

**Termite Ectoparasitic Fungi in Japan:
Distribution, Prevalence, and Molecular Detection**

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2018

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Chapter 1

General introduction

1.1. Research Background

Termites are widely spread eusocial insects with 3,106 known species, of which 183 are considered pests and 83 cause significant damage to wooden structures (Su and Scheffrahn 2000). There are a variety of effective termite control methods, including chemical and physical barriers, population management using a bait system, growth regulation by metabolic inhibitors, and biological control agents (Su and Scheffrahn 2000). The selection of termite control techniques basically depends on the termite species, especially on whether they are subterranean or drywood termites. Detection, prevention and remedial treatment are three phases used to eliminate or reduce termite attacks. One of the remedial treatments is biological control with entomopathogenic fungi, with *Metarhizium anisopliae* Sorokin and *Beauveria bassiana* (Balsamo) Vullemin reported as the most promising candidates (Lai et al. 1982, Su and Scheffrahn 2000).

In the natural environment, subterranean termites live in soil with relatively high humidity. These conditions can expose termites to parasites such as fungi. The relationships between termites and fungi are generally divided into two categories: symbiotic mutualism and pathogenic relationships. Termite-fungus interaction is a topic that has attracted researchers for more than fifty years (Chouvenc et al. 2011). Studies have explored both termite-fungus symbiotic mutualism (Hyodo et al. 2000, Aanen et al. 2009, Matsuura et al. 2009, Rosengaus et al. 2011) and pathogenic relationships (Grace and Zoberi 1992,

Yoshimura et al. 1992, Yoshimura and Takahashi 1998, Strack 2000). Entomopathogenic fungi threaten termite survival, behavior and reproduction (Culliney and Grace 2000). Extensive research has been carried out to reveal the power of entomopathogenic fungi against termite attacks, and 20 species have been tested for their pathogenicity, but none of the researches has focused on ectoparasitic fungi (Lai et al. 1982, Zoberi 1995, Culliney and Grace 2000, Chouvenec et al. 2011). Ectoparasitic fungi are fungi that attach to and live on the body surface of their hosts.

Laboulbenia, *Dimeromyces*, *Cordycepioideus*, *Laboulbeniopsis*, *Coreomycetopsis*, *Amphoromorpha*, *Hormiscioideus*, *Antennopsis*, *Termitaria* and *Mattiollea* are the known genera of ectoparasitic fungi, which comprise 22 species, of which *Laboulbeniopsis termitarius* Thaxt, *Antennopsis gallica* Buchli and Heim, and *Termitaria* sp. are the most commonly found on termite bodies (Blackwell and Rossi 1986). Ectoparasitic fungi as a whole can be found in a wide distribution region, from tropical to temperate areas, but none of the reports has mentioned Japan (Blackwell and Rossi 1986). One purpose of this study is to make a comprehensive survey of termite ectoparasites in Japan.

Ectoparasitic fungi have been reported to have the ability to reduce a termite's lifespan (Buchli 1952). However, the effects of ectoparasitic fungi on termite activity remain unclear due to their inability to grow in laboratory conditions. Infection by the ectoparasitic fungi *L. termitarius* and *A. gallica* is commonly detected manually under a light microscope (Thaxter 1920, Heim and Buchli 1951, Gouger and Kimbrough 1969, Myles et al. 1998, Guswenrivo et al. 2017). Several

hundred termites are required when inspecting the colonies (Guswenrivo et al. 2017). Therefore, this study also focuses on the development of a novel method for effective detection of the ectoparasitic fungi together with trials for cultivation of the fungi in the laboratory.

In recent years, numerous DNA-based methods have been developed to detect fungal infection on plants and insects. Polymerase Chain Reaction (PCR) is the most promising method for detecting fungi infection due to its simplicity, specificity, and sensitivity (Luo and Mitchell 2002). PCR-based methods targeting different genes have been described for identification of mycotoxigenic fungi (Miguel ngel Pavón et al. 2017, Kocsubé and Varga 2017) and even molecular variations in insect pathogenic fungi (Cobb and Clarckson 1993). It has been applied to phytopathogenic fungi and has contributed to solving some of the problems associated with the detection, control and containment of pathogens in plants (Henson and French 1993, Martin et al. 2000).

Multiplex PCR is a method used to amplify several DNA sequences in the same reaction (Henegariu et al. 1997). Since 1988, multiplex PCR has been successfully applied in many areas of DNA testing such as pathogenic identification, high-throughput single nucleotide polymorphisms (SNP) genotyping, mutation analysis, gene deletion analysis, template quantitation, linkage analysis, RNA detection, and forensic studies (Chamberlain et al. 1988, Pérez-Pérez and Hanson 2002, Lehmann et al. 2008, Martínez-Culebras et al. 2017, Yli-Mattila et al. 2017, Hayashi et al. 2017). Termite colonies are not always infected by just one species of ectoparasitic fungus, but sometimes two or three species at a time

(Blackwell 1980). Therefore, the use of multiplex PCR to detect ectoparasitic fungi on termites would be highly efficient. This method is expected to help researchers uncover the effects of ectoparasitic fungi on the activity and behavior of termites on the colony level.

