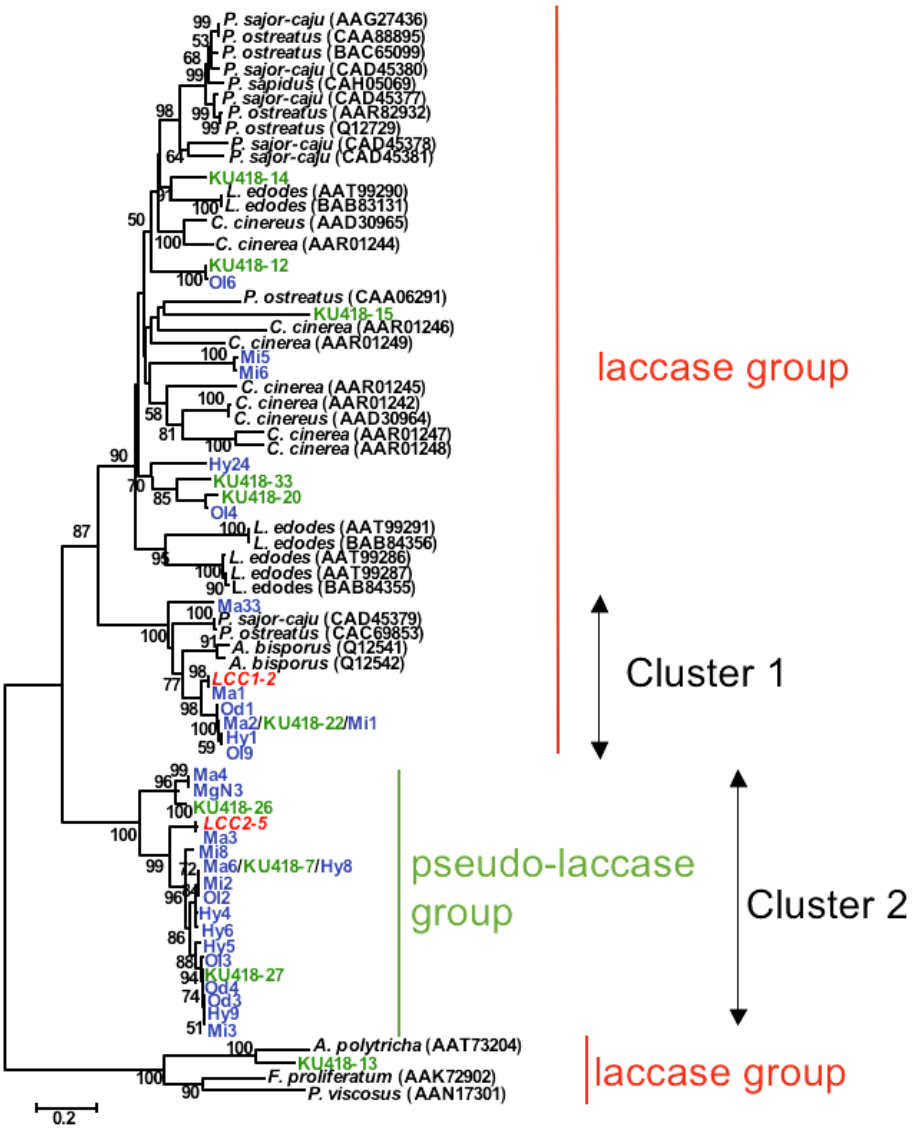

Figure 42 Neighbor-joining tree for amino acid sequences of putative laccases identified from the fungus combs and *Termitomyces* sp. strain KU418 and related proteins. The fungus combcDNA, *Termitomyces* sp. (KU418) cDNA and *Termitomyces* sp.(NS/Mg) full length cDNA were indicated as blue, green and red color , respectively. Clone designations and genus abbreviations are as follows: MaX, *M. gilvus* in Prachinburi; MgN3, *M. gilvus* in PathumThani; OdX, *Odontotermes* sp.; MiX, *Microtermes* sp.; OlX, *O. longignathus*; Hy, *Hypotermes* sp.; KU418-X, *Termitomyces* sp. strain KU418, where X means number; P., *Pleurotus*; L., *Lentinula*; C., *Coprinopsis*; A., *Agaricus*; Au., *Auricularia*; F., *Fusarium*; Pa., *Panorbis*

Phylogenetic analysis placed most of the putative laccase amino acid sequences into two major clusters (cluster 1 and 2) (figure 42). All the sequence from the *M. gilvus* fungus combs from Prachinburi and Pathum Thani were found in these clusters, while five sequences identified from *Microtermes* sp., *Hypotermes* sp., and *O. longignathus* (Mi5, Mi6, Hy24, Ol4, and Ol6) were placed outside these clusters and appeared more closely related to the laccases of known basidiomycetes. All of the sequences in cluster 2 lacked the His residue that is essential for type 3 copper binding [position 129 in *A. bisporus* LCC2 (shown in figure 38)], while other clones possessed His at that position. Among 10 sequences from strain KU418, 3 sequences were found in cluster 2. Sequence that were identical to those of KU418-22 and KU418-7 were identified from the fungus combs [Ma2, Mi1 and Ma6, Hy8, respectively (figure 42)]. The phylogenetic position of sequence KU418-13 was close to that of the ascomycete laccases, although a laccase from the basidiomycete *Auricularia polytricha* also clustered in the same group as the closest relative.

B. Semi-quantitative analysis of gene expression

In this experiment we determine the laccase gene from the cDNA of the fungus comb of *M. gilvus* (PathumThani), *M. gilvus* (Prachinburi) and *Hypotermes* (Saraburi). Concentrations of PCR products were estimated by electrophoresis on an agarose gel stained with SYBR Green I using Molecular Imager FX (Bio-Rad). The minimum cycle number to construct clone library in this experiment is 18 cycles, which the DNA concentration is about 11 pg/ µl of PCR mixture. A clones library was constructed from the PCR product of 18 cycles and done RFLP analysis using *Hha*I (in total 96 clones) and done sequencing.

The relative abundance of putative laccase sequences in each clone library was analyzed semiquantitatively to estimate expression levels of laccase genes in the fungus comb (figure 43). The most abundant clones found in all the three libraries were grouped together in cluster 2 (pseudo-laccase group). Except for the sequences in cluster 2, Ma1, *lcc1-2*, and Hy1 were the most abundant sequences from *M. gilvus*

in Prachinburi, *M. gilvus* in PathumThani, and *Hypotermes* sp. in Saraburi, respectively.

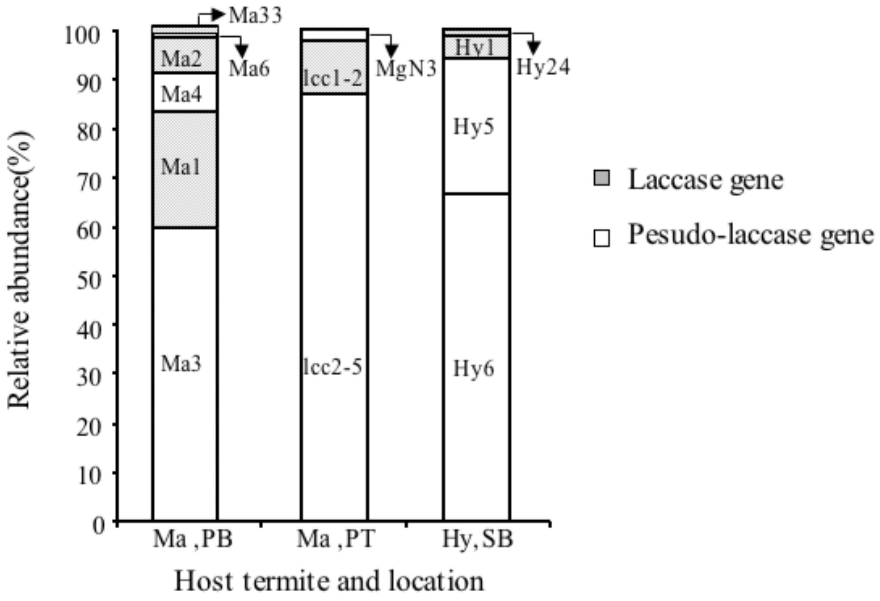

Figure 43 Relative abundance (expression level) of cDNA clones (96 clones) from the fungus combs of *M. gilvus* in Prachinburi (Ma, PB), *M. gilvus* in Pathum Thani (Ma, PT), and *Hypotermes* sp. in Saraburi (Hy, SB).

III. Expressed sequence tag (EST) analysis

Two cDNA libraries for EST analysis were constructed. Poly(A)$^+$RNA extracted from the fungus comb of *M. gilvus* was used to construct the NS cDNA library. To construct the SB cDNA library, the first-strand cDNA used for construction of the NS library was subtracted with driver RNA extracted from *Termitomyces* sp. strain NS/Mg grown on the PDA medium. Partial sequences of the randomly chosen clones in the two libraries were determined from the 5´-ends. After removal of low-quality sequences and contaminants derived from the cloning vector and ribosomal RNA, 2,108 and 505 ESTs were collected from the NS and SB libraries, respectively. Three ESTs similar to a ribosomal RNA gene were found by the BLASTN program (Altschul *et al*. 1997) and removed from our EST database. No

sequence derived from the *E. coli* genome was found. Sequence assembling was performed, resulting in 1,382 and 325 tentative consensus sequences (TCs) for the NS and SB libraries, respectively. The average sequence lengths of TCs from the NS and SB libraries were 629 and 547 bp.

Table 4 lists the 20 most abundant ESTs in the NS library and their results of sequence similarity search against the protein database. All source organisms of BLASTX hits were fungi, of which 60% were basidiomycetes, including *Termitomyces*. TCs NS2E10 and NS11F03 were sequences corresponding to the laccase-related protein cDNA *lcc2-5* (AB201165) and the laccase cDNA *lcc1-2* (AB201164).

Table 4 BLASTX results of the 20 most abundant ESTs in the NS (nonsubtractive) library and a comparison of EST frequency between the NS and SB libraries

TC ID[a]	BLAST *E*-value	Percent identity[b] (aa overlap)	Description (accession no.)	Source organism	Abundance (%)[c] NS	SB
NS3A9[d]	-		No hit		2.0	2.0
NS2E10[d]	0	100 (555)	LCC2-5 (AB201165)	*Termitomyces* sp.	1.9	0.4
NS23H11[d]	2e-27	58.8 (102)	Ribonuclease Pol (P81762)	*Pleurotus ostreatus*	1.4	0.6
NS2A8	3e-19	54 (102)	CipC protein (Q8NKC9)	*Aspergillus indulans*	1.3	6.7
NS14A09[d]	1e-132	63.1 (355)	Acid phosphatase (Q75V97)	*Pholiotanameko*	1.2	0.6
NS1C10	-		No hit		1.2	0.6
NS10H10	2e-58	53.8 (429)	DDR48 (P18899)[e]	*Saccharomyces cerevisiae*	1.0	4.4
NS12E07[d]	1e-115	43.8 (543)	HP[f] (Q7S495) alkaline phosphatase (PF00245)[g]	*Neurospora crassa*	0.9	0.4
NS11F03[d]	0	100 (510)	Laccase LCC1-2 (AB201164)	*Termitomyces* sp.	0.9	0.0
NS11D11[d]	5e-62	51.0 (255)	Endopolygalacturonase (Q9P8M5)	*Chondrostereum purpureum*	0.6	0.2
NS2E5			No hit		0.5	0.8
NS14E10[d]	6e-09	33.3 (180)	4MeS (O13320)	*Metarhizium anisopliae*	0.5	1.0
NS16F03	1e-123	62.5 (363)	Formate dehydrogenase (Q6CDN8)	*Yarrowia lipolytica*	0.5	0.8

Table 4 (Continued)

TC ID[a]	BLAST *E*-value	Percent identity[b] (aa overlap)	Description (accession no.)	Source organism	Abundance (%)[c] NS	SB
NS06A08[d]	8e-18	49.6 (131)	Putative hydrophobin (Q9HGW9)	*Agaricus bisporus*	0.5	3.2
NS22B11			No hit		0.4	0
NS11B04	0	72.0	Phosphate transporter (Q96X52)	*P. nameko*	0.4	0
NS17H08			No hit		0.4	1.8
NS14E02[d]	1e-143	75.0 (328)	Cellobiohydrolase I-II (Q6E5B2)	Volvariella volvacea	0.4	0
NS1B9	0	63.9 (502)	Aldehyde dehydrogenase (O74187)	*A. bisporus*	0.3	0.2
NS04G06	1e-101	58.3 (300)	D-Xylose reductase (Q9P8R5)	*Aspergillus niger*	0.3	0.4

[a] Identification of a tentative consensus sequence
[b]Amino acid identity calculated by FASTA
[c]*Abundance = (ESTs in a TC/ total ESTs in a library)* x 100
[d]Indicates a transcript with a putative signal sequence in its deduced amino acid sequence
[e]DNA damage-responsive protein. Filter-off option was used in BLAST search
[f]Hypothetical protein
[g]Pfam description

The enzyme homologs involved in the degradation of plant cell walls found in the NS and the SB library are summarized in table 5. A total of 70 ESTs (3.3%) and 29 TCs (2.1%) in the NS library showed significant similarity to the genes that encode plant cell wall degrading enzymes. A small number of homologs relevant to cell wall degradation were identified from the SB library. The number of homologs of pectin and hemicellulose-degrading enzymes was 2.5-fold more than the number of cellulose degrading enzymes, such as cellobiohydrolase, endoglucanase and ß-glucosidase. Homologs of endo- and exopolygalacturonase, pectate lyase, rhamnogalacturonan lyase, arabinase, pectin methylesterase, and α-galactosidase were potentially involved in pectin degradation. Among ligninolytic enzymes, lignin peroxidase, manganese peroxidase and laccase, only laccase homologs were identified from our EST database.

Table 5 Putative cell-wall-degrading enzymes identified in NS library

Enzyme	No. of EST		No. of TC		CAZy family[a]
	NS	SB	NS	SB	
Pectin-degrading					
Endopolygalacturonase	17	1	3	1	GH28
Exopolygalacturonase	9	0	2	0	GH28
Pectate lyase	4	0	1	0	PL1, 2
Rhamnogalacturonan lyase	2	0	1	0	PL4
Pectin methylesterase	1	1	1	1	CE8
Rhamnogalacturonan acetylesterase	0	1	0	1	CE12
Other hemicellulose-degrading					
Endo-1,4-beta-xylanase	5	0	5	0	GH10, 11
beta-Mannanase	5	0	2	0	CE5
Acetylxylan esterase	3	0	1	0	CE5
Arabinase	2	0	2	0	GH43
alpha-Galactosidase	1	0	1	0	GH27
Cellulose-degrading					
Cellobiohydrolase	12	1	3	1	GH6, 7
Endoglucanase	6	0	4	0	GH5, 44, 61
beta-Glucosidase	3	0	3	0	GH1, 3

[a] Carbohydrate-active enzymes (CAZy) family

CONCLUSION AND RECOMMENDATION

Conclusion

From the experimental results and discussion of this study, the conclusion of each experiment can be drawn as follow:

The phylogenetic analysis experiment experiment, *Termitomyces*-related symbiotic basidiomycetes in the nest of fungus-growing termites (Macrotermitinae) of several genera in Thailand were cultivated and analyzed phylogenetically based on the DNA sequence of nuclear ribosomal RNA genes.

Among the 39 strains analyzed in this study, 38 strains were related to *Termitomyces*. Comparison of the sequence similarity showed that these 38 strains were significantly related. Among these 38 strains, the DNA sequence of the ITS1-5.8S rDNA-ITS2 region (530-645 bp) showed more than 72% nucleotide identity to one another. Based on the comparison of the ITS1-5.8S rDNA-ITS2 region, these 38 strains were classified into eight groups. Within each group, the DNA sequences showed more than 99% nucleotide identity to one another. The DNA sequence of at least 330 bp of the LSU rDNA region was analyzed in all these 38 strains and showed more than 93% nucleotide identity to one another.

The longer LSU rDNA region (1.2-1.4 kbp) of at least one representative of each of the eight groups was analyzed for their DNA sequences. All the representatives of the eight groups were closely related to one another, showing more than 92% nucleotide identity. The LSU rDNA sequences were compared with the representative basidiomycetes' DNA sequences of Agaricales available in the databases. The phylogenetic analyses of the LSU rDNA indicated that all the eight groups identified from the termite nests were clustered together with *Termitomyces heimii*, *Termitomyces cylindricus*, *Termitomyces* sp. strain BSI sp1 and *Termitomyces microcarpus* (*Podabrella microcarpus* strain PRU3900). The results suggest that all the 38 strains are *Termitomyces*-related basidiomycetes and confirm that

Termitomyces are true symbionts which grow on the fungus comb as fungus nodules. The eight groups probably represent species or subspecies of this genus. Recently, several DNA sequences from fungus nodules in some termite nests have been appeared in the databases (AB051879-AB051890 and AF357023). These DNA sequences of the ITS1-5.8S rDNA-ITS2 region were clearly related to those of the *Termitomyces*-like strains cultivated in this study, but they formed distinct groups from the eight groups identified here.

The only exception strain, KU416, the LSU rDNA analysis clearly indicated that this strain was affiliated to Ascomycota of the family Xylariaceae that includes the genus *Xylaria*. The LSU rDNA of some members of this family showed the highest sequence identity, around 92%, with this strain. It has been reported that *Xylaria*-like fungi inhabit the fungus comb as mycelia. (Thomas, 1987) However, they never produce spores or stroma until the fungus comb is removed from the nest or termites abandon the nest. The presence of termites probably prevents *Xylaria*-like fungi from proliferating on the fungus comb.

Relationships of the *Termitomyces*-related fungi cultivated from the fungus comb with their host termites were not simple. Some the isolated strains appeare to be symbionts of more than one genus of termite. The symbionts are thought to be generalists rather than specialists with respect to the relationship with their hosts. From a single genus of the termite, more than two groups of the symbiotic fungi were identified when the termites of multiple species or multiple locations were examined. The choice of the symbionts by the hosts, at least at the genus level of the termites, is not fixed to a single group of the symbionts. Furthermore, it is noted that a single fungus comb harbored two different groups of the symbiotic fungi in the case of *Microtermes* sp. in Prachinburi. In the case of *Macrotermes annandalei* in Chanthaburi, two groups were obtained from the different nests. The groups of the symbiotic fungi obtained in a single sampling site were also limited so the locality of the host termites seemed to affect the choice of the symbionts. Thus, the locality alone is not crucial for the selection of the symbionts, and some *Termitomyces* lineages of the symbionts and dispersed across the locations.

The laccase experiment, the symbiotic fungi (*Termitomyces*) from termites belonging to the genera *Macrotermes, Odontotermes, Hypotermes* and *Microtermes* and fungus combs (a substrate used to cultivate symbiotic fungi) from termites belonging to the genera *Macrotermes, Odontotermes* and *Microtermes* in Thailand were investigated their phenol-oxidizing enzymes. The result clearly demonstrated that laccase was the sole detectable phenol-oxidizing enzyme in the fungus combs of *Microtermes* sp., *Odontotermes* sp. and *M. gilvus*. No peroxidase activity was detected in the fungus comb and low activity was detected in some culture supernatants of *Termitomyces* spp. strains. The analysis of the fungus comb of *M. gilvus* from PathumThani and its symbiotic fungus revealed that the *lcc1-2* gene of *Termitomyces* sp. strain NS/Mg is the dominant isozyme under symbiotic conditions. The LCC1-2 amino acid sequence contains all of the conserved His and Cys residues required for copper ion binding and an additional conserved Leu residue affecting the redox potential of laccase (figure 38).