This study consists of 6 chapters. The first part, Chapter 1, provides a general introduction, explaining the research background, main purpose of the study, and some previous work done on related topics. The ectoparasitic fungi on termite and DNA-based method on fungi detecting was reviewed. The main body of the study is divided into two parts. The second part (Chapters 2–4) describes the first discovery of ectoparasitic fungi *L. termitarius* and *A. gallica* in Japan, and the results of a national survey on the distribution of the ectoparasitic fungi. The third part, Chapter 5, describes the molecular approach to detection of ectoparasitic fungi *L. termitarius* and *A. gallica* in termite colonies.

1.2. Ectoparasitic fungi

Parasitic nutrition is a mode of heterotrophic nutrition where a parasitic organism lives on the body surface or inside the body of another type of organism (a host) and gets nutrition directly from the body of the host. Since these parasites derive nourishment from their host, this interaction is often described as harmful to the host (Parker and Riches 1993, Quicke 1997). Parasites depend on their host for survival, since the host provides nutrition and protection. As a result, parasites have optimized their strategies to obtain nutrition from the hosts, resulting in survival (Quicke 1997). Parasites are divided into two groups:

endoparasites and ectoparasites. Endoparasites are parasites that live inside the body of the host, whereas ectoparasites are parasites that live on the outer surface of the host (Sibley 2004). Endoparasites and ectoparasites require different adaptations to derive nutrients from their hosts.

Ectoparasites do not have a readily available source of nutrients on the outer surface of the host, so they need adaptations to access host nutrients (Nieto et al. 2007), such as the ability to penetrate the host cuticle or to secrete digestive enzymes and the presence of a gut to digest host-derived nutrients (Geiser et al. 2005). Ectoparasites also have a variety of parasite transporters and permeases to enable them to acquire nutrition from their host across numerous membranes (Geiser et al. 2005, Igoillo-Esteve et al. 2011). Many ectoparasites are known as pathogen vectors, so they transmit these pathogens during nutrient acquisition. Most ectoparasites are fungi (Geiser et al. 2005, Igoillo-Esteve et al. 2011). The geographical distribution of ectoparasitic fungi covers all regions, from tropical to temperate areas (Blackwell and Rossi 1986, Riddick et al. 2009, Espadaler and Santamaria 2012).

Laboulbeniales is a well-defined class within phylum Ascomycota with more than 2,000 described species that have obligate biotrophic associations with arthropods, and it is assumed that large numbers of undescribed species exist (Alexopoulos et al. 1996, Hawksworth et al. 1995). There are some unusual ectoparasitic Ascomycetes that lack mycelium. Instead, these fungi have a developed thallus, and are classified into three major groups: suborder Laboulbeniineae with two families (Peyritsiellaceae, Laboulbeniomycetaceae)

and one family of suborder Ceratomycetaceae (Thaxter 1896). A major taxonomic revision was made based on development status and morphological identification (Tavares 1985). Members of Laboulbeniales produce fruiting bodies (thalli) of determinate growth, usually bearing antheridia and perithecia. These characteristics were used as a mark among species, and identifications were made in the form of the mature thallus (Thaxter 1926). Mature thalli have ascospores within the perithecium (Weir and Beakes 1995). The thalli attach to their host's integument by a basal cell, where the fungi make contact with the host, either through the cuticular pores or by active penetration of the host's integument (Tavares 1985, De Kesel 1996). However, the Laboulbeniales are considered harmless despite their parasitic nature (De Kesel 1996). They spend their entire life cycle on the host, without any free-living stages, and only some are known its sexual reproduction (Tavares 1985, Weir and Beakes 1995, De Kesel 1996). Most species of Laboulbeniales are considered host-specific, and some are even specific to a position on their host, and perhaps their host's sex (Weir and Beakes 1995).