In addition to the *lcc1-2* laccase gene, *lcc2-5* gene was found. The LCC2-5 amino acid sequence showed significant similarity with that of *Agaricus bisporus* laccase 2, but it lacked three His residues required for copper ion binding in the deduced amino acid sequence (figure 38). The laccase molecule has four copper ions distributed among three sites, each of which is defined according to its spectroscopic properties (Solomon *et al*., 1996). The T1 site contains the type 1 blue copper that is responsible for absorption at around 600 nm. The T2 site contains a type 2 copper with a characteristic electron paramagnetic resonance. In the T3 site, the pair of strongly coupled type 3 coppers is electron paramagnetic resonance silent in the presence of dioxygen. The mononuclear T1 site extracts an electron from a reducing substrate and mediates its transfer to the trinuclear T2/T3 center where molecular oxygen is reduced.

Putative cDNA fragments of laccase were amplified using the degenerate PCR primers from the fungus combs of various termite hosts. Their deduced amino acid sequences showed similarity with those of fungal laccases, but some of the sequences had Glu or Gln residues at the site of the conserved His residue corresponding to His

129 of *A. bisporus* laccase 2. Phylogenetic analysis showed that all of these sequences were placed in cluster 2 and that they formed a lineage distinct from those of basidiomycete laccases (figure 42). LCC2-5 was also found in cluster 2, suggesting that while the sequences in cluster 2 are closely related to laccase genes, they do not most certainly encode "true" laccase. Interestingly, these pseudolaccase cDNA sequences were identified from all of the fungus combs tested in this study. Amino acid identities among these pseudolaccases ranged from 61 to 100%. Also, the transcription levels of the pseudolaccase genes were much higher than those of the putative laccase genes (figure 43). From the result of expressed sequence tag analysis for symbiotic fungus of *M. gilvus*, the sequence corresponding to lcc2-5 was one of the abundant sequences. These findings suggest that the pseudolaccase genes are nonetheless essential for either the symbiotic fungi themselves or symbiosis with the host termites.

Multiple laccase genes are often found in single organisms. The saprophytic fungus *Coprinopsis cinerea*, for example, has eight different laccase genes that were reported previously (Hoegger *et.al*., 2004) and one additional gene in the public databases. *Termitomyces* sp. strain KU418 expressed seven putative laccase genes when the fungus was cultured in KB liquid medium under low nitrogen (LN) conditions. These sequences showed higher diversity than those found in either the *C. cinerea* laccases or the sequences from the *M. gilvus* fungus comb that were found only in cluster1 (figure 42). In *M. gilvus* from Prachinburi, only one identical sequence set (Ma2 and KU418-22) was retrieved from the clone library of the fungus comb and strain KU418, although four times more clones were sequenced from the KU418 clone library than from the fungus comb library. This finding could possibly be attributed to differences in culture conditions between the fungus comb and KB medium, because the differential expression of fungal laccase genes is often found depending upon nutritional conditions, for example, copper ion concentrations (Burke and Cairney, 2002; Collins and Dobson, 1997) and the presence of various aromatic compounds (Terron *et. al*., 2004). Katoh *et al*. (2002) demonstrated no genetic variation in symbiotic fungus from a single large nest of the termite *Odontotermes*

formosanus so this would be support the idea that *Termitomyces* fungi have multiple laccase genes.

Putative functional laccase cDNA fragments that clustered outside cluster 2 were identified from the fungus combs of all the termite hosts examined in this study, indicating that laccase is widely distributed among the symbiotic fungi of fungus-growing termites. Phylogenetic analysis showed that the laccase sequences from the fungus comb and *Termitomyces* sp. strain KU418 were closely related to the laccase from *A. bisporus*, *L. edodes*, *Pleurotus* spp., and *C. cinerea*. Like the *Termitomyces* fungi, these fungi belong to the order Agaricales and mostly produce class 2 laccases. From the previous experiment (I), the phylogenetic analysis among *Termitomyces* fungi used in this study, except for strain NS/Mg, showed that fungal strains isolated from *O. longignathus* (Saraburi), *Odontotermes* sp. and *M. gilvus* (Prachinburi) were closely related and shared more than 99% nucleotide identity in partial sequences of the large subunit rRNA genes. In the laccase phylogeny, specific relationships were not found among laccase homologs from those symbiotic fungi. It is uncertain whether this is due to differential expression of multiple laccase genes in each fungus comb as seen in the *M. gilvus* fungus comb and strain KU418 or each symbiotic fungus possessing individual laccase genes. Comprehensive analyses of laccase genes in those symbiotic fungi would be required to answer the question.

In this study, laccase activity was detected and higher laccase activity was detected in the fungus comb and the isolated strain from the nest of *M. gilvus*. Hower, it is known that laccase can oxidize non-phenolic compounds and degrade lignin in the presence of a laccase mediator such as 3-hydroxyanthranilate (Eggert *et al*., 1996). Thus, laccase mediators must be studied to estimate the contribution of laccase to lignin degradation in the fungus comb of the genus *Macrotermes*. Further functional studies on laccase ans laccase-like protein from the symbiotic fungi are necessary to clarify the importance of these enzymes for efficient decomposition of plant material by fungus-growing termites in tropical ecosystem.

Because of the study of *Termitomyces* under the symbiotic conditions is thought to be useful to understand the mechanism of degradation of dead plant materials by fungus growing termites. The expressed sequence tag (EST) analysis showed partial nucleotide sequences of 1,582 tentative unique transcripts expressed mostly in *Termitomyces*, of which 1,580 sequences including 29 transcripts putatively relevant to plant cell wall degradation were newly identified in this study. A large number of homologs relevant to plant cell wall degradation were identified (Table 5), the most abundant were genes for enzymes involved in pectin degradation. Pectin is a heteropolysaccharide containing mainly homogalacturonan and rhamnogalacturonans with arabinan, galactan and arabinogalactan side chains (Willats *et al*., 2001). It is an abundant polysaccharide in the primary cell wall of plants and in the middle lamellae between primary cell walls where it functions in regulating intercellular adhesion. High expression of pectin-degrading enzymes might be affected by the substrate of the *M. gilvus* fungus comb, which consists mainly of leaves and grasses (Johjima *et al*., 2003b). Further studies on cell-wall-degrading enzymes in fungus comb are necessary to understand the efficient degradation.

The laccase gene *lcc1-2* (NS11F03) was one of the abundant ESTs in the NS library (Table 4) whereas no homologs for lignin or manganese peroxidase were identified in this study. This result is consistent with the result from laccase experiment that laccase was the sole detectable phenol-oxidizing enzyme in the fungus comb of *M. gilvus*.

This study, the phylogenetic study indicated that *Termitomyces* spp. are true symbionts in the termite nests and the symbiotic fungi was not specific to the termites host but rather related to the geographic distribution. To study the function of the symbiotic fungi to the termites, the enzymes activity assay and the EST analysis were performed from both of fungus combs and *Termitomyces* spp. The result showed that laccase activity was detected from *Termitomyces* spp. and fungus combs. Thus, symbiotic fungi may play an important role in termite nests, for example the degradation of phenolic compound by laccase produced by these fungi. In addition, pseudolaccase gene showed highest gene expression in the termite nests but it has no

laccase activity. Its amino acid sequence showed significant similarity with that of *A. bisporus* laccase 2, but it lacked three His residues which required for copper ion binding. Thus, this gene might be encoded other protein or enzyme which may play an important role in termite nests.

Recommendation

The further experiment should be focus on pseudolaccase gene to investigate the function of the encoded protein by enzymatic assay using various parameters such as, substrate and assay condition. Furthermore, the result of EST analysis show more interested gene expressed in the fungus comb especially in NS library including pectin-degrading, cellulose-degrading and hemicellulose-degrading gene homologs which may involved in lignocellulolytic degradation for termite which consumed mainly leave and barks. The measurement of cell wall degradation enzymes activities in fungus combs compare with *Termitomyces* spp. should be important to understand the mechanism of the fungi in the termite nests.

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APPENDICES

Appendix A

The information and the solutions for molecular study

1. Information of the samples

Appendix Table A1 The termite hosts and location for molecular phylogenetic analysis used in this study

Location	Host termite
Prachinburi	*Macrotermes gilvus*, *Microtermes* sp., *Odontotermes* sp.
Saraburi	*Ma. carbonarius*, *Hypotermes* sp., *O. longignathus*, *Odontotermes* sp.
Chanthaburi	*Ma. annandalei*, *Microtermes* sp., *Odontotermes* sp.
Nakronratchasima	*Odontotermes* sp.

Appendix Table A2 The fungal strains used in this study.

Strain name	**JCM No.**	**Termite host**	**Location**
KU418	JCM11089	*Macrotermes gilvus*	Prachinburi
KU419	JCM11153	*Macrotermes gilvus*	Prachinburi
KU435	JCM11101	*Macrotermes gilvus*	Prachinburi
KU436	JCM11102	*Macrotermes gilvus*	Prachinburi
KU437	JCM11103	*Macrotermes gilvus*	Prachinburi
NS/Mg	JCM13351	*Macrotermes gilvus*	PathunThani
KU403	JCM11082	*Microtermes* sp.	Prachinburi
KU410	JCM11086	*Microtermes* sp.	Prachinburi
KU413	JCM11088	*Microtermes* sp.	Prachinburi
KU430	JCM11098	*Microtermes* sp.	Prachinburi
KU438	JCM11104	*Microtermes* sp.	Prachinburi
KU439	JCM11105	*Microtermes* sp.	Prachinburi
KU440	JCM11106	*Microtermes* sp.	Prachinburi
KU432	JCM11100	*Odontotermes* sp.	Prachinburi
KU434	JCM11157	*Odontotermes* sp.	Prachinburi
KU428	JCM11096	*Hypotermes* sp.	Saraburi
KU429	JCM11097	*Hypotermes* sp.	Saraburi
KU444	JCM11110	*Hypotermes* sp.	Saraburi
KU423	JCM11091	*Odontotermes longignathus*	Saraburi
KU424	JCM11092	*Odontotermes longignathus*	Saraburi
KU425	JCM11093	*Odontotermes longignathus*	Saraburi
KU426	JCM11094	*Odontotermes longignathus*	Saraburi
KU446		*Macrotermes gilvus*	Prachinburi

2. Solutions for DNA Extraction

TE (Tris-EDTA) buffer

Trizma-base (Tris [hydroxymethyl] aminomethane)	12.11	g
EDTA·Na_2 (Ethylenediaminetetraacetic acid)	1.86	g

Trizma-base (Tris [hydroxymethyl] aminomethane) was dissolved in 800 ml of MilliQ water and the solution was adjusted to the pH 8.0 by adding concentrated HCl. EDTA·Na_2 (Ethylenediaminetetraacetic acid) were added. The volume of the solution was adjusted to 1 liter with MilliQ water and sterilized by autoclaving 121°C for 15 min.

100 mg/ ml of RNase A

One-hundred mg/ ml of RNase A was prepared by dissolving 100 mg of RNase A in 1 ml of TE (Tris-EDTA) buffer. The solution was stored at –20°C.

3 M sodium acetate (pH 5.2)

Sodium acetate (40.81 g) was dissolved in 60 ml of MilliQ water and adjusted the pH to 5.2 with glacial acetic acid. The volume of the solution was adjusted to 100 ml with MilliQ water. The solution was sterilized by autoclaving at 121°C for 15 min.

3. Solutions for Determination of Extracted DNA Quality by PCR Amplification

50x Tris- Acetate- EDTA (TAE) buffer

Trizma base (Tris (hydroxymethyl) aminomethane)	121	g
EDTA. Na_2 (Ethylenediaminetetraaceticacid)	18.6	g
Glacial acetic acid	28.55	g

The reagents were dissolved and adjusted to 500 ml with MilliQ water. After the solution was autoclaved at 121°C for 15 min, the solution volume was adjusted to 500 ml again with sterile MilliQ water.

1x Tris –Acetate -EDTA (TAE) buffer

The working solution of 1x TAE buffer was prepared from the stock solution, 50x TAE buffer. The 20 ml of 50x TAE buffer was adjusted to 1 liter (1: 50 v/v) with MilliQ water.

Ethidium bromide (EtBr) solution (0.5 mg/ ml)

Five ml of 10 mg/ ml ethidium bromide solution (Nacali Tesque) was adjusted to 100 ml with 1x TAE buffer. The solution was stored in light- tight container at room temperature.

1% and 3% agarose gel

The 1% agarose gel (Nacalai Tesque) in 1x TAE (Tris-Acetate EDTA) buffer was prepared by adding 1 g of powder agarose with <100 ml of 1x TAE. After mixing, the slurry was heated in a microwave oven and then stirred. The volume was adjusted to 100 ml with MilliQ water. The agarose solution was cooled to 45°C and then poured into the mold and a comb was set. The agarose gel should be between 3-5 mm thick and presence of air bubble was avoided. The gel was allowed to set at room temperature for 30 min. After that the comb was carefully removed. 3% agarose gel was prepared with 3 g of powder agarose using the same method.

SOC medium

2% w/v Tryptone	10 mM $MgCl_2$	2.5 mM KCl
0.5% w/v Yeast extract	20 mM $MgSO_4$	

10 mM NaCl 20 mM Glucose

The reagents; tryptone, yeast extract and NaCl were dissolved with 1 liter of deionized distilled water and stirred. After autoclaved at 121 °C for 15 min at 15 lb/ in^2 and cooled at room temperature, the remaining reagents were added and mixed. The solution volume was adjusted to 1 litter with sterile deionized distilled water. The final pH should be 7.0.

4. Media for Bacterial Cultivation and Bacterial Stock

Luria-Bertani (LB) agar plate containing ampicillin (50 mg/ml)

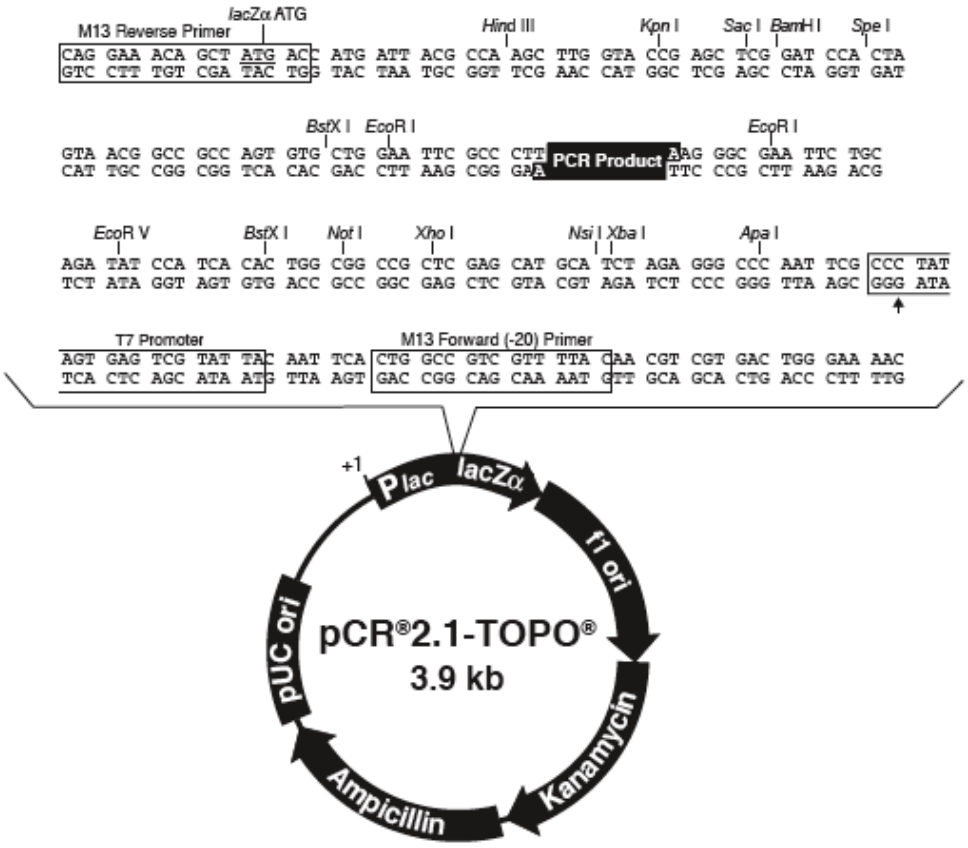
Tryptone	10	g
Yeast extract	5	g
NaCl	5	g
Becto™ agar (Difco)	15	g

The reagents were dissolved in 1 liter of MilliQ water. After mixing, the solution was autoclaved at 121°C for 15 min and cooled to 45°C. Then, it was added with 1 ml of 50 mg/ml ampicillin that prepared by dissolving 5 g of ampicillin (D(-)-α-Aminobenzylpenicillin) (SIGMA) in 10 ml of sterile deionized distilled water and sterilized by filtration through a 0.22-mm membrane filter. The stock solution was stored at –20°C. So, the final ampicillin concentration in the medium was 50 mg/ ml. The medium can be stored at 4°C for up to 1 month.

Appendix B

Plasmids used in this study

pCR®2.1-TOPO Vector of TOPO TA Cloning Kit (Invitrogen)



Comments for pCR®2.1-TOPO®
3931 nucleotides

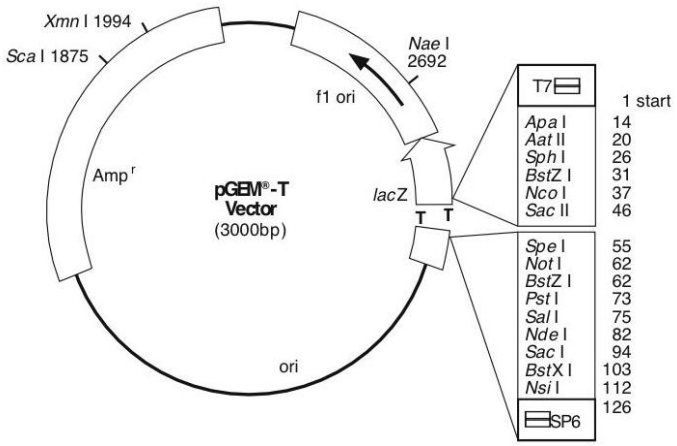
LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Appendix Figure B1 The structure of pCR®2.1-TOPO vector and details of promoter and multiple cloning sequences.