Laboulbeniales are obligate external parasites of arthropods (especially insects), and there have been numerous reports regarding these insect ectoparasitic fungi (Richards and Smith 1955, Tavares 1979, Santamaria 2001, Kaur and Mukerji 2006, Espadaler and Santamaria 2012). Several ectoparasitic fungi are known to be obligate with insects, such as *Aegeritella* Balazy & J. Wiś, *Hormiscium myrmecophilum* Thaxter, *Rickia wasmannii* Cavara, *Laboulbenia camponoti* S.W. T. Batra, and *Laboulbenia formicarum* Thaxt, which have been

found on the body surface of ants (Espadaler and Santamaria 2012). Four other species of Laboulbeniales in the genus *Hesperomyces* were found on lady beetles (Thaxter 1931, Riddick et al. 2009). Information regarding the relationship and interaction between these ectoparasitic fungi and their host insects has not been properly ascertained, although a reduced life duration or activity level of the host has been suggested (Buchli 1952, Ch'erix 1982, Wi'sniewski and Buschinger 1982). Some reports have concluded that ectoparasitic fungi are neutral to their hosts, beside the fact that they penetrate into cuticle to the hemocoel and can attain an extremely high abundance on the host (Weir and Beakes 1996, Santamaria 2001, Gemenio et al. 2004, Kaur and Mukerji 2006, Espadaler and Santamaria 2012).

However, it was reported that ectoparasitic fungi caused small or harmful effects to their hosts (Tavares 1979, Weir and Beakes 1995). *Rickia wasmannii* was reported to reduce the lifespan of the ant *Myrmica scabrinodis* significantly, and to increase the allogrooming of the colony (Csata et al. 2014). A high abundance of ectoparasitic fungi will give a mortality effect to the host as is observed with *A. gallica* on *Reticulitermes lucifungus* Rossi (Buchli 1952). *Hesperomyces virescens* Thaxter caused premature mortality of *Chilocorus bipustulatus* L. under field conditions, and the insects were attacked by several predatory lady beetles (Kamburov et al. 1967, Tavares 1985, Weir and Beakes 1996, Christian, 2001). On the other hand, very little information is available on the biology and ecology of Laboulbeniales ectoparasitic fungi in relation to host insects. All species of ectoparasitic fungi require a living host for survival and

reproduction, and up until now, all have proven unable to grow in laboratory conditions (Henk et al. 2003).

Termites have been reported to host 22 species of ectoparasitic fungi, with *Laboulbeniopsis*, *Antennopsis*, and *Termitaria* being the genera of ectoparasitic fungi most commonly found on the termite body (Blackwell and Rossi 1986) (Fig 1.1).

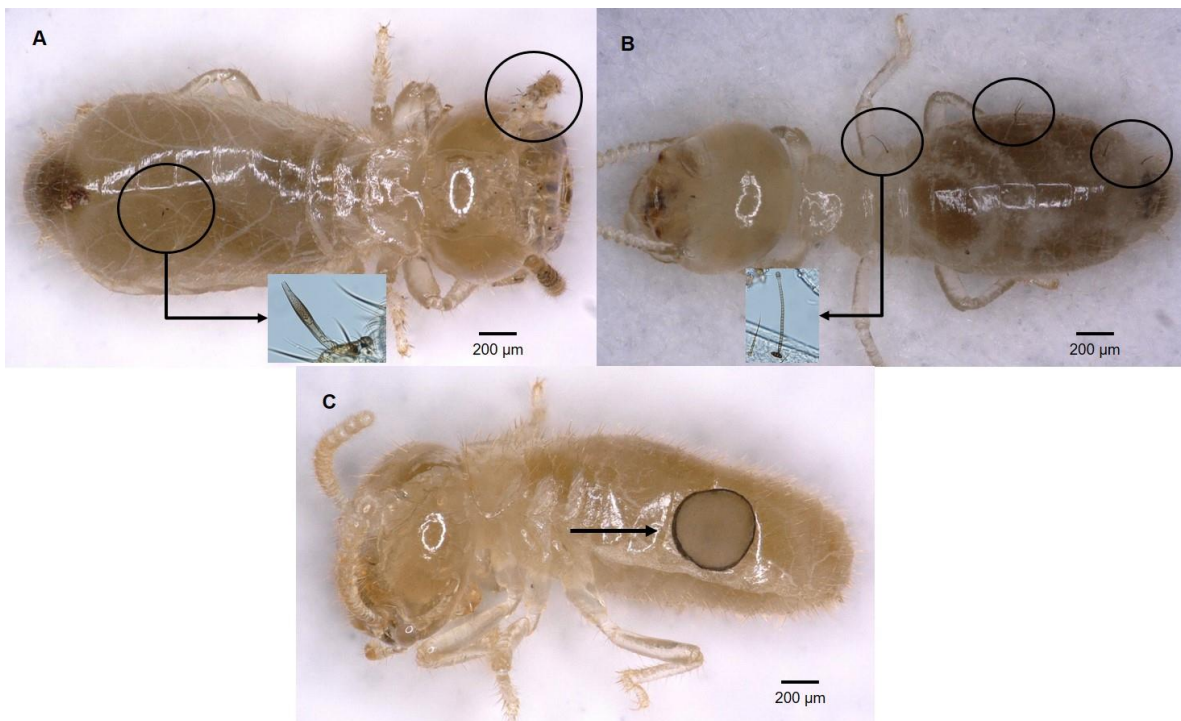


Fig. 1.1. Three common ectoparasitic fungi, *Laboulbeniopsis termitarius* Thaxt (A), *Antennopsis gallica* Buchli and Heim (B) and *Termitaria* sp. (C), infecting workers of *Reticulitermes speratus* (Kolbe).

The presence of ectoparasitic fungi on termites might interrupt both the molting process and termite behavior. Certain ectoparasitic fungi have been suspected of causing the death of termite colonies. Lenz and Kimbrough (1982) discovered that termites infected by the ectoparasitic fungus *Termitaria* sp. tended

to have lower survival than non-infected termites. Similarly, *Nasutitermes corniger* Motschulsky and *N. ephratae* Holmgren in Panama infected by *Mattirolella crustosa* Thaxt died rapidly compared with non-infected individuals (Thorne and Kimbrough 1982). Buchli (1952, 1960a, 1960b) reported that termites showed worsened health conditions with the presence of *A. gallica*, and died when the infection rate was high. He concluded that these effects were due to the adhesive compounds produced when *A. gallica* attaches to the host, which interfered with termite movement and feeding. Information about ectoparasitic fungi has led us to the idea that we can use these fungi as biological agents against termites. The mortality of termites infected by ectoparasitic fungi might in fact be due to a combination of fungal toxins, physical obstruction of blood circulation, nutrient depletion and invasion of organs (Goettel and Inglis 1997).

1.3. DNA-based method of fungi detection

Fungi detection methods have mainly relied on conventional tools: isolation, culturing, and morphological observation. The conventional methods are time consuming, laborious, and may require days or weeks for isolation. Therefore, in recent years, DNA-based methods have been developed and have been shown to be preferable for overcoming those problems. RFLP (restriction fragment length polymorphism), SSR (simple sequence repeats or just microsatellites), RAPD (randomly amplified polymorphic DNA), AP-PCR (arbitrarily primed PCR), ISSR (Inter-simple sequence repeats), AFLP (amplified fragment length polymorphism), SNPs (single nucleotide polymorphisms), and DarT (diversity array technology)

are molecular-based techniques used in genetic diversity studies (Abdel-Mawgood 2012). In addition, amplified ribosomal DNA (rDNA) sequencing, amplified rDNA restriction analysis, and temperature and denaturing gradient gel electrophoreses (TGGE and DGGE) of rDNA are other molecular-based techniques that have been used to elucidate microbial population structures in the environment (Hiorns et al. 1995, Borneman et al. 1996, Heuer et al. 1997, Kowalchuk et al. 1997, Smit et al. 1999). The use of molecular techniques has led to an increase in our understanding and knowledge of fungal ecology (Liesack and Stackebrandt 1992).

Among all molecular techniques, PCR-specific amplification is a rapid method used for the direct detection of DNAs or RNAs of microorganisms, both in clinical and environmental samples, to obtain accurate and quantitative data sets (Wu et al. 2003). Polymerase chain reaction (PCR) is a promising method because of its simplicity, specificity, and sensitivity (Luo and Mitchell 2002). PCR is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (Saiki et al. 1985). There are three main steps in DNA-based methods: DNA isolation or extraction, PCR amplification of DNA fragments containing the appropriate genetic markers, and detection of the DNA profiles defined by the markers (Pereira et al. 2013). Even though DNA of microbial cultures can be directly amplified using PCR, performing DNA extraction first is preferable for filamentous fungi and yeasts (Lie et al. 2000).

The process of DNA extraction might eliminate the presence of interfering substances in the biological materials, and has an important role in ensuring consistent results (Lie et al. 2000). The DNA extraction could be done using a single spore, or a boiled mycelium with a grinder (with or without liquid nitrogen) (Cenis 1992, Lie et al. 2000). The amplification of PCR typically includes target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium of PCR amplification (Promega online). For example, DNA extraction (and subsequent amplification) was used to detect entomopathogenic fungus *Beauveria bassiana* on the infected grasshopper *Melanoplus sanguinipes* in Canada (Hegedus and Khachatourians, 1996). It was also used in detecting *Metarhizium anisopliae* var. *anisopliae* infecting larvae of the sugarcane borer *Diatraea saccharalis* in Brazil (Destéfano et al. 2014).

In this study, a fast, efficient and specific targeted molecular assay was created in order to detect ectoparasitic fungi *L. termitarius* and *A. gallica* on *Reticulitermes* spp.

Chapter 2

The first record of *Laboulbeniopsis termitarius* Thaxter, an ectoparasitic fungus on the termite *Reticulitermes speratus* (Kolbe)

2.1. Introduction

As described in “General introduction”, ectoparasitic fungi is a group of fungi that attach and grow onto insects host body surface. Laboulbeniomyces (Ascomycota) are the largest class of ectoparasitic fungi associated with arthropods, mostly insects. The Laboulbeniomyces consists about 2,050 species in about 140 genera, and they produce thalli instead of mycelium (Haelewaters et al. 2012). In Japan, 168 species in 22 genera of Laboulbeniomyces have been reported mainly from beetles (Katamoto 2010). For termites, only *Termitaria* species, belonging to the different class Sordariomycetes (Ascomycota), were found in Japan by Hojo et al. (2001, 2002) from three termite species so far.