Source: Invitrogen Corporation (2002)

pGEM-T vector (Promega) for cloning

(A)



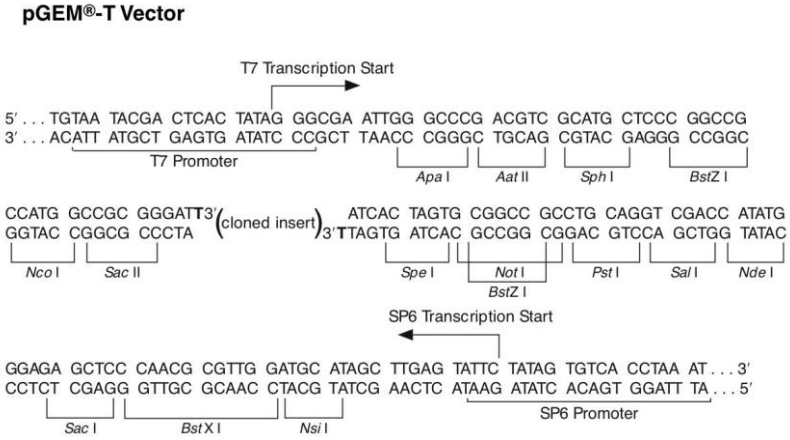
(B)

pGEM®-T Vector sequence reference points:	
T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–113
SP6 RNA polymerase promoter (–17 to +3)	124–143
SP6 RNA polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161–177
*lac*Z start codon	165
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lac operon sequences	2821–2981, 151–380
pUC/M13 Forward Sequencing Primer binding site	2941–2957
T7 RNA polymerase promoter (–17 to +3)	2984–3

Appendix Figure B2 The structure of pGEM-T vector (A), sequence refernce points (B) and the promoter and multiple cloning sequences (C). The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

Source: Promega technical manual for pGEM-T and pGEM-T easy vector systems (1998)

(C)



Appendix Figure B2 (Continued)

Appendix C

Media and reagent for enzyme activities measurement

1. Medium for maintenance of fungal cultures

Potato Dextrose Agar (PDA) (Nissui)

PDA powder 39 g adjust volume to 1 liter with MilliQ water. Autoclaved at 121 °C for 15 min at 15 lb/ in^2.

2. Culture Medium for enzyme activities measurement

Kirk's medium (Tien and Kirk, 1988)

- Stock Reagents

2.1 Basal III medium (per liter):

KH_2PO_4	20 g
$MgSO_4$	5 g
$CaCl_2$	1 g

2.2 Trace element solution (per liter, filter sterilized);

$MgSO_4$	3 g
$MnSO_4$	0.5 g
NaCl	1.0 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CoCl_2$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
$CuSO_4$	0.1 g
$AlK(SO_4)_2 \cdot 12H_2O$	10 mg
H_3BO_3	10 mg
Nitrilotriacetate	1.5 g

Nitrilotriacetate 1.5 g was dissolved with 750 ml MiliQ water, then adjusted pH to 6.5 with 1N KOH or 1N HCl, remaining chemicals were added and mixed and adjust volume to 1000 ml with MiliQ water, then kept at 4 °C.

2.3 10% glucose (glucose 10 g in MiliQ water 100 ml, autoclaved)

2.4 Thiamin (100 mg/liter stock, filter sterilized)

2.5 120 mM Ammonium tartrate (22 g/liter stock)

- Medium Composition (per liter);

Basal III medium 100 ml , 120 mM Ammonium tartrate 10 ml (for low nitrogen source) or 100 ml (for high nitrogen source) and/or agar 15 g were mixed and adjust volume to 790 ml with MilliQ water and autoclaved. After autoclaved at 121 °C for 15 min at 15 lb/ in^2 and cooled at room temperature, 100 ml Trace elements solution, 100 ml of 10% glucose and 10 ml Thiamin were added and mixed.

3. Solutions for enzyme activities measurement

Sodium tartrate pH 3.0-3.5 (200 mM stock)

Sodium tartrate (2.3 g) was used to prepare the stock solution by adding 30 ml of MilliQ water and adjusted pH to 3.0-3.5 with 1N NaOH, then adjust volume to 50 ml. The solution was stored at 4 °C.

Sodium malonate pH 4.5 (500 mM stock)

Sodium malonate (7.4 g) was used to prepare the stock solution by adding 60 ml of MilliQ water and adjusted pH to 4.5 with 1N NaOH, then adjust volume to 100 ml. The solution was stored at 4 °C.

Veratryl alcohol (VA) (50 mM stock)

35.61 µl of veratryl alcohol was adjusted to 5 ml with sterilized MiliQ water. The solution was stored in light- tight container at 4 °C.

H_2O_2 (10 mM stock)

7.75 µl of H_2O_2 was adjusted to 10 ml with sterilized MiliQ water. The solution was stored at 4 °C.

$MnCl_2$ (50 mM stock)

$MnCl_2$ (0.099 g) was used to prepare the stock solution by adding MilliQ water, mixed and adjusted to 10 ml. The solution was stored at 4 °C.

2,6-dimethoxyphenol (DMP) (50 mM stock)

DMP 0.077 g was dissolved in acetone 500 µl and adjusted volume to 10 ml using sterilied MiliQ water. The solution was stored in light- tight container at 4 °C.

Appendix D

Experimental data

Appendix Table D1 The results of the screening for peroxidative fungi from *Termitomyces* strains. Growth and red zone produced by fungi grown on Kirk medium with 1.2 mM Ammonium tertrate for 4 weeks.

Strain name	Growth (cm)	red zone (cm)
KU 403	1.2	2.2
KU 410	1.2	2.4
KU 413	1	2.1
KU 418	1.7	2.3
KU 419	1.9	2.2
KU 423	2.1	2.8
KU 424	1.5	2.6
KU 425	1.4	2
KU 426	1.4	2
KU 428	1.2	1.8
KU 429	1.2	2.1
KU 430	1.6	2.5
KU 432	1.4	1.8
KU 434	1.4	0
KU 435	2.1	1.4
KU 436	1.5	2.1
KU 437	1.6	2.1
KU 438	1.2	2.3
KU 439	1.4	2.4
KU 440	1.3	2.1
KU 444	1.8	2
KU 446	2.1	3

Appendix Table D2 The results of the screening for peroxidative fungi from *Termitomyces* strains. Growth and red zone produced by fungi grown on Kirk medium with 12 mM Ammonium tertrate for 4 weeks.

Strain name	Growth (cm)	red zone (cm)
KU 403	1.3	2.1
KU 410	1.3	2.5
KU 413	1.1	2.3
KU 418	1.8	2.6
KU 419	1.7	2.2
KU 423	1.7	3
KU 424	2.8	0
KU 425	1.3	2.4
KU 426	2.1	2.9
KU 428	1.6	2.6
KU 429	1.3	0
KU 430	1.6	2.5
KU 432	1.8	2.3
KU 434	1.6	0
KU 435	1.8	2.9
KU 436	2	2.5
KU 437	1.8	2.8
KU 438	1.3	2.4
KU 439	1.3	2.1
KU 440	1.2	2.3
KU 444	1.7	0S
KU 446	1.7	2.3

Appendix Table D3 The results of the screening for ligninolytic enzyme production from *Termitomyces* strains. Decolorization of RBBR by the ligninolytic enzyme from *Termitomyces* strains grown on Kirk medium under low nitrogen source condition.

	culture strains and absorbance(nm)																	
days		KU418			KU432			KU446			KU426			KU430			KU428	
	592	500	592/500	592	500	592/500	592	500	592/500	592	500	592/500	592	500	592/500	592	500	592/500
0	0.112	0.029	3.86	0.119	0.032	3.72	0.115	0.033	3.49	0.12	0.032	3.75	0.119	0.032	3.72	0.119	0.032	3.72
2	0.112	0.033	3.39	0.118	0.036	3.28	0.108	0.031	3.48	0.113	0.035	3.23	0.06	0.027	2.22	0.109	0.032	3.41
5	0.114	0.033	3.46	0.123	0.039	3.15	0.112	0.033	3.39	0.107	0.033	3.24	0.031	0.023	1.35	0.086	0.024	3.58
7	0.121	0.04	3.025	0.13	0.046	2.83	0.112	0.035	3.2	0.104	0.029	3.59	0.022	0.021	1.05	0.082	0.03	2.73
9	0.125	0.053	2.36	0.142	0.064	2.22	0.108	0.035	3.27	0.1	0.028	3.57	0.019	0.02	0.95	0.069	0.021	3.3
12	0.08	0.026	3.077	0.107	0.034	3.147	0.103	0.032	3.22	0.084	0.024	3.5	0.01	0.014	0.71	0.066	0.028	2.4
14	0.082	0.028	2.93	0.1	0.031	3.23	0.104	0.032	3.25	0.072	0.023	3.13	0.007	0.012	0.58	0.057	0.023	2.5
16	0.081	0.032	2.53	0.098	0.033	2.97	0.106	0.035	3.03	0.061	0.019	3.2	0.004	0.01	0.4	0.06	0.031	3.83
19	0.052	0.023	2.26	0.086	0.028	3.07	0.103	0.033	3.12	0.05	0.017	2.94	0.007	0.015	0.47	0.052	0.028	1.9
21	0.025	0.02	1.25	0.089	0.035	2.54	0.101	0.036	2.81	0.04	0.009	4.4	0.003	0.011	0.3	0.052	0.032	1.6
23	ND	ND	ND	0.081	0.03	2.7	0.053	0.023	2.3	0.045	0.017	2.65	0.007	0.015	0.5	0.046	0.028	1.6
26	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.045	0.019	2.4	0.009	0.019	0.5	0.043	0.026	1.7
27	ND	ND	ND	0.074	0.029	2.55	0.01	0.013	0.77	ND	ND	ND	ND	ND	ND	ND	ND	ND
28	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.04	0.016	2.5	0.008	0.018	0.4	ND	ND	ND
30	ND	ND	ND	0.072	0.031	2.32	ND	ND	ND	0.04	0.018	2.2	0.007	0.018	0.4	ND	ND	ND
33	ND	ND	ND	ND	ND		ND	ND	ND	0.038	0.015	2.5	0.006	0.016	0.4	ND	ND	ND
34	0.021	0.03	0.7	0.07	0.033	2.12	0.014	0.02	0.7	ND	ND	ND	ND	ND	ND	ND	ND	ND
36	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
37	0.019	0.028	0.68	0.068	0.03	2.27	0.008	0.015	0.53	ND	ND	ND	ND	ND	ND	ND	ND	ND
41	0.011	0.021	0.52	0.06	0.025	2.4	0.003	0.011	0.27	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND= no data

Appendix Table D4 The results of the screening for ligninolytic enzyme production from *Termitomyces* strains. Decolorization of RBBR by the ligninolytic enzyme from *Termitomyces* strains grown on Kirk medium under high nitrogen source condition.

	culture strains and absorbance(nm)																	
days		KU418			KU432			KU446			KU426			KU430			KU428	
	592	500	592/500	592	500	592/500	592	500	592/500	592	500	592/500	592	500	592/500	592	500	592/500
0	0.115	0.034	3.38	0.114	0.034	3.35	0.112	0.032	3.5	0.119	0.033	3.61	0.114	0.032	3.56	0.113	0.032	3.53
2	0.109	0.031	3.52	0.106	0.03	3.53	0.106	0.03	3.53	0.11	0.032	3.44	0.076	0.028	2.71	0.118	0.043	2.74
5	0.111	0.033	3.36	0.112	0.034	3.29	0.107	0.036	2.97	0.107	0.032	3.34	0.049	0.029	1.69	0.07	0.025	2.8
7	0.121	0.038	3.18	0.119	0.041	2.9	0.109	0.036	3.03	0.105	0.034	3.09	0.035	0.027	1.3	0.053	0.029	1.83
9	0.131	0.055	2.38	0.159	0.089	1.79	0.102	0.032	3.19	0.094	0.03	3.13	0.024	0.023	1.04	0.044	0.027	1.63
12	0.04	0.016	2.5	0.07	0.037	1.892	0.101	0.038	2.66	0.049	0.018	2.7	0.019	0.024	0.79	0.045	0.039	1.2
14	0.006	0.008	0.75	0.021	0.017	1.24	0.093	0.032	2.91	0.015	0.01	1.5	0.015	0.024	0.63	0.034	0.029	1.2
16	0.01	0.012	0.83	0.02	0.019	1.05	0.082	0.035	2.34	0.002	0.003	0.67	0.003	0.013	0.23	0.042	0.039	1.1
19	0.007	0.009	0.78	0.019	0.02	0.95	0.008	0.018	0.44	0.006	0.009	0.67	0.003	0.012	0.25	0.042	0.04	1.1
21	0.012	0.014	0.86	0.027	0.028	0.96	0.008	0.017	0.47	0.002	0.004	0.5	-0.002	0.006	-0.3	0.042	0.04	1.1
23	ND	ND	ND	0.025	0.025	1	ND	ND	ND	0.004	0.008	0.5	0.004	0.014	0.29	0.043	0.043	1
26	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.006	0.009	0.7	0.004	0.015	0.27	0.041	0.039	1.1
27	ND	ND	ND	0.027	0.027	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
28	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.007	0.01	0.7	0.005	0.017	0.29	ND	ND	ND
30	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.01	0.013	0.8	0.006	0.018	0.3	ND	ND	ND
33	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.009	0.011	0.8	0.006	0.019	0.3	ND	ND	ND
34	0.016	0.02	0.8	0.027	0.031	0.87	0.016	0.027	0.59	ND	ND	ND	ND	ND	ND	ND	ND	ND
36	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
37	0.012	0.015	0.8	0.029	0.033	0.88	0.009	0.021	0.43	ND	ND	ND	ND	ND	ND	ND	ND	ND
41	0.007	0.011	0.64	0.021	0.026	0.81	0.005	0.017	0.29	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND= no data

Appendix Table D5 The enzymes activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU418 strain isolated from *M. gilvus* in Prachinburi under low (LN) and high (HN) nitrogen conditions.

	enzyme and its activity (Units/ml [(µmole/min.ml)/ml]					
days	LiP, HN	LiP, LN	MnP, HN	MnP, LN	Laccase, HN	Laccase, LN
2	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	0	0	0	0
9	0	0	0	0	0	0
12	0	0	0	0	6.0484E-05	0
14	0	5.3763E-05	0	0	2.7218E-04	2.0161E-06
16	0	5.3763E-05	0	0	3.0242E-04	7.0565E-05
19	0	5.3763E-05	0	0	2.2177E-04	6.0484E-04
21	0	5.3763E-05	0	0	2.2177E-04	1.1391E-03
23	0		0	0	1.7137E-04	1.1190E-03
27	0		0	0	1.6129E-04	1.4113E-03
30	0		0	0	1.3105E-04	1.3609E-03
34	0					1.1089E-04
HN = high nitrogen source medium				LiP = lignin peroxidase activity		
LN = low nitrogen source medium				MnP = manganese peroxidase activity		

Appendix Table D6 The enzymes activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU446 strain isolated from *M. gilvus* in Prachinburi under low (LN) and high (HN) nitrogen conditions.

	enzyme and its activity (Units/ml [(µmole/min.ml)/ml]					
days	LiP, HN	LiP, LN	MnP, HN	MnP, LN	Laccase, HN	Laccase, LN
2	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	0	0	0	0
9	0	0	0	0	0	0
12	0	0	0	0	0	0
14	0	0	0	0	0	0
16	0	0	5.8824E-06	0	1.0081E-05	0
19	0	0	1.1765E-05	0	1.0081E-05	0
21	0	0	1.1765E-05	0	5.0403E-05	0
23	0	0	1.1765E-05	0	7.0565E-05	0
27	0	0	2.9412E-05	0	1.2097E-04	0
30	0	0	1.1765E-05	0	1.2097E-04	0
34	0	0	1.1765E-05	0	8.0645E-05	0
HN = high nitrogen source medium				LiP = lignin peroxidase activity		
LN = low nitrogen source medium				MnP = manganese peroxidase activity		

Appendix Table D7 The enzymes activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU430 strain isolated from *Microtermes* sp. in Prachinburi under low (LN) and high (HN) nitrogen conditions.

	enzyme and its activity (Units/ml [(µmole/min.ml)/ml]					
days	LiP, HN	LiP, LN	MnP, HN	MnP, LN	Laccase, HN	Laccase, LN
2	0	0	-1.0588E-03	0	0	2.0161E-05
5	2.1505E-04	-5.3760E-05	-2.9410E-04	-5.2941E-04	-1.0081E-05	-1.0081E-04
7	-4.8390E-04	-1.6129E-04	0	0	1.0081E-05	0
9	0	0	0	0	-2.5202E-04	-2.0161E-04
12	7.5269E-04	-5.3760E-05	0	-1.4706E-03	-1.0081E-05	-1.0081E-05
14	-5.3760E-05	1.0753E-04	-5.8820E-05	-8.8235E-04	2.0161E-05	0
16	-4.3010E-04	-1.6129E-03	0	-1.7647E-03	1.0081E-04	0
19	1.6129E-04	1.0753E-04	-1.7650E-04	5.8824E-05	-2.7218E-03	1.0081E-05
21	-2.1505E-03	-1.0753E-04	-5.8820E-05	0	1.0081E-05	1.0081E-05
23	5.3763E-05	1.6129E-04	-8.8240E-04	4.7059E-04	5.0403E-05	2.0161E-05
26	5.3763E-05	-2.6882E-04	-2.6471E-03	-2.3529E-04	1.0081E-04	1.0081E-05
28	0	0	-3.5290E-04	-5.8824E-05	0	4.0323E-05
30	0	2.6882E-04	-3.5294E-03	5.8824E-05	0	0
33	0	-5.3760E-05	-1.4706E-03	-5.8824E-05	0	1.0081E-05
HN = high nitrogen source medium				LiP = lignin peroxidase activity		
LN = low nitrogen source medium				MnP = manganese peroxidase activity		

Appendix Table D8 The enzymes activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU432 strain isolated from *Odontotermes* sp. in Prachinburi under low (LN) and high (HN) nitrogen conditions.

	enzyme and its activity (Units/ml [(µmole/min.ml)/ml]					
days	LiP, HN	LiP, LN	MnP, HN	MnP, LN	Laccase, HN	Laccase, LN
2	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	0	0	0	0
9	0	0	0	0	7.0565E-05	0
12	0	0	0	0	6.0484E-05	0
14	0	0	0	0	1.0081E-04	1.0081E-05
16	0	0	0	5.8824E-05	1.4113E-04	2.0161E-05
19	0	0	0	0	1.0081E-04	2.0161E-05
21	0	0	0	5.8824E-05	9.0726E-05	4.0323E-05
23	0	0	0		5.0403E-05	6.0484E-05
27	0	0	0		3.0242E-05	7.0565E-05
30	0	0	0		3.0242E-05	8.0645E-05
34	0	0	0		5.0403E-05	1.0081E-05
HN = high nitrogen source medium				LiP = lignin peroxidase activity		
LN = low nitrogen source medium				MnP = manganese peroxidase activity		

Appendix Table D9 The enzymes activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU426 strain isolated from *Odontotermes longignathus* in Saraburi under low (LN) and high (HN) nitrogen conditions.

	enzyme and its activity (Units/ml [(µmole/min.ml)/ml]					
days	LiP, HN	LiP, LN	MnP, HN	MnP, LN	Laccase, HN	Laccase, LN
2	0	0	1.1765E-04	-7.6471E-04	-1.0081E-05	0
5	-8.0650E-04	-2.6882E-03	5.8824E-05	-2.3529E-03	2.0161E-04	0
7	-1.5054E-03	-1.2903E-03	-1.7647E-04	5.8824E-04	-1.0081E-05	0
9	-2.9570E-03	-2.4194E-03	-5.8824E-05	5.8824E-05	-5.0403E-04	6.5524E-04
12	-1.6130E-04	5.3763E-05	0	3.5294E-04	2.0161E-05	0
14	0	-1.6129E-04	0	4.1177E-04	2.0161E-05	0
16	5.3760E-05	5.3763E-05	5.8824E-05	5.8824E-05	2.0161E-05	0
19	5.3760E-05	0	-1.1765E-04	0	1.0081E-04	0
21	-5.3760E-05	0	7.6471E-04	0	4.0323E-05	-1.0081E-05
23	5.3760E-05	0	5.8824E-05	5.8824E-04	1.0081E-04	1.0081E-05
26	0	-5.3763E-04	-1.0588E-03	-1.7647E-04	2.0161E-05	-1.0081E-05
28	-1.6130E-04	0	3.5294E-04	-1.7647E-04	2.0161E-05	0
30	1.0750E-04	5.3763E-05	6.4706E-04	-1.1765E-04	2.0161E-05	0
33	0	-1.6129E-04	-5.8824E-05	0	-2.0161E-05	-5.0403E-05
HN = high nitrogen source medium				LiP = lignin peroxidase activity		
LN = low nitrogen source medium				MnP = manganese peroxidase activity		

Appendix Table D10 The enzymes activities (LiP, MnP and Laccase) in the culture supernatant of *Termitomyces* sp. KU428 strain isolated from *Hypotermes* sp. in Saraburi under low (LN) and high (HN) nitrogen conditions.

	enzyme and its activity (Units/ml [(µmole/min.ml)/ml]					
days	LiP, HN	LiP, LN	MnP, HN	MnP, LN	Laccase, HN	Laccase, LN
2	0	-2.6882E-03	2.9412E-04	0	-2.0160E-04	7.5605E-04
5	-3.2260E-04	-1.0750E-04	0	5.8824E-04	0	2.0161E-05
7	-5.3760E-05	-9.6770E-04	0	5.8824E-04	0	-2.0160E-05
9	-2.1510E-04	-5.9140E-04	-8.8240E-04	-5.8820E-05	0	0
12	1.0753E-04	-2.9570E-03	-4.7060E-04	4.1176E-04	1.0081E-05	0
14	-5.3760E-05	0	-2.3530E-04	1.1765E-04	-1.0080E-05	-1.0080E-05
16	2.1505E-04	-5.9140E-04	-5.2941E-03	-5.8820E-04	-5.0400E-05	1.0081E-05
19	0	5.3763E-05	-1.2941E-03	5.8824E-04	5.0403E-05	1.0081E-05
21	0	-4.3010E-04	2.3529E-04	0	2.0161E-05	3.0242E-05
23	-1.0750E-04	-5.3760E-05	2.3529E-04	0	-3.0240E-05	0
26	-1.0750E-04	0	9.4118E-04	-5.8820E-05	0	-2.5200E-04
HN = high nitrogen source medium				LiP = lignin peroxidase activity		
LN = low nitrogen source medium				MnP = manganese peroxidase activity		

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PUBLICATIONS :

Taprab Y., M. Ohkuma, T. Johjima, Y. Maeda, S. Moriya, T. Inoue, P. Suwanarit, N. Noparatnaraporn and T. Kudo. 2002. Molecular Phylogeny of Symbiotic Basidiomycetes of Fungus-growing Termites in Thaialand and Their Relationship with the Host. **Biosci. Biotechnol. Biochem.** 66 (5): 1159-1163.

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Note

Molecular Phylogeny of Symbiotic Basidiomycetes of Fungus-growing Termites in Thailand and Their Relationship with the Host

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***Termitomyces*-related symbiotic basidiomycetes in the nests of fungus-growing termites (Macrotermitinae) of several genera in Thailand were cultivated and analyzed phylogenetically based on the DNA sequence of nuclear ribosomal RNA genes. The relationships of the symbiotic fungi with host termites and their locality were apparently complex, supporting intricate mechanisms for the termites to acquire the symbionts.**

Key words: symbiosis; termite; basidiomycete; *Termitomyces*; phylogeny

Termites of the subfamily Macrotermitinae, so-called fungus-growing termites, have a sophisticated and highly efficient symbiotic relationship with fungi. Fungus-growing termites are abundant in Asian and African tropics and have a great impact on the decomposition of dead plant material in those ecosystems.[1,2] The symbiotic fungi are responsible for the decomposition. In the nests of the termites, the symbiotic fungi grow on a sponge-like structure (called a fungus comb) constructed by the termites from litter. They are found as mycelia and white round structures (called fungus nodules) on the fungus comb surface. Both the fungi and the fungus comb are consumed by the termites. The symbiotic fungi have been proposed to play a ligninolytic role to improve digestibility of cellulose for the termites,[3,4] to supply cellulase and xylanase which work synergistically with endogenous enzymes,[5–7] and to concentrate nutrients, particularly nitrogen, for the termites.[8]

In some cases, mushrooms appear on the termite nests in a particular season. These mushrooms are unique in nature, blooming only from the termite nests, and are commercially fascinating due to their prized edibility. They have been placed within the genus *Termitomyces* (Basidiomycota, Agaricales, Tricholomataceae),[9] and twelve species of *Termitomyces* from southeast Asia have been described.[10] Since these *Termitomyces* mushrooms have long pseudorhiza which connects to the surface of the fungus comb, they have been considered to be symbiotic fungi found on the fungus comb as mycelia and fungus nodules. To our knowledge, however, there has been no report about developing the fruiting body from mycelia of the symbiotic fungi in a laboratory. Moreover, the fruiting body has never found associated with some species of fungus-growing termites. Since mycelia give poor information about the taxonomy based on morphology, molecular sequences are expected to be useful to identify the symbiotic fungi grown on the fungus comb. Also, a reliable phylogeny of the symbiotic fungi is important in order to understand the symbiotic relationship with termites. In this study, we cultivated symbiotic fungi from the fungus combs of fungus-growing termites of several genera in Thailand. We analyzed the fungal symbionts based on nuclear ribosomal DNA sequences consisting of internal transcribed spacers (ITS1 and ITS2), 5.8S rDNA, and partial large subunit (LSU) rDNA. We compared the phylogeny of symbiotic fungi in relation to the termite hosts and their locality.

The termite hosts and their locations for the sampling are listed in Table 1. The sampling sites were located at distances of at least 70 km to one another in Thailand. For the samplings, we used a single fungus comb in a nest in each case except for one (we cultivated two strains from different nests in the case of *Macrotermes annandalei* in Khao Kitchagoot). Fungus-nodules on fungus combs were carefully picked up with using sterile forceps, rinsed with an about 0.6% sodium hypochlorite solution, washed with sterile water, and cultivated on Potato-Dextrose-Agar (Nissui) in darkness at room temperature.

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Table 1. Number of the Strains among the 38 *Termitomyces*-related Basidiomycetes in Each of the Eight Phylogenetic Groups

location	Host termite	Group 1	2	3	4	5	6	7	8
Prachinburi	*Macrotermes gilvus*					5			
Prachinburi	*Microtermes* sp.		6			1			
Prachinburi	*Odontotermes* sp.					5			
Saraburi	*Macrotermes carbonarius*	5							
Saraburi	*Hypotermes* sp.			3					
Saraburi	*Odontotermes longignathus*				6				
Saraburi	*Odontotermes* sp.				2				
Khao Kitchagoot	*Macrotermes annandalei*[*]					1			1
Khao Kitchagoot	*Microtermes* sp.							1	
Khao Kitchagoot	*Odontotermes* sp.		1						
Nakronratchasima	*Odontotermes* sp.						1		

[*] The two strains were from different nests of this termite species. In the other cases, strains of each termite host were from only one fungus comb.

From four out of five genera of fungus-growing termites found in Thailand, a total of 39 strains were successfully cultivated, which included one strain cultivated from the fruiting body of *Termitomyces* sp. (inoculated with the tissue of the internal part of the stipe) blooming from the nest of *Odontotermes* sp. in Nakronratchasima. The strains cultured in this study have been deposited in the Japan Collection of Microorganisms under the accession numbers JCM11082-JCM11106, JCM11110, JCM11115, and JCM11153-JCM11164.

Mycelia were grown for three weeks and their DNA was extracted using an ISOPLANT kit (Nippon Gene). The DNA region consisting of ITS1, 5.8S rDNA, ITS2, and partial LSU rDNA was amplified by PCR with rTaq DNA polymerase (Toyobo). The PCR primers used were ITS5[11] and LR7.[12] The PCR condition was for 35 cycles at 94°C for 30 sec, 55°C for 45 sec, and 72°C for 2 min. The PCR products (2.1 kbp) were purified using a Wizard PCRpreps DNA purification system (Promega). The purified PCR products were used for either direct DNA sequencing as templates or cloning into a pGEM-T vector (Promega). Plasmid DNA of the clones was firstly analyzed by restriction fragment length polymorphism with *Hae*III and *Hha*I, confirming that the clones from a single strain were equivalent to one another. Among the strains, we could not detect any heterokaryons, which, if present, would have given heterogeneity of the DNA sequence. Then, the DNA sequence of a representative clone from each strain was analyzed by automated sequence analyzers (ABI model 3700 and 377) using the sequencing primers ITS2, ITS3, ITS4, ITS5,[11] LROR, LR3, LR3R, LR5, and LR16.[12] The nucleotide sequence data found in this study will appear in the databases under accession numbers AB073496-AB073545 and AB073739.

The DNA sequence data were aligned using the CLUSTAL W package[13] and checked manually. Nucleotide positions of ambiguous alignments were omitted from the subsequent phylogenetic analyses. The programs implemented in PHYLIP 3.5c (distributed by Felsenstein, J., Department of Genetics, University of Washington, Seattle) were used to infer the neighbor-joining and the parsimony trees and to obtain bootstrap confident estimates. The program PUZZLE 4.0[14] was used with 10,000 puzzling steps to infer the quartet-puzzling maximum likelihood tree.

Comparison of the sequence similarity showed that 38 strains from the fungus-nodules and from the fruiting body of *Termitomyces* sp. were significantly related. Among the 38 strains, the DNA sequence of the ITS1-5.8S rDNA-ITS2 region (530–645 bp) showed more than 72% nucleotide identity to one another. Based on the comparison of the ITS1-5.8S rDNA-ITS2 region, the 38 strains were classified into eight groups. Within each group, the DNA sequences showed more than 99% nucleotide identity to one another. Strains between the groups 4 and 5 showed approximately 97% nucleotide identity to each other, whereas those between the other groups showed less than 90% nucleotide identity to one another. The grouping was supported by phylogenetic analyses of this DNA region (data not shown), in which the branching order of the groups was not strongly supported except for the clustering of groups 1 and 2, and that of groups 4 and 5. The DNA sequence of at least 330 bp of the LSU rDNA region was analyzed in all the 38 strains and showed more than 93% nucleotide identity to one another. The comparison of the LSU rDNA region indicated that the strains within each group showed more than 99% nucleotide identity to one another, being consistent with the grouping based on the ITS regions. However, this DNA region was too similar to distinguish the grouping in some cases. The strains between groups 1 and 2, and those between groups 4 and 5, showed more than 99% nucleotide identity to each other, respectively.

The longer LSU rDNA region (1.2–1.4 kbp) of at least one representative of each of the eight groups was analyzed for their DNA sequences. All the representatives of the eight groups were closely relat-

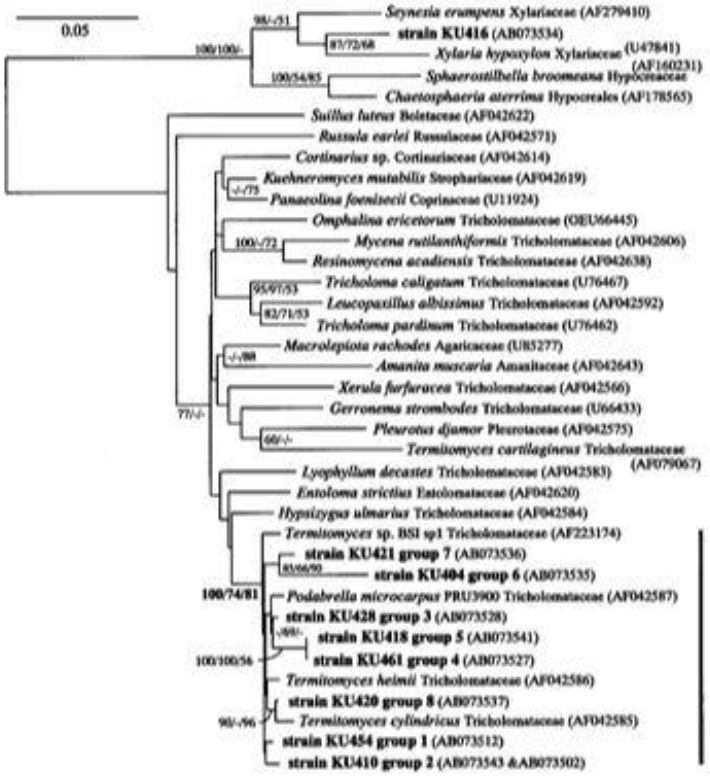

Fig. 1. Phylogenetic Positions of the Strains Symbiotic with Fungus-Growing Termites.

The tree was inferred by the neighbor-joining method based on the LSU rDNA sequence. The bootstrap values for the neighbor-joining and parsimony methods and percent occurrence in the puzzling-steps (divided by slashes, in this order) are shown at node when they are above 50%. The bar indicates 0.05 nucleotide substitutions per position. The cluster consisting of *Termitomyces* and the basidiomycete strains cultivated in this study are indicated by the vertical bar on the left. The fungal family are described after the name of species. In the case of *Chaetosphaeria aterrima* (class Hypocreales), the family is unclassified. The database accession number is given in parenthesis.

ed to one another, showing more than 92% nucleotide identity. The LSU rDNA sequences were compared with the representative basidiomycetes' DNA sequences of Agaricales available in the databases. The phylogenetic analyses of the LSU rDNA indicated that all the eight groups identified from the termite nests were clustered together with *Termitomyces heimii, Termitomyces cylindricus*, and *Termitomyces* sp. strain BSI sp1. This cluster also contained *Podabrella microcarpus* strain PRU3900 (Tricholomataceae). The close relationship between *Termitomyces* and *Podabrella* has already been established by molecular phylogenetic analysis,[12] and in fact, the same strain has recently appeared as *Termitomyces microcarpus* in the database (AF357023). The clustering was fully supported by the statistical analyses, showing 100% and 74% bootstrap values for the neighbor-joining and the parsimony methods, respectively, and 81% occurrence in the quartet-puzzling steps. The results suggest that all the 38 strains are *Termitomyces*-related basidiomycetes and confirm that *Termitomyces* are true symbionts which grow on the fungus comb as fungus nodules. The eight groups probably represent species or subspecies of this genus. Close relationships were found in the cases between groups 4 and 5, between groups 6 and 7, and between group 8 and *T. cylindricus*, which were supported by the statistical analyses, respectively.

Recently, several DNA sequences from fungus nodules in some termite nests have been appeared in the databases (AB051879-AB051890 [Kato, H. *et al.*] and AF357023 [Hofstetter, V. *et al.*]). These DNA sequences of the ITS1-5.8S rDNA-ITS2 region were clearly related to those of the *Termitomyces*-like strains cultivated in this study, but they formed dis-

tinct groups from the eight groups identified here.

Among the 39 strains analyzed in this study, 38 strains were related to *Termitomyces*. The only exception was strain KU416, cultivated from *Odontotermes* sp. in Khao Kitchagoot. The LSU rDNA analysis clearly indicated that this strain was affiliated to Ascomycota of the family Xylariaceae that includes the genus *Xylaria*. The LSU rDNA of some members of this family showed the highest sequence identity, around 92%, with this strain. The ITS1-5.8S rDNA-ITS2 analysis (data not shown) also supported the closer relationship with members of the family Xylariaceae. It has been reported that *Xylaria*-like fungi inhabit the fungus comb as mycelia.[15] However, they never produce spores or stroma until the fungus comb is removed from the nest or termites abandon the nest. As discussed previously,[15] the presence of termites probably prevents *Xylaria*-like fungi from proliferating on the fungus comb. Since cultivation often introduces some biases, we are now investigating the composition of the fungal inhabitants on the fungus comb without cultivation of them.

Relationships of the *Termitomyces*-related fungi cultivated from the fungus comb with their host termites were not simple. The groups 2 and 5 of the symbiotic fungi were identified from the termites of different genera. Also, the group 4 was identified from the different species of *Odontotermes*. Some species of *Termitomyces*, such as *T. eurhizus* and *T. medius*, are reported as symbionts of several species of more than two genera of termites, respectively.[16] The symbionts are thought to be generalists rather than specialists with respect to the relationship with their hosts. From a single genus of the termite, more than two groups of the symbiotic fungi were identified when the termites of multiple species or multiple locations were examined. From the genus *Microtermes*, for example, the symbionts of groups 2, 5, and 7 were identified. Also, from the genus *Odontotermes*, those of groups 2, 4, 5, and 6 were identified. The choice of the symbionts by the hosts, at least at the genus level of the termites, is not fixed to a single group of the symbionts. Furthermore, it is noted that a single fungus comb harbored two different groups of the symbiotic fungi in the case of *Microtermes* sp. in Prachinburi. In the case of *Macrotermes annandalei* in Khao Kitchagoot, two groups were obtained from the different nests.

The locality of the host termites seemed to affect the choice of the symbionts because the groups of the symbiotic fungi obtained in a single sampling site were limited, at least in Prachinburi and Saraburi. In Prachinburi, only the groups 2 and 5 were found. In the case of two *Odontotermes* species in Saraburi, only the group 4 was found. However, more than two groups were identified from a single location in each of the three locations where the multiple termite genera were investigated. In the cases of groups 2 and 5 of the symbionts, they were identified from both Prachinburi and Khao Kitchagoot. Thus, the locality alone is not crucial for the selection of the symbionts, and some *Termitomyces* lineages of the symbionts are dispersed across the locations.

Probably, these complex phylogenetic relationships between the host termites and their symbiotic fungi are reflected by the methods to acquire the symbionts during the establishment of a new colony of the termites. Two methods are discussed to have evolved within fungus-growing termites.[17,18] One is that the symbiont is carried over by reproductive alates of the termites, and the other is the collection of the symbiont by foraging workers of the termites in the early stage of colony foundation. The apparently complex relationships cannot be explained if the only one of the two methods is used by the termites. More extensive study is necessary to clarify what kinds of methods are used by each termite species, taking differences of their behavior prominently into account. The DNA sequences reported in this study can be the basis for such further study.

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Symbiotic Fungi Produce Laccases Potentially Involved in Phenol Degradation in Fungus Combs of Fungus-Growing Termites in Thailand†

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Fungus-growing termites efficiently decompose plant litter through their symbiotic relationship with basidiomycete fungi of the genus *Termitomyces*. Here, we investigated phenol-oxidizing enzymes in symbiotic fungi and fungus combs (a substrate used to cultivate symbiotic fungi) from termites belonging to the genera *Macrotermes*, *Odontotermes*, and *Microtermes* in Thailand, because these enzymes are potentially involved in the degradation of phenolic compounds during fungus comb aging. Laccase activity was detected in all the fungus combs examined as well as in the culture supernatants of isolated symbiotic fungi. Conversely, no peroxidase activity was detected in any of the fungus combs or the symbiotic fungal cultures. The laccase cDNA fragments were amplified directly from RNA extracted from fungus combs of five termite species and a fungal isolate using degenerate primers targeting conserved copper binding domains of basidiomycete laccases, resulting in a total of 13 putative laccase cDNA sequences being identified. The full-length sequences of the laccase cDNA and the corresponding gene, *lcc1-2*, were identified from the fungus comb of *Macrotermes gilvus* and a *Termitomyces* strain isolated from the same fungus comb, respectively. Partial purification of laccase from the fungus comb showed that the *lcc1-2* gene product was a dominant laccase in the fungus comb. These findings indicate that the symbiotic fungus secretes laccase to the fungus comb. In addition to laccase, we report novel genes that showed a significant similarity with fungal laccases, but the gene product lacked laccase activity. Interestingly, these genes were highly expressed in symbiotic fungi of all the termite hosts examined.