Laboulbeniopsis termitarius Thaxt is the most commonly found ectoparasitic fungi on termite body with varieties of termite species as its hosts (Blackwell and Rossi 1986). It is reported to be found in wide areas (Thaxter 1920, Blackwell and Rossi 1986, Gouger and Kimbrough 1969), but none of the report has mentioned about Japan. *Laboulbeniopsis termitarius* morphologically can be characterized by the unique shape and size. They have small body size, barely longer than termite setae, and attach to body surface of termites. *Laboulbeniopsis termitarius* can be identified from three main body structures namely: foot cell, stalk, and sporogonium. For the first approach for ectoparasitic fungi of termites, *Reticulitermes speratus* (Kolbe), the most widely distributed common species, which is also one of the most economically important wood pest insect in Japan was examined. From the results,

the number of termites to be required for discovering the fungi and the season for collection were discussed.

2.2. Materials and Methods

2.2.1. Sample preparation

Eight colonies of *R. speratus* were collected from Uji City, Kyoto Prefecture, Japan in two locations: 34°53'11.5" N 135°49'3.1" E and 34°54'33.0" N 135°47'49.6" E on September 5, 2015 and May 15, 2016, respectively. The nesting tree branches on the ground were wrapped with papers and kept inside of plastic bags, then brought back to the laboratory. A subsample consisting of 500 workers and 20 soldiers was separated from the nests and debris, and then frozen for 2 h prior to examination. The ratio in the subsamples between workers and soldiers was based on the actual ratio of the colonies. The termites were individually examined under a dissecting microscope (S8AP0, Leica, Wetzlar, Germany). Termites that were infected by ectoparasite fungi were separated and kept in a refrigerator at 4°C.

2.2.2. Morphological identification

The mounting samples were prepared for morphological identification by the method of Dring (1971). A clean slide glass was prepared with 100 µL of lacto phenol (phenol 10 g, lactic acid 10 g, glycerin 20 g and distilled water 10 g) solution at the center. Fungi thalli were removed from the termite body using an entomological pin (5ST 11252-00, Dumont, Heidelberg, Germany) and were directly embedded in lacto

phenol. A cover glass was placed on the lacto phenol solution, and ringed with nail varnish. The infected body parts of the termite were cut, dissected, and treated with the same way. The fungus was observed using a light microscope (BX51, Olympus, Tokyo) and a digital microscope (VHX-5000, Keyence, Osaka, Japan). The sizes of the thallus, sporogonium, foot, stalk, cell and the spore of the fungi were measured and recorded.

2.2.3. Molecular identification

Ensuring the fungi species, identification by DNA analysis was carried out. Genomic DNA of *L. termitarius* was extracted from infected termite using the Gentra Puregene cell and tissue kit (Qiagen, USA) according to the manufacturer's instructions and stored at 20 °C upon usage. Partial of small subunit (SSU) ribosomal RNA 18s were amplified by using designed specific primers LterSpeF2 (TATGGCCTTTGGCTGACGC), Lter SpeR2 (CTCTGACCATTGAATACTGATGC), LterSpeF4 (TCACATGCTTTTGACGGGTA) and LterSpeR4 (CACCAGACTTGCCCTTCAGT). These specific primers was designed based on the sequencing of universal primers NS17 (Gargas and Taylor 1992) and NS4 (White et al. 1990) from GeneBank.

A polymerase chain reaction (PCR) mixture was set up in a reaction volume of 20 µL using Takara EmeraldAmp Max PCR Master Mix (Takara, Japan). The PCR conditions include an initial denaturation step at 94 °C (3 min) followed by 35 cycles of 94 °C (30 s), 50 °C (30 s), 72 °C (40 s) and a final extension phase 72 °C (5 min). All PCR products were sequenced in both directions by DNA Sequencing Core – Uji campus, Kyoto University (Kyoto, Japan) by using applied Biosystems 3130xl

genetic analyzer. Sequence data from both directions were assembled and checked using Sequencer 4.9 (GeneCodes). The generated sequences were aligned by using MUSCLE as implemented in MEGA 6 with default settings (Tamura et al. 2013).

2.3. Results

2.3.1. Morphological identification

The thallus of *L. termitarius* was observed and attached to the any parts of termite body surface (abdomen, head, antennae and legs) (Fig. 2.1). The morphological identification showed that it is consisted of a foot cell, a stalk and a sporogonium, 77.0–121.11 μm in total length (Fig. 2.2.A). Foot cell pale brown, elliptical, 17.9–35.6 $\times$ 8.0–15.1 μm , with a dark black flat basal area attached to the termite cuticle (Fig. 2.3.A–B). The connecting part of stalk and foot cell consist of a septum (Sp) (Fig. 2.3.C). Stalk consisted of two cylindrical cells, the lower cell adjoining the foot cell, longer, 20.2–35.8 $\times$ 6.5–8.0 μm , and the cell adjoining the sporogonium, shorter, 11.2–22.7 $\times$ 7.0–9.5 μm (Fig. 2.2.A, C). Sporogonium, straight to slightly curved, 38.0–65.3 $\times$ 9.0–13.3 μm , slightly inflated below, tapering to the distal end (Fig. 2.2.A–B). Spores broad elliptical, 2.5–5.8 $\times$ 2.0–3.0 μm (Fig 2.2. D–E), released through an apical pore surrounded by dark brown ring of cell wall (Fig. 2.2.D).

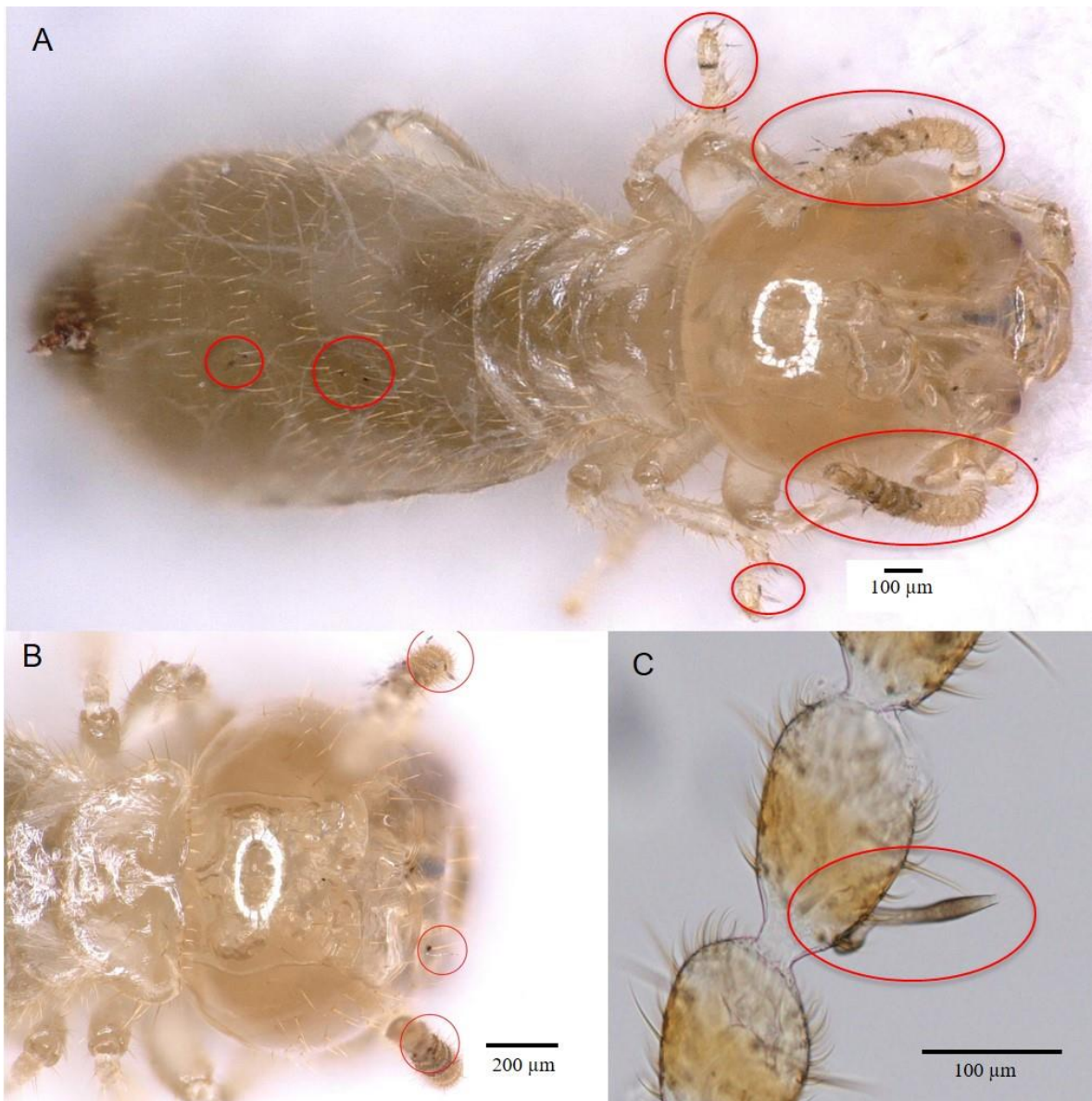


Fig. 2.1. A and B: *Reticulitermes speratus* infected by *Laboulbeniopsis termitarius*. Thalli of *L. termitarius* on head, leg, abdomen, and antenna of a *R. speratus* worker (red circle). **C:** A thallus of *L. termitarius* on termite antenna.