Fungus-growing termites (subfamily, *Macrotermitinae*) are distributed throughout tropical Africa and Asia, where they are the dominant soil invertebrates (1, 41). The *Macrotermitinae* have a highly efficient system for digesting plant litter due to their symbiotic relationship with basidiomycete fungi of the genus *Termitomyces* (order, *Agaricales*; family, *Tricholomataceae*). These termites have a great impact on plant litter decomposition and carbon cycling in tropical ecosystems (42, 43). For example, Buxton (5) demonstrated that fungus-growing termites consumed 90% of the dry woody litter in an arid tropical area of Kenya.

Because of their unique characteristics, such as symbiosis with *Termitomyces* fungi and sophisticated division of labor, the *Macrotermitinae* have been the subject of extensive research (for reviews, see references 8, 31, and 42). Fungus-growing termites cultivate symbiotic fungi in their nest on a special substrate composed of dead plant material known as the fungus comb or fungus garden. In most *Macrotermes* species, termite workers ingest dead plant material and deposit undigested or partially digested feces on the top rim of the fungus comb. Thus, there is an age gradient within the fungus comb. As the fungus comb ages, decolorization from the top to the bottom of the fungus comb is observed. After a certain period, the aged part of the fungus comb is eaten by the host termites (42). Several roles of symbiotic fungi have been proposed, including the provision of glycosyl hydrolases (26), enrichment of nitrogen (6, 27), and lignin degradation (14, 19, 30), with the significance of each role apparently varying in importance among host termite species (20, 31).

Previously, we demonstrated that water-soluble phenolic compounds in the fungus comb of *Macrotermes gilvus* were degraded during fungus comb aging (21). Higher plants synthesize and accumulate a variety of phenolic compounds as secondary metabolites. Although physiological functions of plant phenolic compounds are not yet fully understood, it is thought that they contribute to plant defenses against pests and pathogens, and therefore, they also influence the decomposition of plant litter by microorganisms in the detritus food chain (33). Consequently, phenol degradation in the fungus comb is considered to be important for improving palatability of termite food, especially that containing high phenol content such as fallen leaves and bark.

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The ability of white rot basidiomycetes to degrade a variety of aromatic compounds, such as lignin (23) and aromatic pollutants (29), has been extensively studied. It has been shown that the extracellular phenol-oxidizing enzymes lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), and laccase (EC 1.10.3.2) are responsible for the depolymerization of lignin (13, 23). Lignin peroxidase catalyzes the oxidation of various aromatic compounds to form aryl cation radicals (23) while manganese peroxidase oxidizes Mn(II) to Mn(III), which diffuses from the enzyme and oxidizes various phenolic compounds. These enzymes require hydrogen peroxide for their activities. Laccase also catalyzes the oxidation of various phenolic compounds and aromatic amines, but this reaction is coupled with the reduction of molecular oxygen to water. Because of their broad substrate specificities, they can be also involved in the degradation of a variety of plant phenols. Although Mora and Lattaud (28) reported the presence of the oxidation activity of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and syringaldazine in the fungus combs of several fungus-growing termites in Africa, and although those authors considered that these reactions were catalyzed by laccase, there is no molecular evidence for the presence of laccase in the fungus comb, and there is no information on the other phenol-oxidizing enzymes such as the peroxidases described above. To better understand phenol degradation in the fungus comb and the contribution of symbiotic fungi to its degradation process, further studies on phenol-oxidizing enzymes in the fungus comb are required.

In this study, we investigated phenol-oxidizing enzymes in fungus combs of fungus-growing termites in Thailand. Laccase was the sole detectable phenol-oxidizing enzyme, and laccase cDNA sequences were identified directly from the fungus combs, showing the distribution and diversity of laccase genes in symbiotic fungi. We also found laccase-like sequences that did not encode laccase and that were highly expressed in fungus combs.

MATERIALS AND METHODS

Fungus combs, microbial strains, and culture conditions. The fungus combs of five fungus-growing termites were collected from May 2000 to October 2001 in Thailand. The termite hosts and locations sampled in this study were *M. gilvus*, *Microtermes* sp. and *Odontotermes* sp. from the Prachinburi Province, *Odontotermes longignathus* and *Hypotermes* sp. from the Saraburi Province, and *M. gilvus* from the Pathum Thani Province. The sampling locations were separated from each other by at least 50 km. The fungus combs were stored at −80°C until use. *Termitomyces* strains described previously (34) (JCM accession no. 11082, 11086, 11088, 11089, 11091 to 11094, 11096 to 11098, 11100 to 11106, 11110, 11153, and 11157) and strain NS/Mg (JCM accession no. 13351), which was newly isolated from a fungus comb of *M. gilvus* in the Pathum Thani Province, were maintained on a potato dextrose agar medium (0.4% potato extract, 2% glucose; Nissui) at 30°C in our laboratory (a complete list of the strains examined in this study is given in Table S1 of the supplemental material). The partial rRNA gene sequence of strain NS/Mg was determined as described previously (34) and submitted to the DDBJ database (accession no. AB202123). The partial large-subunit rRNA gene sequence (1.2 kbp) of this strain was closely related to that of *Termitomyces* sp. group 1 (accession no. AB073514), showing 94% nucleotide identity. For determination of enzyme activity, *Termitomyces* spp. strains were also cultured in KB liquid medium (1% glucose, 1.2 mM [low nitrogen {LN}] or 12 mM [high nitrogen {HN}] ammonium tartrate, and 100 ml of Kirk's basal III mineral medium [37] per liter). Four mycelial plugs (4 mm) from each of the *Termitomyces* strains grown on potato dextrose agar medium were transferred to a 500-ml flask containing 120 ml of KB liquid medium under LN and HN conditions and cultured with agitation (120 rpm) at 28°C.

Plate assay of phenol-oxidizing enzyme. *Termitomyces* spp. strains were incubated at 30°C for 4 weeks on KB agar medium containing 0.01% guaiacol under HN and LN conditions. A brownish pigment was observed if the fungi produced a phenol-oxidizing enzyme(s).

Enzyme activity. The activities of phenol-oxidizing enzymes in fungus combs and in a liquid culture of *Termitomyces* spp. were examined. The fungus comb was gently ground using a mortar and pestle. Approximately 0.1 g of the ground comb was added to a 1.5-ml polypropylene tube containing 1 ml MilliQ water and subsequently mixed. The supernatant was recovered by centrifugation (10,000 × *g* for 5 min at 4°C), and enzyme activities were determined. Lignin peroxidase and manganese peroxidase activities were measured as described previously (37, 39) using 3,4-dimethoxybenzyl alcohol and Mn(II) as substrates, respectively. Laccase activity was measured spectrophotometrically by following the oxidation of 2,6-dimethoxyphenol (DMP) or ABTS in sodium citrate at pH 5.0 and 4.5, respectively. The oxidation rates of DMP and ABTS were determined using a $\Delta\varepsilon_{469}$ of 49.6 mM^{-1} cm^{-1} and a $\Delta\varepsilon_{415}$ of 36.0 mM^{-1} cm^{-1}, respectively (39, 40).

RNA extraction. Poly(A)$^+$ RNA was extracted from the fungus comb that was ground into a fine powder with a mortar and pestle under liquid nitrogen. Approximately 0.1 g of this fungus comb powder was then transferred to a polypropylene tube containing 0.2 ml of extraction buffer (4 M guanidine thiocyanate, 0.1 M Tris-HCl, pH 7.5, 1% 1,4-dithiothreitol, 0.5% lauroylsarcosine). After mixing thoroughly, 0.6 ml of dilution buffer (0.1 M Tris-HCl, pH 7.5, 0.4 M LiCl, and 20 mM EDTA) was added. The supernatant containing RNA was separated from the debris by centrifugation (17,000 × *g*). Poly(A)$^+$ RNA was isolated from the supernatant using an Oligotex mRNA kit according to the manufacturer's protocol (Takara). Total RNA was extracted from a 23-day-old *Termitomyces* culture (strain KU418) according to the method described previously by Han et al. (15), and poly(A)$^+$ RNA was extracted with an Oligotex mRNA kit. First-strand cDNA was synthesized from 0.1 μg (fungus comb samples) or 1 μg (strain KU418) of poly(A)$^+$ RNA using Superscript II (Invitrogen) and poly(T) primer 5′-TTTACCTCTTCAGC$(T)_{16}$-3′ at 42°C for 50 min and subjected to PCR.

Isolation of the laccase genes and cDNA. Degenerate primers, primer 1 (5′-GGMACSTTCTGGTAYCAY-3′) and primer 2 (5′-CCRTGCARRTGGAAKGGRTG-3′), targeting a copper binding domain of basidiomycete laccases, were designed to amplify laccase-like sequences from the fungus comb and *Termitomyces* sp. strain KU418. PCR was carried out using a PTC-200 thermocycler (MJ Research) and Ex-*Taq* (Takara). Thermal cycling consisted of 35 cycles with an initial denaturation step at 94°C for 30 s, an annealing step at 50°C for 30 s, and an extension step at 72°C for 1 min. PCR products (ca. 900 bp) were purified from 1% agarose gel using a Minelute gel extraction kit (QIAGEN) before being ligated into the pGEM-T vector (Promega) to construct laccase cDNA libraries from five fungus combs and *Termitomyces* sp. strain KU418. Approximately 30 clones of each library were randomly selected, and their insert DNA sequences were amplified by PCR using a universal primer set of the vector and sorted into groups by restriction fragment length polymorphism (RFLP) using either HaeIII or HhaI. The DNA sequences of representative clones from each library were determined using M13 forward and reverse primers on a DNA sequencer (ABI model 377) and a BigDye Terminator cycle sequencing kit (Applied Biosystems).

Full-length cDNA sequences of *lcc1-2* and *lcc2-5* were determined using rapid amplification of cDNA ends (RACE)-PCR. Primer 3 (5′-GGTGCAAAGCAGCCTCAAGATCT-3′) and primer 4 (5′-TGGAACATCAATAATGTTTCGTAC-3′) were used for amplification of the 3′-end sequences of *lcc1-2* and *lcc2-5*, respectively. The PCR conditions for *lcc1-2* were 35 cycles of 94°C for 30 s followed by 65°C for 1.5 min. The PCR conditions for *lcc2-5* were 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min. For the amplification of the 5′ end, reverse transcription reactions for *lcc1-2* and *lcc2-5* were performed with primer 5 (5′-TACCAGTCGGCAAGAG-3′) and primer 6 (5′-ATGCTTGAGTTGTGCC-3′), respectively, using a 5′ RACE-PCR kit version 2 (Gibco BRL). The PCR conditions were 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. PCR products from the 3′ and 5′ RACE-PCR were cloned and sequenced as described above.

The coding regions of the *lcc1-2* and *lcc2-5* genes were amplified from genomic DNA extracted from the *Termitomyces* sp. strain NS/Mg using an Isoplant kit (Nippon Gene). The 5′- and 3′-flanking regions of the *lcc1-2* and *lcc2-5* genes were obtained using inverse PCR (38) with primer 7 (5′-CCTCAAGATCTCCTTCCTTCT-3′) and primer 8 (5′-AGATCCTTCAGAGGATCGTTC-3′) for *lcc1-2* and primer 9 (5′-TCTGTCATTGAGGTCGACTTC-3′) and primer 10 (5′-TAAATGAGCCGAAGGATCTTCG-3′) for *lcc2-5*. Genomic DNA (0.5 μg) from *Termitomyces* sp. strain NS/Mg was digested with either PstI or EcoRI for the *lcc1-2* and *lcc2-5* amplifications, respectively. PCR conditions were 30 cycles of 94°C for 20 s and 67°C for 8 min or 5 min for *lcc1-2* and *lcc2-5*, respectively.

TABLE 1. Laccase activity in fungus combs

Termite species (location)	Activity (μkat/g of comb)[a]
M. gilvus (Prachinburi)	32.6
M. gilvus (Pathum Thani)	33.3
Odontotermes sp. (Prachinburi)	9.58
Microtermes sp. (Prachinburi)	5.15

[a] The data shown are mean values of duplicate experiments.

PCR products were purified using a PCR purification kit (QIAGEN) and analyzed by direct sequencing.

Semiquantitative analysis of gene expression. In order to estimate expression levels of laccase genes semiquantitatively, we optimized PCR conditions by changing parameters related to the PCR cycle, annealing temperature, and extension time (reference 17 and references therein). The concentrations of the first-strand cDNA and primers 1 and 2 were 0.1 ng/μl and 0.5 μM, respectively. Thermal cycling consisted of an initial denaturation step at 95°C for 2 min followed by 18 or 20 cycles of 95°C for 30 s, 50°C for 1 min, and 72°C for 3 min and, subsequently, a final extension step at 72°C for 10 min. Concentrations of PCR products were estimated by electrophoresis on an agarose gel stained with SYBR green I using Molecular Imager FX (Bio-Rad). Approximately 0.2 to 0.3 nM of PCR products (ca. 900 bp) was obtained. PCR products were purified using a Minelute PCR purification kit (QIAGEN) and cloned into a pCR 2.1 vector using a TOPO TA cloning kit (Invitrogen). Ninety-six randomly chosen clones from the library were sorted by comparing RFLP. Expression levels were expressed as the clone abundance (percent) of each RFLP group.

Phylogenetic analysis. Taking PCR error into consideration, clones isolated from a sample with more than 99% nucleotide sequence identity were considered sufficiently similar and were grouped together. Representative sequences were used for phylogenetic analysis. Based on sequence alignment and the "GT-AG" rule, some sequences appeared to contain introns. In these cases, putative introns were manually removed and subsequently used for further analyses. All of the sequences were checked for the presence of chimera using the Bellerophon server (http://foo.maths.uq.edu.au/~huber/bellerophon.pl) (18). No chimera sequences were found. A similarity search was conducted by using BLASTX (2) against the nonredundant protein database (May 2005). The protein sequences were aligned using ClustalX version 1.8 (36). A neighbor-joining tree was constructed using the MEGA package with a PAM matrix (24). The sampling variance of the distance values was estimated from 1,000 bootstrap resamplings of the alignment columns. The signal peptide sequence was predicted using the SignalP program (3). GENETYX version 10.1 (Software Development) was used to calculate sequence identity.

Partial purification of laccase from the fungus comb. Approximately 100 g of *M. gilvus* fungus comb from Pathum Thani was gently ground with 1.0 liter of distilled water by using a mortar and pestle. Laccase was precipitated by ammonium sulfate at 65% saturation and collected by centrifugation (10,000 × *g*, 30 min). The precipitate was dissolved in approximately 60 ml of MilliQ water. The fungus comb of *M. gilvus* contains considerable amounts of dissolved organic matter (DOM) (21). DOM appeared to contain acidic macromolecular compounds and interfered with enzyme purification. To remove the DOM, 0.8 g of DEAE-Sephadex A50 powder (Amersham) was added to the crude fungus comb extract. Most of the laccase did not bind to the DEAE-Sephadex powder under these conditions. The solution separated from Sephadex was dialyzed against 10 mM sodium acetate (pH 4.0). After 16 h, the solution was adjusted to pH 6.0 and subsequently applied to a column (1.5 by 10 cm) of DEAE-Toyopearl (Tosoh) equilibrated with 10 mM phosphate (pH 6.0). The column was washed with the starting buffer until all of the DOM had been removed. Elution with 20 mM sodium acetate (pH 4.0) resulted in 77% laccase recovery in this step. The enzyme solution was then applied to a Superdex 200HR 10/30 column (Amersham) with a 50 mM sodium acetate–0.15 M ammonium sulfate buffer (pH 4.0) at a flow rate of 0.25 ml/min. The enzyme was further purified using a HiTrap-phenyl Sepharose HP column (Amersham). A sample containing 2 M ammonium sulfate was applied to the column and eluted using a linear gradient of 1.5 to 0.7 M ammonium sulfate in 100 mM acetate (pH 4.0). The fractions exhibiting laccase activity were pooled and reapplied to the same column a total of four times. The N-terminal amino acid sequences were determined using an automated sequencer (ABI model 494cLC).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the DDBJ database under the following accession numbers: AB201126 to AB201165.

TABLE 2. Inhibitory effect of chemicals on laccase activity from *M. gilvus* comb in Pathum Thani[a]

Inhibitor	Concn (mM)	Inhibition (%)[b]		
		Fungus comb	*P. cinnabarinus*[c]	*C. hirsutus*[c]
DTT[d]	1.0	100	100	100
NaN_3	0.1	97	100	100
EDTA	5.0	11	0[e]	0[e]

[a] DMP was used as a substrate.
[b] Zero inhibition refers to 16 nkat of laccase activity.
[c] Laccases from *Pycnoporus cinnabarinus* and *Coriolus hirsutus* (12).
[d] DTT, dithiothreitol.
[e] EDTA, 4.0 mM.

RESULTS

Phenol-oxidizing enzyme activity in the fungus comb. We examined a series of phenol-oxidizing enzymes, laccase, lignin, and manganese peroxidases in the fungus combs of *Microtermes* sp., *Odontotermes* sp., and *M. gilvus*. Of these enzymes, only laccase activity was detected in all of the fungus combs examined (Table 1). The laccase activity monitored by DMP oxidation was not affected by the addition of H_2O_2 (data not shown), indicating the absence of horseradish peroxidase type enzyme in the fungus comb because horseradish peroxidase can oxidize DMP in the presence of H_2O_2. Inhibitory effects of several chemicals on the laccase activity in the fungus comb of *M. gilvus* were similar to those on laccases from known basidiomycete isolates (Table 2). The laccase activity was also significantly inhibited in a nitrogen atmosphere (data not shown), indicating that oxygen is required for this activity. The optimum pH for DMP oxidation was pH 5.0.

Laccase activity from *Termitomyces* strains. The ability of the symbiotic fungi to produce phenol-oxidizing enzymes was examined in 22 *Termitomyces* strains from fungus combs of various termite species (34) using a plate assay method. Phenol-oxidizing activity was observed under both HN and LN

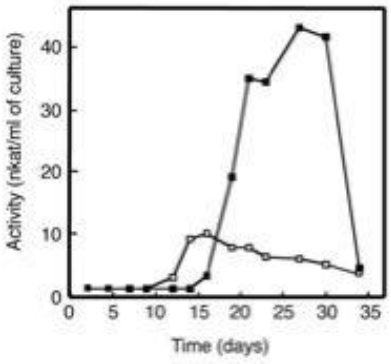

FIG. 1. Laccase activity in the culture supernatant of *Termitomyces* sp. strain KU418 isolated from *M. gilvus* under low-nitrogen (closed squares) and high-nitrogen (open squares) conditions. The reaction mixture contained 0.5 mM DMP and 100 μl of culture supernatant in 50 mM sodium citrate, pH 5.0.