The fungi specimen were examined and collected at Kosai, Uji (close to Uji river) (34°53'11.5" N 135°49'3.1" E) and Kyoto University Uji Campus (34°54'33.0" N 135°47'49.6" E), Uji-shi, Kyoto Prefecture, Japan. Total of eight colonies were

collected from the sampling location and all had/ harbored *L. termitarius*. Host Insect: *Reticulitermes speratus* (Kolbe). The voucher slide has been deposited in the National Museum of Nature and Science, Japan, TNS-F-54007.

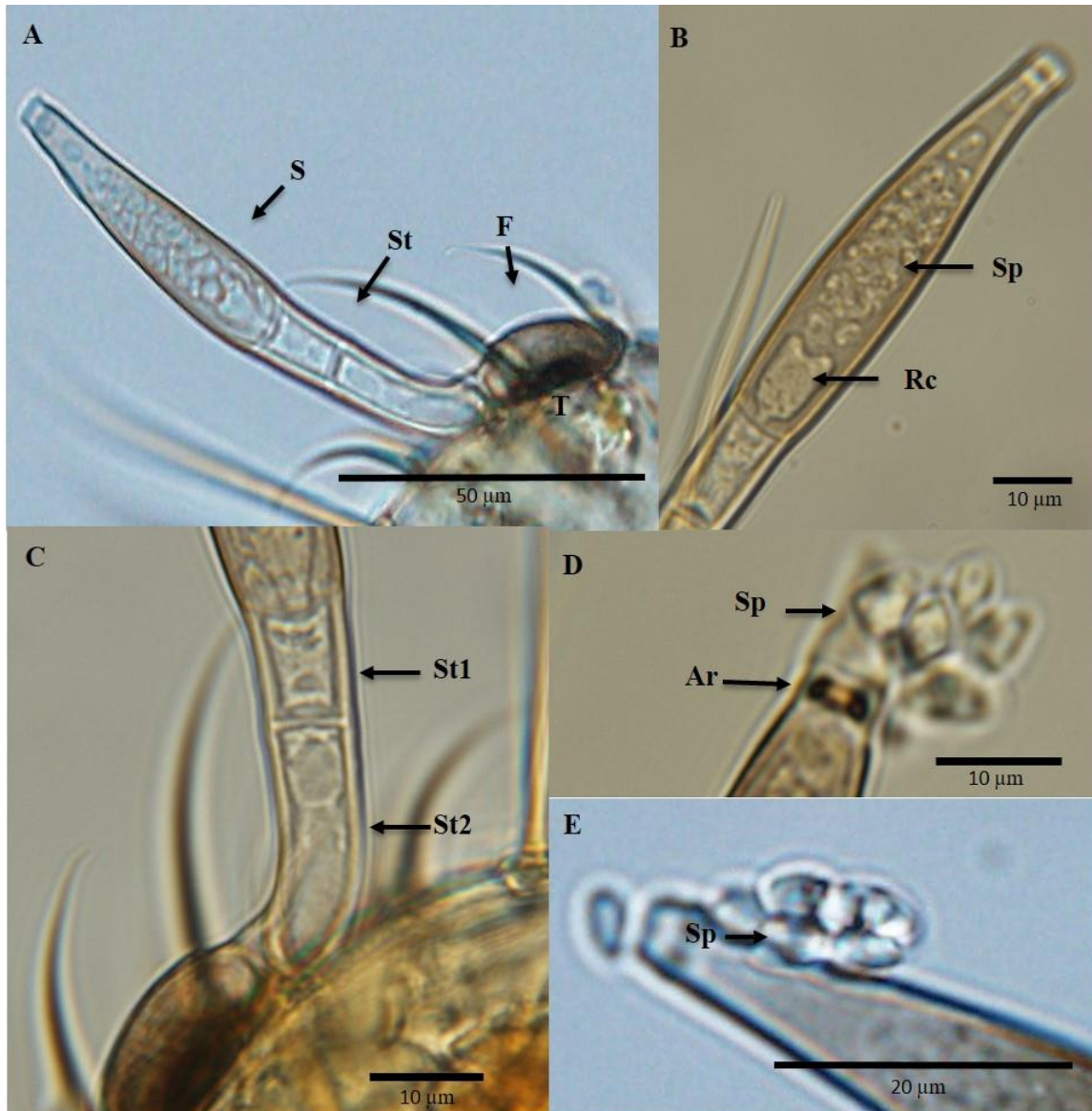


Fig. 2.2. **A:** Entire mature thallus of *Laboulbeniopsis termitarius* (A) consist of sporogonium (S), stalk (St), and foot cell (F). **B:** Sporogonium. Spores (Sp) waiting for their release with residual cytoplasm (Rc) at the base of sporogonium (B). **C:** Two cylindrical cells (C) of 2 stalks: St1 is connected to sporogonium, and St2 is connected to foot cells. **D** and **E:** showed the spores (Sp) came out from the apical ring (Ar).