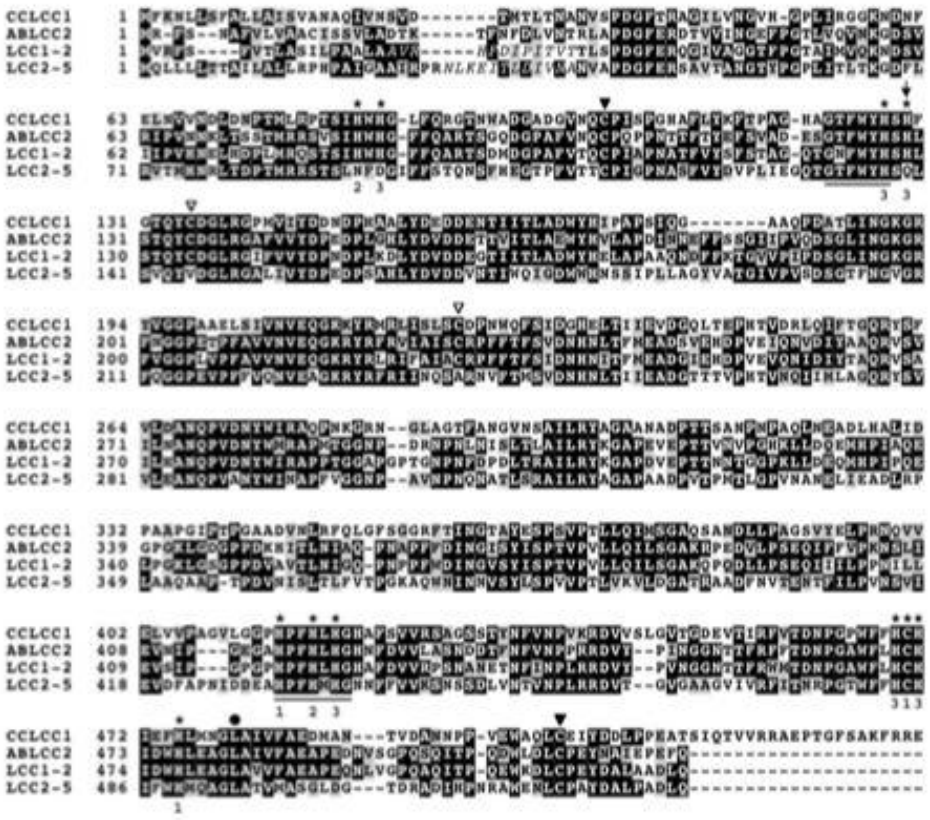

FIG. 2. Sequence comparison among *C. cinerea* laccase 1 (CCLCC1) (DDBJ accession no. AAD30964), *A. bisporus* laccase 2 (ABLCC2) (accession no. Q12542), LCC1-2, and LCC2-5. Identical and similar amino acids are shaded. The conserved amino acid residues potentially involved in copper ion binding are marked by asterisks, and position 129 of *A. bisporus* laccase 2 is also marked with an arrow. Numbers under the His and Cys residues indicate types of copper ions that bind to each residue. The amino acid sequences that are used to design the degenerate primers are underlined. The Cys residues that form disulfide bonds in *C. cinerea* laccase 1 are marked with closed (Cys 103-Cys 505) and open (Cys 135-Cys 222) inverted triangles (9). A closed circle indicates the Leu residue that influences a laccase redox potential. Experimentally determined N-terminal amino acid sequences of LCC1-2 and LCC2-5 are italicized.

conditions in most of the strains assayed (data not shown), except for two strains from *Hypotermes* sp. and one strain from *O. longignathus* that only exhibited the activity under LN conditions. No activity was observed from one *Odontotermes* sp. strain under any condition. The strains that exhibited higher activity were cultured in KB liquid medium, and the series of phenol-oxidizing enzymes were examined. Only laccase activity was found, and no lignin or manganese peroxidase activities were detected in any strain. As shown in Fig. 1, significant laccase activity was detected under LN conditions in strain KU418 that was isolated from the fungus comb of *M. gilvus*. Weak laccase activities (less than 6.7 nkat/ml) were also detected in other strains isolated from the fungus combs of *Hypotermes* sp. (strain KU428), *Microtermes* sp. (strain KU430), *Odontotermes* sp. (strain KU432), and *O. longignathus* sp. (strain KU426).

Cloning and characterization of putative laccase genes. In order to identify laccase genes expressed in the fungus comb, reverse transcription (RT)-PCR was carried out on RNA extracted from the fungus comb of *M. gilvus* from Pathum Thani with degenerate primers targeting the highly conserved copper binding domains II and III (Fig. 2). RT-PCR products of an appropriate size (ca. 900 bp) were obtained and cloned. The sequences of the clones showed a significant similarity with fungal laccases. The full-length cDNAs of two representative clones (*lcc1-2* and *lcc2-5*) were isolated from the fungus comb RNA. The corresponding gene sequences were also identified from genomic DNA of the symbiotic fungal strain NS/Mg isolated from the same fungus comb. Comparison between the genomic and cDNA sequences revealed that the coding regions of genomic *lcc1-2* and *lcc2-5* were interrupted by 23 and 21 introns and encoded 524 and 534 amino acids, respectively.

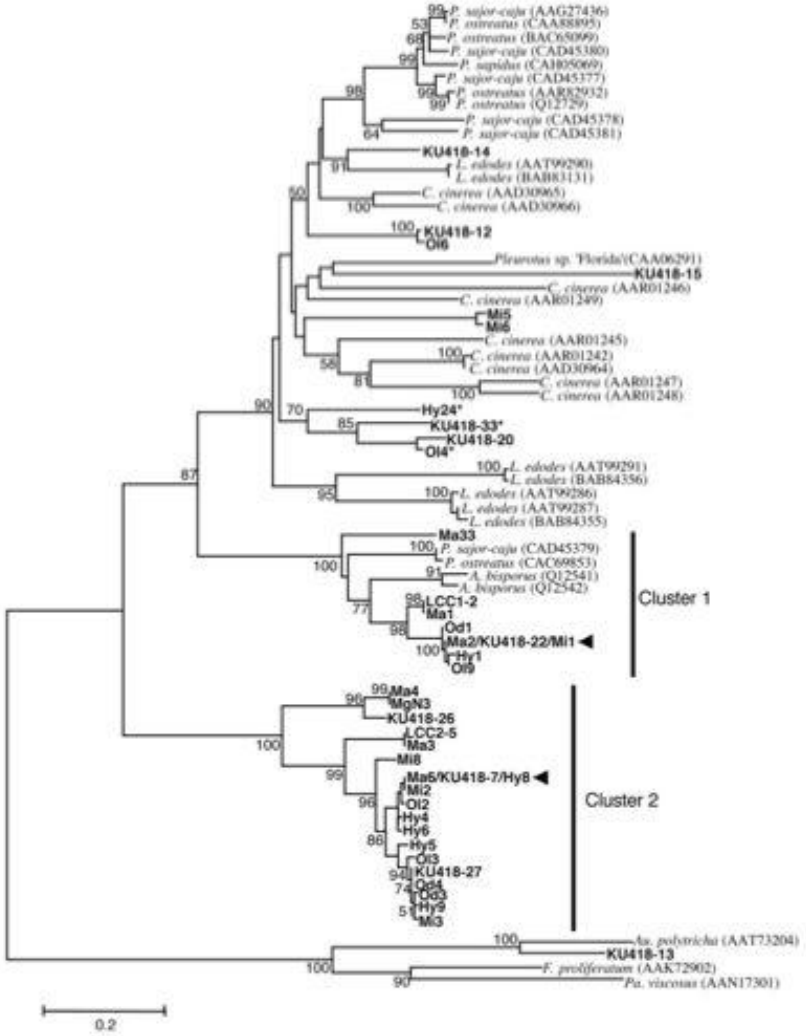

FIG. 3. Neighbor-joining tree for amino acid sequences of putative laccases identified from the fungus combs and *Termitomyces* sp. strain KU418 and related proteins. The scale bar represents 0.2 amino acid substitutions per position. Accession numbers are shown after the names of organisms. Bootstrap confidence values greater than 50 are indicated at the nodes. Branches containing identical sequences are indicated with arrowheads. Clone designations and genus abbreviations are as follows: MaX, *M. gilvus* in Prachinburi; MgN3, *M. gilvus* in Pathum Thani; OdX, *Odontotermes* sp.; MiX, *Microtermes* sp.; OlX, *O. longignathus*; Hy, *Hypotermes* sp.; KU418-X, *Termitomyces* sp. strain KU418, where X means number; *P.*, *Pleurotus*; *L.*, *Lentinula*; *C.*, *Coprinopsis*; *A.*, *Agaricus*; *Au.*, *Auricularia*; *F.*, *Fusarium*; *Pa.*, *Panorbis*. Asterisks indicate the clones that contain a putative intron(s).

Deduced amino acid sequences from the *lcc1-2* and *lcc2-5* cDNAs contained 6 and 10 potential N-glycosylation sites (N-X-S/T), respectively. Putative signal peptides were detected from the first Met to Ala 18 of LCC1-2 and to Gly 21 of LCC2-5, respectively. The LCC1-2 amino acid sequence contained all of the amino acid residues that are essential for copper ion binding in laccase, whereas the copper binding domains in LCC2-5 were incomplete (Fig. 2). The LCC2-5 amino acid sequence lacked 3 of 11 amino acid residues involved in copper ion binding. Two disulfide bonds, Cys 103-Cys 505 and Cys 135-Cys 222, were found in the crystal structure of laccase 1 from *Coprinus cinerea* (9). The equivalent four Cys

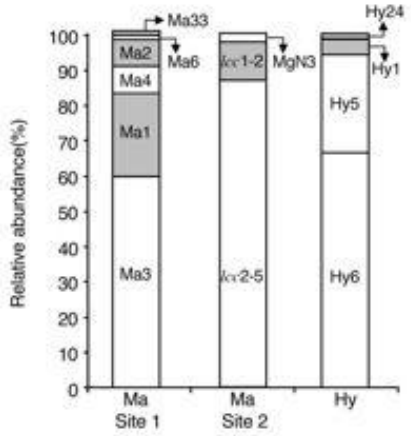

FIG. 4. Relative abundance of cDNA clones from the fungus combs of *M. gilvus* in Prachinburi (site 1), *M. gilvus* in Pathum Thani (site 2), and *Hypotermes* sp. in the Saraburi Province. Clone designations are shown in the legend of Fig. 3. The sequences possessing a conserved His residue at position 129 of *A. bisporus* laccase 2 are shaded.

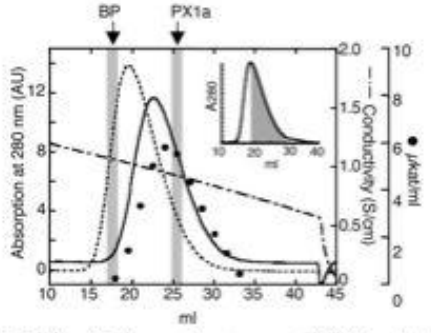

FIG. 5. Phenyl Sepharose chromatogram of BP (broken line) and PX1a (solid line). The fractions indicated with shaded squares were subjected to N-terminal amino acid sequencing. The closed circles indicate laccase activity of PX1a fractions. The inset shows a typical chromatogram of PX1 on Phenyl Sepharose. The fractions with laccase activity are shaded. Further details are described in Results. AU, absorbance units.

residues were also conserved in the LCC1-2 sequence, while one set of Cys residues was present in the LCC2-5 sequence. A BLAST search showed that best hits for LCC1-2 and LCC2-5 amino acid sequences were laccase 1 (Q12541) and laccase 2 (Q12542), respectively, from the basidiomycete *Agaricus bisporus*. Amino acid identities and similarities between LCC1-2 and laccase 1 were 66 and 76%, while those between LCC2-5 and laccase 2 were 47 and 62%, respectively.

Laccase cDNA fragments from various fungus combs. We have thus far demonstrated that our primer set designed here was capable of amplifying laccase cDNA from the fungus comb of *M. gilvus*. Therefore, laccase cDNAs in the fungus comb of different termites were analyzed using the same method. RT-PCR products with expected sizes were obtained from all the fungus comb samples. A total of 71 sequences were identified from the fungus combs of five termite species and from strain KU418 grown in KB liquid culture (Fig. 1). Of these, 69 sequences showed significant similarities to basidiomycete laccases (BLAST scores and E values ranged from 441 to 234 and 1e–122 to 2e–60, respectively). Termination codons were found in putative open reading frames of two sequences from the fungus comb of *Odontotermes* sp. and one sequence from strain KU418, and these were not analyzed further. In addition to the *lcc1-2* and *lcc2-5* genes, the numbers of distinct cDNA sequences eventually identified were six from *M. gilvus*, three from *Odontotermes* sp., and six from *Microtermes* sp. in Prachinburi; seven from *Hypotermes* sp. and five from *O. longignathus* in Saraburi; one from *M. gilvus* in Pathum Thani; and nine from strain KU418.

Phylogenetic analysis placed most of the putative laccase amino acid sequences into two major clusters (clusters 1 and 2) (Fig. 3). All of the sequences from the *M. gilvus* fungus combs from Prachinburi and Pathum Thani were found in these clusters, while five sequences identified from *Microtermes* sp., *Hypotermes* sp., and *O. longignathus* (Mi5, Mi6, Hy24, Ol4, and Ol6) were placed outside these clusters and appeared more closely related to the laccases of known basidiomycetes. All of the sequences in cluster 2 lacked the His residue that is essential for type 3 copper binding (position 129 in *A. bisporus* LCC2 [shown in Fig. 2]), while other clones possessed His at that position. Among 10 sequences from strain KU418, 3 sequences were found in cluster 2. Sequences that were identical to those of KU418-22 and KU418-7 were identified from the fungus combs (Fig. 3). The phylogenetic position of sequence KU418-13 was close to that of the ascomycete laccases, although a laccase from the basidiomycete *Auricularia polytricha* also clustered in the same group as the closest relative.

The relative abundance of putative laccase sequences in each clone library was analyzed semiquantitatively to estimate expression levels of laccase genes in the fungus comb (Fig. 4). The most abundant clones found in all the three libraries were grouped together in cluster 2. Except for the sequences in cluster 2, Ma1, *lcc1-2*, and Hy1 were the most abundant sequences from *M. gilvus* in Prachinburi, *M. gilvus* in Pathum Thani, and *Hypotermes* sp. in Saraburi.

Partial purification of laccase from the fungus comb. Crude enzyme from the fungus comb of *M. gilvus* from Pathum Thani was separated into two fractions containing laccase activity using a DEAE-Toyopearl column. One fraction (PX1) that was eluted with 20 mM sodium acetate (pH 4.0) contained laccase activity that was three times higher than that of the other fraction that was eluted with a salt gradient. Although sodium dodecyl sulfate-polyacrylamide gel electrophoresis of PX1 gave a single band with a molecular mass of 75 kDa, analysis of the N-terminal amino acid sequence indicated that this band contained two proteins. PX1 was further purified using gel filtration and subsequent hydrophobic-interaction chromatography. When PX1 was subjected to a Phenyl Sepharose column, a broad single peak appeared, where laccase activity was found in the shaded part of the peak (Fig. 5, inset), indicating

that PX1 consisted of laccase and nonlaccase proteins. The fractions with laccase activity were pooled, and the chromatographic separation with the same column was repeated. Figure 5 shows the chromatograms of the separated laccase and nonlaccase fractions (designated PX1a and BP, respectively) after chromatographic runs were performed four times. The purified BP fraction did not exhibit the oxidation activities for ABTS, DMP, potassium ferrocyanide, and *o*- and *p*-catechol under the laccase assay conditions. The N-terminal amino acid sequence of BP was determined to be NLKEITLDIVNA. Since the PX1a fraction still contained BP protein, the N-terminal amino acid sequence of the protein with laccase activity was estimated to be AVRHFDIPXTVT by subtracting the BP amino acid sequence. This protein is tentatively called PX1a in this paper. The N-terminal amino acid sequences of PX1a and BP were found in the deduced amino acid sequences of LCC1-2 and LCC2-5, respectively (Fig. 2). Thus, it is highly likely that the gene *lcc1-2* encodes laccase PX1a and that *lcc2-5* is not a laccase gene.

DISCUSSION

A number of phenol-oxidizing enzymes have been characterized in basidiomycete fungi by culturing them in the laboratory. However, to our knowledge, this is the first detailed molecular study of such an enzyme characterized directly in a natural environment without cultivation. We consider the naturally occurring condition of *Termitomyces* very important in order to understand the real nature of the symbiotic relationship with the host termite and their efficient decomposition of plant litter. Here, we clearly demonstrated that laccase was the sole detectable phenol-oxidizing enzyme in the fungus combs of *Microtermes* sp., *Odontotermes* sp., and *M. gilvus*. No peroxidase activity was detected in either the fungus comb or culture supernatants of *Termitomyces* spp. strains, although many white rot basidiomycetes produce extracellular peroxidases. A detailed analysis of the fungus comb of *M. gilvus* from Pathum Thani and its symbiotic fungus revealed that the *lcc1-2* gene of *Termitomyces* sp. strain NS/Mg encodes laccase PX1a and that this is the dominant isozyme under symbiotic conditions. The LCC1-2 amino acid sequence contains all of the conserved His and Cys residues required for copper ion binding and an additional conserved Leu residue affecting the redox potential of laccase (Fig. 2). According to the classification of Eggert et al. (10), LCC1-2 belongs to the class 2 laccases that have a moderate redox potential (0.71 to 0.47 V) (25). A Met or Phe residue is located at the same position in the class 1 or 3 laccase sequence. All the results found in this study indicated that laccase in the fungus comb of *M. gilvus* had the common characteristics of laccase in its catalytic properties and primary structure.

In addition to the *lcc1-2* laccase gene, we identified a novel gene, *lcc2-5*, and its gene product, BP, from the fungus comb of *M. gilvus*. The discovery of the BP protein was attributed to its chromatographic behavior, which was markedly similar to that of laccase PX1a. The LCC2-5 amino acid sequence showed significant similarity with that of *A. bisporus* laccase 2, but it lacked three His residues required for copper ion binding in the deduced amino acid sequence (Fig. 2), and the gene product, BP, also lacked laccase activity. The laccase molecule has four copper ions distributed among three sites, each of which is defined according to its spectroscopic properties (32). The T1 site contains the type 1 blue copper that is responsible for absorption at around 600 nm. The T2 site contains a type 2 copper with a characteristic electron paramagnetic resonance. In the T3 site, the pair of strongly coupled type 3 coppers is electron paramagnetic resonance silent in the presence of dioxygen. The mononuclear T1 site extracts an electron from a reducing substrate and mediates its transfer to the trinuclear T2/T3 center where molecular oxygen is reduced. However, as shown in Fig. 2, while LCC2-5 (BP protein) possesses the necessary residues for type 1 copper binding, absorption at around 600 nm was not observed (data not shown), suggesting a distortion of the tertiary structure of a potential T1 site in LCC2-5.

Putative cDNA fragments of laccase were amplified using the degenerate PCR primers from the fungus combs of various termite hosts. Their deduced amino acid sequences showed similarity with those of fungal laccases, but some of the sequences had Glu or Gln residues at the site of the conserved His residue corresponding to His 129 of *A. bisporus* laccase 2. Phylogenetic analysis showed that all of these sequences were placed in cluster 2 and that they formed a lineage distinct from those of basidiomycete laccases (Fig. 3). LCC2-5 was also found in cluster 2, suggesting that while the sequences in cluster 2 are closely related to laccase genes, they do not most certainly encode "true" laccase. Interestingly, these pseudolaccase cDNA sequences were identified from all of the fungus combs tested in this study. Amino acid identities among these pseudolaccases ranged from 61 to 100%. Also, the transcription levels of the pseudolaccase genes were much higher than those of the putative laccase genes (Fig. 4). Expressed sequence tag analysis for symbiotic fungus of *M. gilvus* is in progress in our laboratory, and the sequence corresponding to *lcc2-5* was one of the abundant sequences (T. Johjima, unpublished data). These findings suggest that the pseudolaccase genes are nonetheless essential for either the symbiotic fungi themselves or symbiosis with the host termites. Although laccase gene sequences from plants and bacteria have been deposited in public databases, their phylogenetic positions were distinct from those of fungal laccase clusters (data not shown), suggesting that the putative laccase and laccase-like cDNA sequences from the fungus combs were certainly of fungal origin.

Multiple laccase genes are often found in single organisms. The saprophytic fungus *C. cinerea*, for example, has eight different laccase genes that were reported previously (16) and one additional gene in the public databases. *Termitomyces* sp. strain KU418 expressed seven putative laccase genes when the fungus was cultured in KB liquid medium under LN conditions. These sequences showed higher diversity than those found in either the *C. cinerea* laccases or the sequences from the *M. gilvus* fungus comb that were found only in cluster 1 (Fig. 3). In *M. gilvus* from Prachinburi, only one identical sequence set (Ma2 and KU418-22) was retrieved from the clone library of the fungus comb and strain KU418, although four times more clones were sequenced from the KU418 clone library than from the fungus comb library. This finding could possibly be attributed to differences in culture conditions between the fungus comb and KB medium, because the dif-

ferential expression of fungal laccase genes is often found depending upon nutritional conditions, copper ion concentrations (references 4 and 7 and references therein), and the presence of various aromatic compounds (35). It is unlikely that more than one species of symbiotic fungi are associated with a fungus comb, and consequently, the multiple laccase genes were identified, since Katoh et al. (22) demonstrated no genetic variation in symbiotic fungus from a single large nest of the termite *Odontotermes formosanus*. We have also analyzed fungal community structures in the fungus combs of *Odontotermes* sp., *Microtermes* sp., and *M. gilvus* from Prachinburi, showing that single or closely related *Termitomyces* species almost exclusively grew on each fungus comb (S. Moriya, unpublished data). These findings support the idea that *Termitomyces* fungi have multiple laccase genes.

Putative functional laccase cDNA fragments that clustered outside cluster 2 were identified from the fungus combs of all the termite hosts examined in this study, indicating that laccase is widely distributed among the symbiotic fungi of fungus-growing termites. Phylogenetic analysis showed that the laccase sequences from the fungus comb and *Termitomyces* sp. strain KU418 were closely related to the laccases from *A. bisporus*, *L. edodes*, *Pleurotus* spp., and *C. cinerea*. Like the *Termitomyces* fungi, these fungi belong to the order *Agaricales* and mostly produce class 2 laccases. Previously, we analyzed phylogenetic relationships among *Termitomyces* fungi used in this study, except for strain NS/Mg (34), and showed that fungal strains isolated from *O. longignathus* (Saraburi Province), *Odontotermes* sp., and *M. gilvus* (Prachinburi Province) were closely related and shared more than 99% nucleotide identity in partial sequences (1.2 to 1.4 kbp) of the large-subunit rRNA genes. In the laccase phylogeny, specific relationships were not found among laccase homologs from those symbiotic fungi. It is uncertain whether this is due to differential expression of multiple laccase genes in each fungus comb as seen in the *M. gilvus* fungus comb and strain KU418 or each symbiotic fungus possessing individual laccase genes. Comprehensive analyses of laccase genes in those symbiotic fungi would be required to answer the question.

Hyodo et al. (20) analyzed the chemical composition of fungus combs from *Odontotermes* spp., *Hypotermes makhamensis*, *Ancistrotermes pakistanicus*, *Pseudacanthotermes militaris*, and four *Macrotermes* spp. and found that lignin was preferentially degraded compared to carbohydrates in only *Macrotermes* fungus combs. Those authors noted that the *Macrotermes* species examined in their study tended to use leaf litter for construction of the fungus comb, while the other genera, with the exception of *P. militaris*, appeared to use predominantly wood, and therefore, the different material of the fungus comb possibly affects lignin degradation in fungus combs. In this study, laccase activity was detected and higher laccase activity was found in the fungus combs of *M. gilvus* than in *Odontotermes* sp. and *Microtermes* sp. (Table 1). Does the higher laccase activity promote preferential lignin degradation in this genus? This study provides insufficient information on lignin degradation because laccase is basically incapable of oxidation of nonphenolic lignin moieties that comprise up to 85% of the lignin polymer because of the low redox potential of laccase. However, it is known that laccase can oxidize nonphenolic compounds and degrade lignin in the presence of a laccase mediator such as 3-hydroxyanthranilate (11). Thus, laccase mediators must be studied to estimate the contribution of laccase to lignin degradation in the fungus comb of the genus *Macrotermes*. Further functional studies on laccase and laccase-like protein from the symbiotic fungi are necessary to clarify the importance of these enzymes for efficient decomposition of plant material by fungus-growing termites in tropical ecosystems.

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GENOMICS AND PROTEOMICS

Large-scale identification of transcripts expressed in a symbiotic fungus (*Termitomyces*) during plant biomass degradation

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Abstract Fungus-growing termites have a symbiotic relationship with the basidiomycetes of the genus *Termitomyces*. This symbiotic system is able to degrade dead plant material efficiently. We conducted expressed sequence tag (EST) analysis of a symbiotic *Termitomyces* fungus degrading plant material in a field nest of the termite *Macrotermes gilvus*. A subtractive cDNA library was also investigated to facilitate the discovery of genes expressed specifically under the symbiotic conditions. A total of 2,613 ESTs were collected and resulted in 1,582 nonredundant tentative consensus sequences, of which approximately 59% showed significant similarity to known protein sequences. A number of homologous sequences to genes involved in plant cell wall degradation were identified and a majority of them encoded putative pectinolytic enzymes. Real-time quantitative reverse transcriptase polymerase chain reaction analyses confirmed significant upregulation of putative stress response genes under symbiotic conditions. The present ESTs database provides a valuable resource for molecular biological study of plant material degradation in the symbiosis between termites and fungi.

Keywords Fungus-growing termite · *Termitomyces* · Symbiosis · Expressed sequence tag · Lignocellulose degradation

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Introduction

Biomass is recognized as a renewable resource for the production of bioenergy and biomaterial (Ragauskas et al. 2006). However, developing new technologies for efficient conversion of biomass would be required to substitute for petroleum-based energy and chemicals. Thus, catabolism and utilization of biomass in nature are of great interest because of their sophisticated mechanisms and safety and mildness to the environment.

Termites belonging to subfamily Macrotermitinae cultivate basidiomycete fungi of the genus *Termitomyces*. The fungus-growing termites are distributed throughout tropical and subtropical areas in Africa and Asia. They play a significant role in the decomposition of dead plant materials and have a great impact on carbon cycling in tropical ecosystems (Abe and Matsumoto 1979; Wood and Sands

1978). In particular, the contribution of *Termitomyces* fungi on carbon mineralization is very large (Yamada et al. 2005).

Termitomyces fungi are cultivated on a special substrate called a fungus comb in the termite nest. In most termites of the genus *Macrotermes*, the fungus comb is made from their primary feces composed of almost undigested dead plant matter (Wood and Thomas 1989). After the growth of *Termitomyces*, the host termites feed on the mature parts of the fungus comb and aggregated asexual spores, called fungal nodules, for their nutrition. Dead plant materials are thought to be degraded efficiently and strongly in the symbiotic system because fungus-growing termites, unlike many other termites, do not produce final feces rich in organic matter (Rouland-Lefèvre and Bignell 2001). Thus, the symbiotic fungi seem to be responsible for the efficient decomposition of plant materials by fungus-growing termites.

Several roles of *Termitomyces* in the decomposition of plant materials were proposed, including the provision of glycosyl hydrolases for the termite hosts (Martin and Martin 1978; Matoub and Rouland 1995) and lignin (phenol) degradation during fungus comb maturation (Grassé and Noirot 1958; Hyodo et al. 2000; Johjima et al. 2003; Taprab et al. 2005). However, the mechanisms are not fully understood and basic information on their biological activities under the symbiosis, especially at the molecular level, is also very limited. This is most probably due to the difficulty of developing and maintaining a colony and/or a nest of fungus-growing termites under laboratory conditions. Thus, we have employed modern molecular biological approaches to study *Termitomyces* without rearing termites in the laboratory, and have characterized the genes encoding laccases and laccase-related proteins that are expressed in *Termitomyces* under the symbiotic conditions (Taprab et al. 2005).

Recently, expressed sequence tag (EST) analyses were applied for fungal species (Skinner et al. 2001). Because this method is demonstrated to be rapid and cost-effective for gene discovery and for estimating approximate gene expression levels, it is considered advantageous particularly for the study of organisms with little or no genetic research history, such as *Termitomyces*. We previously demonstrated that *Termitomyces* is almost the sole fungus growing on a fungus comb in any of several termite species examined, and that genetically unique species of *Termitomyces* occurs predominantly on fungus combs in a nest (Moriya et al. 2005). Therefore, it is expected that the most information obtained from the EST analysis of the fungus comb is derived from a single *Termitomyces* species.

In this study, we constructed a cDNA library from RNA extracted directly from a naturally occurring fungus comb of *Macrotermes gilvus* in Thailand and presented an overview of the EST database. Because we have established mycelial pure cultures of *Termitomyces* (Taprab et al. 2002, 2005), a subtractive cDNA library was also constructed and investigated to screen the genes specifically expressed in *Termitomyces* under the symbiotic conditions. Levels of gene expression under the symbiotic and cultured conditions were also examined in some cases by real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Aspects of polysaccharide decomposition and utilization in the fungus comb were discussed based on the present results. This study is the first report for large-scale identification of fungal transcripts that expressed during plant material degradation.

Materials and methods

Fungus comb, fungal strain, and culture media

Fungus combs of *M. gilvus* were collected from an orchard in Pathum Thani Province, Thailand (June 2001). Termites were completely removed from the fungus combs, which were immediately frozen with liquid nitrogen in the field. The fungus combs were stored at −80°C until used. *Termitomyces* sp. strain NS/Mg (JCM13351), previously isolated from a fungus comb in the same termite nest (Taprab et al. 2005), was maintained on potato dextrose agar (PDA; 0.4% potato extract, 2% glucose; Nissui, Tokyo, Japan) at 30°C. The strain was also cultured on KB medium (Taprab et al. 2005) with 1.2 mM ammonium tartrate, 1.5% agar, and various carbon sources such as 0.1% xylose or 1% glucose, carboxymethyl cellulose (CMC), pectin (from citrus), and xylan (from oat spelt) purchased from Nacalai Tesque (Kyoto, Japan). Growth was inhibited when strain NS/Mg was cultured on 1% xylose medium.

Construction of cDNA libraries

We used a modification of the single-strand linker ligation method (Shibata et al. 2001) to construct a subtractive cDNA library from relatively small amounts of RNA. This method used DNA ligase to add a double-strand linker to first-strand cDNA. The linkers have random 6-bp (dN_6 or dGN_5) 3′ overhangs that can ligate to any cDNA sequence. The synthesized cDNAs have tag sequences at the 5′- and 3′-ends that originated from the linker and oligo(dT) primer for reverse transcription, respectively. This means that the cDNA sequence between these tags can be amplified by PCR. Whole fungus comb was subjected to poly(A)$^+$RNA extraction by using oligo(dT) latex (Takara, Otsu, Japan) according to previously described method (Taprab et al. 2005). First-strand cDNA was synthesized from approximately 2 μg of poly(A)$^+$RNA by using superscript II reverse transcriptase (Invitrogen) and primer 1 (5′-TTTAAG CAGTGGTATCAACGCAGAGAAGCT$_{16}$VN-3′). The dou-

ble-strand linkers GN5 and N6 were prepared by mixing oligonucleotides A (5′-AAGCAGTGGTATCAACGCAGA GAATTC$\underline{GN_5}$-3′) and B (5′-GAATTCTCTGCGTTGATAC CACTGCTT-3′, 5′-end phosphorylated), and oligonucleotides B and C (5′-AAGCAGTGGTATCAACGCAGA GAATTC$\underline{N_6}$-3′), respectively. The double-strand linker was ligated with the first-strand cDNA according to the method of Shibata et al. (2001). The first-strand cDNA with the linker was dissolved in TE (pH 8.0) and used to construct nonsubtractive (NS) and subtractive (SB) cDNA libraries. Subtractive hybridization was carried out with a super subtraction kit (Sawady Technology, Tokyo, Japan). With this method, driver RNA was immobilized on the surface of a carbodiimide-coated microplate. Driver RNA/cDNA hybrid was formed on the surface of the microplate and unbound cDNAs were recovered from the aqueous phase. Driver RNA was prepared from 21-day-old mycelium of *Termitomyces* sp. strain NS/Mg grown on a PDA plate. Poly $(A)^+$ RNA was extracted with the same method used for the fungus comb sample. Approximately 7 μg of poly$(A)^+$ RNA was immobilized, and one third of the first-strand cDNA with the double-strand linker was used for the hybridization. Subtractive hybridization was carried out twice at 65°C for 24 h according to the manufacturer's instructions. Unbound cDNAs were purified with a MicroSpin S400HR (Amersham, Buckingghamshire, UK) followed by ethanol precipitation and was subsequently dissolved in 30 μl of 10 mM Tris–HCl, pH 8.0. The cDNAs were amplified with primer 1 (5′-AAGCAGTGGTATCAACGCAGAGAATTC-3′) and 2 (5′-TTTAAGCAGTGGTATCAACGCAGAGAAGCT-3′). PCR was performed using a PTC-200 thermal cycler (MJ Research, Watertown, USA) and Ex-*Taq* (Takara). Thermal cycling consisted of 94°C for 2 min followed by 22 cycles of 95°C for 20 s and 68°C for 6 min. The PCR reaction mixture contained 10 μl of the subtractive cDNA or 2,000 times diluted first-strand cDNA solution (nonsubtractive) and 0.2 mM deoxyribonucleotide triphosphate containing 5-methyl deoxycytidine triphosphate. The PCR product was size-fractionated (>400 bp) with a SizeSep400 spun column (Amersham) and directionally cloned into pBlueScript (KS+) vector (Stratagene) at the *Eco*RI/*Hin*dIII sites (Meissner et al. 1987), before being transferred into XL10-gold cells (Stratagene, La Jolla, USA). The NS and SB libraries contained 3.5×10^4 and 2.5×10^4 individual clones.

DNA sequencing, data processing, and annotation

Sequencing templates were prepared using PCR with universal primers for the vector from *Escherichia coli* colonies. The PCR products were purified with an ExoSAP-IT kit (USB, Cleveland, USA). Plasmids were also used for sequencing when the amplification of insert cDNA was difficult. The DNA sequences of the 5'-end of the cDNA were determined using T7HT primer (Toyobo, Osaka, Japan) with DNA sequencers (ABI models 3700 and 377; Applied Biosystems Japan, Tokyo). The resultant sequences were processed with the Staden package version 1.4.1 (https://sourceforge.net/projects/staden/). Sequence chromatograms were trimmed to eliminate low-quality regions with a threshold of 18, and vector and linker sequences were removed using the pregap4 program in the Staden package. Cleaned sequences were assembled using a pregap4 shotgun assembler with default parameters. Three ESTs similar to a ribosomal RNA gene were found by the BLASTN program (Altschul et al. 1997) and removed from our EST database. No sequence derived from the *E. coli* genome was found.

The ESTs were annotated based on the results of BLASTX with an *E* value of less than 1e−5 (Altschul et al. 1997). BLASTX searches for assembled sequence sets were performed against Uniprot/swiss-prot and Uniprot/TrEMBLE (Release 22.0, October 2004). Gene ontology (GO) annotations and GO–slims terms associated with each Uniprot entry were obtained from the GOA web site (http://www.ebi.ac.uk/GOA/) and were mapped to each BLAST hit. GO is a dynamically controlled vocabulary of over 16,000 terms used to describe molecular function, process, and protein location. Putative amino acid sequences were generated from unique EST sequences using the prot4EST software version 2.0.2b (Wasmuth and Blaxter 2004), which is a prediction pipeline employing methods such as ESTScans and DECODER. Signal peptides in the deduced amino acid sequences were searched for at the SignalP web site (Bendtsen et al. 2004). Putative extracellular proteins were manually inspected based on the results of both SignalP and BLASTX searches. Protein domains were also searched for at the Pfam web site (Bateman et al. 2004). Carbohydrate-active enzymes (CAZy) classification to putative cell-wall-degrading enzymes was assigned based on similarity to entries in CAZy database (Coutinho and Henrissat 1999). Bioinformatic works such as BLAST result parsing and GOA term mapping were conducted by using custom Perl scripts with Bioperl modules (Stajich et al. 2002).

Real-time quantitative RT-PCR

Termitomyces sp. strain NS/Mg was grown on the synthetic media containing various carbon sources until the mycelia reached to approximately 3 cm in diameter (22- to 49-day cultures). Poly$(A)^+$RNA was extracted from three mycelial mats of the strain and the fungus comb by using an Oligotex direct mRNA purification kit (Qiagen GmbH, Hilden, Germany) with sodium dodecyl sulfate–urea solution. After treatment with RNase-free DNase I (Promega, Madison, USA), the first-strand cDNA was synthesized by using the oligo(dT) primer as described above. Gene-specific primer

sets were designed by using Primer express version 2.0 (Applied Biosystems) based on contig sequences generated from this EST analysis (Supplementary Table S1). Quantification was performed using the 7300 real-time PCR system and a SYBR Green PCR core reagent kit (Applied Biosystems) according to the manufacturer's instructions. Plasmids containing target cDNA fragments were used as external standards. Relative expression levels were normalized to mRNA for eukaryotic translation initiation factor 5A (eIF, NS05G01). Statistical analysis was performed by using STATISTICA version 4.1 (StatSoft Japan, Tokyo).

Nucleotide accession numbers

Nucleotide sequences of the tentative consensus sequences (TCs) were submitted to DNA Data Bank of Japan under the following accession numbers: BW633614–BW634995 (NS library) and BW633289–BW633613 (SB library).

Results

Overview

Two cDNA libraries for EST analysis were constructed in this study. Poly(A)$^+$RNA extracted from the fungus comb of *M. gilvus* was used to construct the NS cDNA library. To facilitate the discovery of the genes that were expressed specifically in *Termitomyces* grown on the fungus comb (symbiotic conditions), the first-strand cDNA used for construction of the NS library was subtracted with driver RNA extracted from *Termitomyces* sp. strain NS/Mg grown on the PDA medium. Subsequently, the SB cDNA library was constructed. Partial sequences of the randomly chosen clones in the two libraries were determined from the 5′-ends. After removal of low-quality sequences and contaminants derived from the cloning vector and ribosomal RNA, 2,108 and 505 ESTs were collected from the NS and SB libraries, respectively. Sequence assembling was performed, resulting in 1,382 and 325 TCs for the NS and SB libraries, respectively. The average sequence lengths of TCs from the NS and SB libraries were 629 and 547 bp. A total of 1,582 novel cDNA sequences were eventually identified by assembling ESTs from the two libraries.

All the TCs were subjected to BLASTX similarity searches, which revealed that 941 out of 1,582 TCs showed significant similarities (E value <1e−5) to protein-encoding genes in the Uniprot database. The BLASTX results for all the TCs are shown in Supplementary Tables S2 and S3. Analysis of the organisms represented by the BLASTX best hits for the TCs showed that more than 70% were fungal species. Table 1 shows the functional assignments of the ESTs classified with "biological process" in GO–slim terms based on BLASTX results. The ESTs associated with the GO term "response to stimulus" were markedly more abundant in the SB library than in the NS library, while ESTs with the other GO terms were more abundant in the NS library.

Abundant transcripts under symbiotic conditions

Table 2 lists the 20 most abundant ESTs in the NS library and their results of sequence similarity search against the protein database. All source organisms of BLASTX hits in Table 2 were fungi, of which 60% were basidiomycetes, including *Termitomyces*. SignalP analysis indicated that half of the 20 sequences in Table 2 possessed putative signal sequences in the N termini of their deduced amino acid sequences, suggesting that their localization was extracellular. TCs NS2E10 and NS11F03 were sequences corresponding to the laccase-related protein cDNA *lcc*2–5 (AB201165) and the laccase cDNA *lcc*1–2 (AB201164), which were previously identified from the same fungus

Table 1 GO–slim annotations associated with biological process (GO: 0008150)

GO term	Percent of total ESTs		Percent of total TCs[a]	
	NS[b]	SB[c]	NS	SB
Cell communication (GO: 0007154)	0.95	0	1.23	0
Cell growth and maintenance (GO: 0008151)	1.47	0.79	1.95	0.92
Transport (GO: 0006810)	10.7	3.17	11.6	7.08
Metabolism (GO: 0008152)	24.2	17.2	22.1	18.8
Response to stimulus (GO: 0050896)	2.47	10.9	0.43	0.62
Unknown functions (GO: 0000004)	25.9	21.4	23.7	21.5
Others	0.19	0.4	0.12	0.62
No hit[d]	34.1	46.1	38.7	50.5

[a] Tentative consensus sequences (=unique transcripts)
[b] Nonsubtractive cDNA library
[c] Subtractive cDNA library
[d] No homolog in the Uniprot database

comb and *Termitomyces* sp. strain NS/Mg (Taprab et al. 2005). Deduced amino acid sequence of *lcc*2–5 showed significant similarity to those of laccase *lcc*1–2 and other fungal laccases, but the *lcc*2–5 protein does not exhibit laccase activity. Genes induced by phosphate deficiency (Tasaki et al. 2004), such as putative genes for extracellular ribonuclease (NS23H11), phosphatases (NS14A09 and NS12E07), and phosphate transporters (NS11B04) are also found in Table 2.

The abundance value in Table 2 is expected to be higher in the SB library than in the NS library when a gene is expressed specifically under symbiotic conditions. The abundance values of CipC (concanamycin A-inducible protein) and DDR48 (DNA damage-responsive) homologs (NS2A8 and NS10H10) were higher in the SB library than in the NS library, and these were the two most abundant ESTs in the SB library. The GO term response to stimulus was tentatively assigned to these proteins in this study, and this was the main reason for the higher proportion of transcripts associated with response to stimulus in the SB library (Table 1). Greater abundance values were also observed in TCs NS06A08 and NS2D10, which were homologs for hydrophobin and cyclophilin, respectively (Tables S2 and S3). The ten most abundant ESTs of the SB library consisted of homologs for CipC, DDR48, hydrophobin, cyclophilin, unknown protein (4MeS), and ESTs with no significant similarity to known proteins.

Genes putatively relevant to plant cell wall degradation

The enzyme homologs involved in the degradation of plant cell walls found in the NS and the SB library are summarized in Table 3. A total of 70 ESTs (3.3%) and 29 TCs (2.1%) in the NS library showed significant similarity to the genes that encode enzymes acting on macromolecules consisting of the plant cell wall. Protein family search in the Pfam database was conducted for further gene annotation of plant cell-wall-degrading enzymes. This analysis is important especially for carbohydrate-degrading enzymes because sequence similarity with, for example, cellulase, might be due to similarity with a part of a cellulose-binding domain. A small number of homologs relevant to cell wall degradation were identified from the SB library. The number of homologs of pectin and hemicellulose-degrading enzymes

Table 2 BLASTX results of the 20 most abundant ESTs in the NS library and a comparison of EST frequency between the NS and SB libraries

TC ID[a]	BLAST *E*-value	Percent identity[b] (aa overlap)	Description (accession no.)	Source organism	Abundance (%)[c]	
					NS	SB
NS3A9[d]	–		No hit		2.0	2.0
NS2E10[d]	0	100 (555)	LCC2-5 (AB201165)	*Termitomyces* sp.	1.9	0.4
NS23H11[d]	2e−27	58.8 (102)	Ribonuclease Po1 (P81762)	*Pleurotus ostreatus*	1.4	0.6
NS2A8	3e−19	54 (102)	CipC protein (Q8NKC9)	*Aspergillus nidulans*	1.3	6.7
NS14A09[d]	1e−132	63.1 (355)	Acid phosphatase (Q75V97)	*Pholiotamameko*	1.2	0.6
NS1C10	–		No hit		1.2	0.6
NS10H10	2e−58	53.8 (429)	DDR48 (P18899)[e]	*Saccharomyces cerevisiae*	1.0	4.4
NS12E07[d]	1e−115	43.8 (543)	HP[f] (Q7S495) alkaline phosphatase (PF00245)[g]	*Neurospora crassa*	0.9	0.4
NS11F03[d]	0	100 (510)	Laccase LCC1-2 (AB201164)	*Termitomyces* sp.	0.9	0
NS11D11[d]	5e−62	51.0 (255)	Endopolygalacturonase (Q9P8M5)	*Chondrostereum purpureum*	0.6	0.2
NS2E5	–		No hit		0.5	0.8
NS14E10[d]	6e−09	33.3 (180)	4MeS (O13320)	*Metarhizium anisopliae*	0.5	1.0
NS16F03	1e−123	62.5 (363)	Formate dehydrogenase (Q6CDN8)	*Yarrowia lipolytica*	0.5	0.8
NS06A08[d]	8e−18	49.6 (131)	Putative hydrophobin (Q9HGW9)	*Agaricus bisporus*	0.5	3.2
NS22B11	–		No hit		0.4	0
NS11B04	0	72.0 (546)	Phosphate transporter (Q96X52)	*P. nameko*	0.4	0
NS17H08	–		No hit		0.4	1.8
NS14E02[d]	1e−143	75.0 (328)	Cellobiohydrolase I-II (Q6E5B2)	*Volvariella volvacea*	0.4	0
NS1B9	0	63.9 (502)	Aldehyde dehydrogenase (O74187)	*A. bisporus*	0.3	0.2
NS04G06	1e−101	58.3 (300)	D-Xylose reductase (Q9P8R5)	*Aspergillus niger*	0.3	0.4

[a] Identification of a tentative consensus sequence
[b] Amino acid identity calculated by FASTA
[c] *Abundance* = (*ESTs in a TC*/*total ESTs in a library*) × 100
[d] Indicates a transcript with a putative signal sequence in its deduced amino acid sequence
[e] DNA damage-responsive protein. Filter-off option was used in BLAST search
[f] Hypothetical protein
[g] Pfam description

was 2.5-fold more than the numbers of cellulose degrading enzymes, such as cellobiohydrolase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4), and β-glucosidase (EC 3.2.1.21). Homologs of endo- and exopolygalacturonase, pectate lyase, rhamnogalacturonan lyase, arabinase, pectin methylesterase, and α-galactosidase were potentially involved in pectin degradation. Putative signal peptides were present at the deduced N-terminal amino acid sequences of half of the enzyme homologs in Table 3. Among well-studied ligninolytic enzymes from basidiomycetes, such as lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), and laccase (1.10.3.2), only laccase homologs were identified from our EST database.

Real-time quantitative RT-PCR analysis of selective abundant transcripts

Expression levels of the genes encoding CipC, DDR48, and NAD(P)H-xylose reductase (EC 1.1.1.21) were determined using real-time quantitative RT-PCR under the symbiotic conditions, and were compared to those under nonsymbiotic conditions where *Termitomyces* were grown on several synthetic media with various carbon sources. PCR products in this analysis were sequenced, and it was confirmed that identical PCR products were obtained from the fungus comb and *Termitomyces* sp. strain NS/Mg, indicating that *Termitomyces* was the origin of these genes.

The comparative analysis of EST abundance between NS and SB libraries showed that the abundance of TCs NS2A8 (CipC) and NS10H10 (DDR48) were much higher in the SB library than in the NS library (Table 2), suggesting that the gene expression should be higher when *Termitomyces* was grown on fungus comb than on PDA. To confirm this, expression levels of the genes were compared between the two culture conditions (Fig. 1a). Expression levels of both genes for the fungus comb were significantly higher, 6.0- and 2.2-fold, than those for the PDA medium (Mann–Whitney U test, $P<0.05$). Similar results were obtained when the gene for elongation factor 2 (NS11H10) instead of eIF was used to normalize the mRNA levels of the target genes (data not shown).

TC NS04G06 was a homolog to the gene encoding NAD(P)H-dependent D-xylose reductase that catalyzes the first step of D-xylose catabolism by fungi. Statistically significant differences in the expression level of the gene were observed between the fungus comb and every synthetic medium used in this study (one-way ANOVA followed by Tukey's Honestly Significantly Different (HSD) test, $P<0.05$; Fig. 1b). Expression of the gene was 3.1-fold higher on xylose medium than on glucose medium, while the highest expression among the synthetic media was observed in a pectin medium.

Discussion

EST analysis was successfully applied to the study of a symbiotic fungus of a fungus-growing termite using naturally occurring sample. We consider that the study of *Termitomyces* under the symbiotic conditions is very important for understanding the mechanism underlying

Table 3 Putative cell-wall-degrading enzymes identified in NS library

Enzyme	No. of EST		No. of TC		CAZy family[a]
	NS	SB	NS	SB	
Pectin-degrading					
Endopolygalacturonase	17	1	3	1	GH28
Exopolygalacturonase	9	0	2	0	GH28
Pectate lyase	4	0	1	0	PL1, 2
Rhamnogalacturonan lyase	2	0	1	0	PL4
Pectin methylesterase	1	1	1	1	CE8
Rhamnogalacturonan acetylesterase	0	1	0	1	CE12
Other hemicellulose-degrading					
Endo-1,4-β-xylanase	5	0	5	0	GH10, 11
β-Mannanase	5	0	2	0	CE5
Acetylxylan esterase	3	0	1	0	CE5
Arabinase	2	0	2	0	GH43
α-Galactosidase	1	0	1	0	GH27
Cellulose-degrading					
Cellobiohydrolase	12	1	3	1	GH6, 7
Endoglucanase	6	0	4	0	GH5, 44, 61
β-Glucosidase	3	0	3	0	GH1, 3

[a] Carbohydrate-active enzymes (CAZy) family

efficient degradation of dead plant materials by fungus-growing termites. This study provided partial nucleotide sequences of 1,582 tentative unique transcripts expressed mostly in *Termitomyces*, of which 1,580 sequences including 29 transcripts putatively relevant to plant cell wall degradation were newly identified in this study. The fact that only two kinds of protein-encoding genes were identified in *Termitomyces* spp. underscores the value of the present sequencing effort. Recently, genome sequences of two basidiomycetes, *Phanerochaete chrysosporium* and *Cryptococcus neoformans*, were published in which 11,777 and 6,500 genes were discovered, respectively (Loftus et al. 2005; Martinez et al. 2004). On the basis of these numbers, the present TCs, are assumed to represent 13–24% of the total genes in *Termitomyces*. This assumption is probably an overestimate because our study identified a large number of singletons, many of which may simply not overlap despite being transcribed from the same gene.

Subtractive hybridization is a powerful and popular method to enrich upregulated transcripts, but a subtractive library often contains nondifferentially expressed transcripts (so-called false positives and false negatives). Therefore, we confirmed differential expression by using real-time quantitative RT-PCR for some genes that were abundant in the subtractive cDNA library. The genes for CipC and DDR48 (NS2A8 and NS10H10) showed higher abundance in the SB library than in the NS library (Table 2). The results of real-time quantitative RT-PCR in Fig. 1a also indicated higher expression (6.0- and 2.2-fold) under the symbiotic conditions than under the nonsymbiotic conditions (culture on a PDA medium), suggesting that the subtractive cDNA library in this study is a useful resource for an initial screening of differentially expressed genes in *Termitomyces*. Unexpected results were also found in comparison of EST abundance between the NS and SB library. Only small numbers of cell-wall-degrading enzymes and laccase were identified in the SB library. It is uncertain whether the genes for these enzymes were also expressed in *Termitomyces* growing on a PDA medium or lower abundance of these genes is due to false negative transcripts. Consequently, differential expression of genes of interest must be confirmed by using, for example, DNA microarray or quantitative PCR.

The CipC protein is identified from *Aspergillus nidulans* where its expression is upregulated in the presence of the antibiotic concanamycin A (Melin et al. 2002). The molecular function of CipC has been unclear to date. The *ddr*48 gene of *Saccharomyces cerevisiae* is induced by treatments that produce DNA lesions or induce heat-shock stress and was thought to involve the production or recovery of mutations in *S. cerevisiae* (Treger and McEntee 1990). The high expression levels of the genes imply the presence of stress in the fungus comb, even in a symbiotic

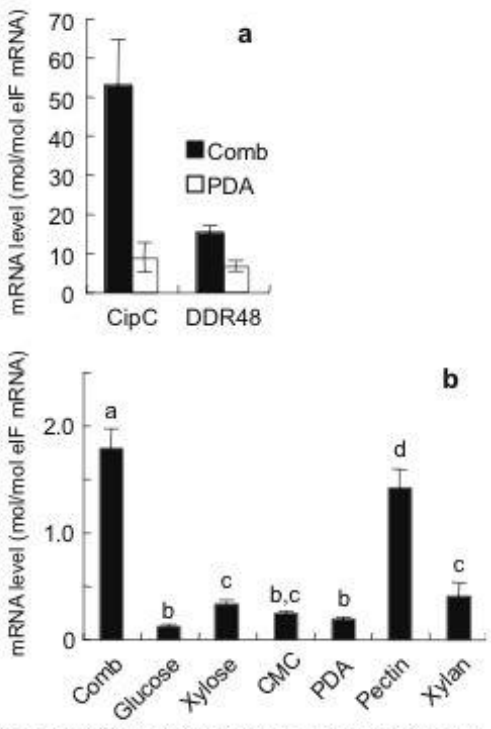

Fig. 1 Quantitative RT-PCR analysis of the gene expressions for **a** CipC and DDR48, **b** NAD(P)H-dependent D-xylose reductase when *Termitomyces* was grown on a fungus comb (*comb*), a potato dextrose agar medium (*PDA*), and other synthetic media varying in carbon sources, carboxymethyl cellulose (*CMC*), glucose, pectin, xylose, and xylan. The expression levels are shown in terms of mRNA copies of target genes per copy of mRNA for eIF. The data are calculated means ±SD values (n=4). The different letters (*a*–*d*) over the bars in subpanel **b** indicate significant differences between the bars (one-way ANOVA followed by Tukey's HSD test, P<0.05)

environment. Stress response was documented in some symbiotic systems, such as plant–arbuscular mycorrhizas. Plant defense responses are induced not only during early stages of plant–fungi interaction, but also in arbuscule formation in roots (Garcia-Garrido and Ocampo 2002). The role of defense genes is suggested to be the control of hyphal spread in the root. There are potential sources of stress to *Termitomyces* fungi in the fungus comb. For example, grazing of fungal mycelium by host termites, bacteria-inhabiting fungus combs, and antimicrobial compounds from plant material may all be stresses to *Termitomyces*. There are several differences in cultivation conditions between symbiotic and nonsymbiotic conditions, such as humidity, availability of nutrient (starvation), light

intensity, and gas compositions and these might be also possible factors that influence expression of stress-responsive genes in *Termitomyces*.

It is generally assumed that the transcript showing abundant ESTs are highly expressed. This hypothesis seems to be applied to the NS library. Homologs of CipC, DDR48, and xylose reductase were abundant ESTs in the NS library (Table 2) and expression levels of those genes verified by real-time quantitative PCR were higher than that of eIF whose EST abundance was 0.2% (Fig 1).

A large number of homologs relevant to plant cell wall degradation were identified in this study (Table 3), the most abundant of which were the genes for enzymes involved in pectin degradation. Pectin is a heteropolysaccharide containing mainly homogalacturonan and rhamnogalacturonans with arabinan, galactan, and arabinogalactan side chains (Willats et al. 2001). It is an abundant polysaccharide in the primary cell wall of plants and in the middle lamellae between primary cell walls where it functions in regulating intercellular adhesion. To our knowledge, little or no information is available on pectin degradation in fungus combs. High expression of pectin-degrading enzymes might be affected by the substrate of the *M. gilvus* fungus comb, which consists mainly of leaves and grasses (Johjima et al. 2003; Kalshoven 1956). Further studies on cell-wall-degrading enzymes in fungus comb are necessary to understand the efficient degradation.

Compared to the genes for xylanase (5 ESTs), more ESTs for a series of cellulases (20 ESTs) were found in the NS library. Conversely, considerably higher numbers of ESTs were involved in intracellular D-xylose catabolism than in that of D-glucose. The gene homolog for NAD(P)H-dependent D-xylose reductase (NS04G06, seven ESTs), xylulose reductase (EC 1.1.1.9; NS21D09, 1 EST), xylulokinase (EC 2.7.1.17; NS14G12, 1 EST), and ribulose-phosphate 3-epimerase (EC 5.1.3.1; NS13B07, 1 EST) were identified from the NS library, whereas two singletons (NS2F11 and NS26G11) involved in glucose catabolism were identified. These two singletons encode putative glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49), which is involved in the first step of the pentose phosphate pathway. Therefore, this enzyme is also involved in galactose catabolism. To obtain further information on carbohydrate catabolism in *Termitomyces*, the expression level of the putative D-xylose reductase gene was examined in relation to the culture conditions of *Termitomyces*. The expression of the D-xylose reductase gene in *Termitomyces* grown on pectin medium was the highest among those grown on the synthetic media and was 20% lower than that on the fungus comb (Fig. 1b). This result suggested that expression of the D-xylose reductase gene was stimulated when *Termitomyces* degraded or catabolized pectin.

The laccase gene *lcc1–2* (NS11F03) was one of the abundant ESTs in the NS library (Table 2), whereas no homologs for lignin or manganese peroxidase were identified in this study. This result is consistent with a previous finding that laccase was the sole detectable phenol-oxidizing enzyme in the fungus comb of *M. gilvus* (Taprab et al. 2005). It is uncertain whether laccase in fungus comb is involved in lignin degradation because most of ligninolytic basidiomycetes produce lignin and/or manganese peroxidases along with laccase, and laccase is unable to catalyze the oxidation of nonphenolic lignin moieties. Further discussion on lignin degradation in fungus comb can be found in Rouland-Lefèvre et al. (2006) and Taprab et al. (2005).

In conclusion, we showed the construction of cDNA libraries directly from fungus comb and the identification of many novel transcripts. Putative functional annotations were assigned to approximately 40% of TCs. Regarding polysaccharide catabolism, ESTs relevant to pectin degradation and D-xylose assimilation were abundantly obtained from the libraries. The functions of the remaining 60% of TCs, including TC NS3A9, which was the most abundant in the NS library, are still uncertain. Nevertheless, the present EST database will be an important resource for future research on the functions of symbiotic fungi.

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