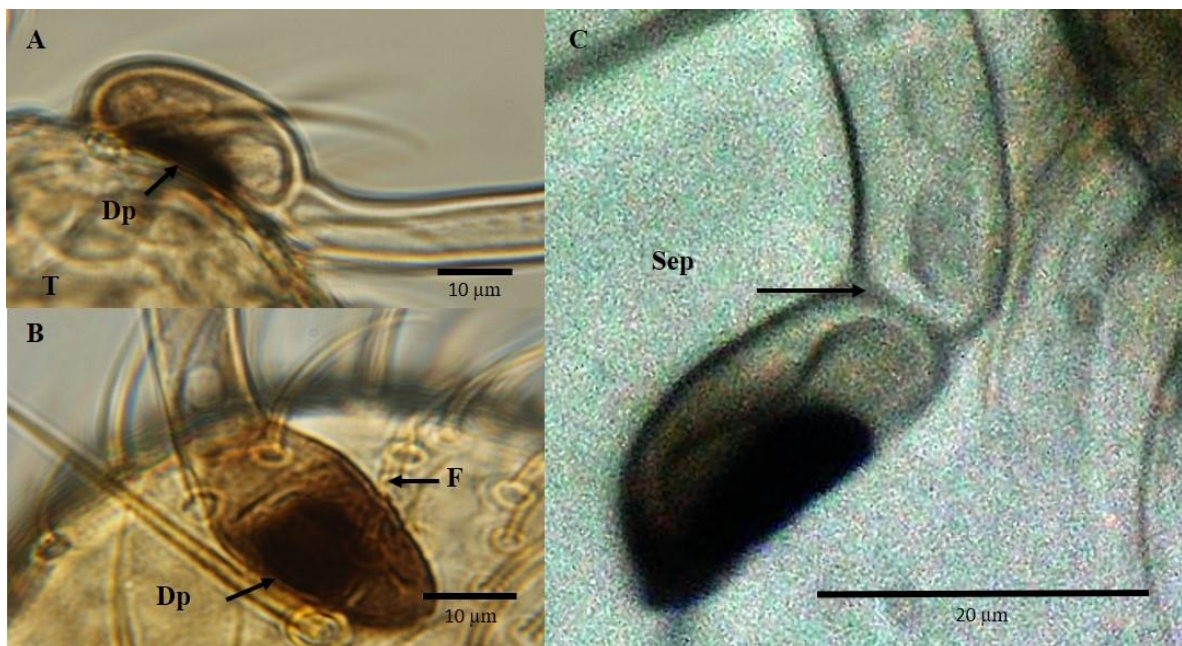


Fig. 2.3. **A** and **B**: The foot cell of *Laboulbeniopsis termitarius* from side (A) and top (B) view with dense pad in black color (Dp). **C**: A foot cell showing the septum (Sep) between foot cell and stalk.

2.3.2. Molecular identification

The morphological characters of the mature thallus of *L. termitarius* on termites in Japan was typically same as that of original description by Thaxter (1920) for the British West Indies collection and other description by Kimbrough and Gouger (1970) for the Florida collection. The partial 18S rDNA gene (602 bp) of *L. termitarius* was recovered from this study. However, there was 34 bp insertion or deletion of nucleotide sequence (indel) between our sample and the reference sequence from Louisiana. The *p*-distance between these two sequences was 2.3 % excluding indel. The difference of indel value might because the location distinction of *L. termitarius*. The sequence was aligned with previously published sequence in GenBank (accession number AY212810).

The sequence data has been deposited in GenBank with accession numbers MF785100. The infection level of *L. termitarius* in termite colonies and individual termite can be characterized by the infection rate and thallus number grown on each termite. There were 3 to 18 termite workers (0.6%–3.6% infection rate) from 500 individuals and no soldiers from 20 individuals were infected by *L. termitarius* from each of the eight colonies in this study (Table 2.1). There was no significant difference between the two collection sites with two different season ($P < 0.05$). Both locations showed a low infection rate, regardless of the season. Furthermore, the infection strength of *L. termitarius* is characterized by the thallus number grown per termite. We classified the thallus number per individual into 5 groups: 1–5; 6–10; 11–15; 16–20; >20. Numbers of thalli grown on an individual were from a single thallus up to 16 (Table 2.1). The infection strength from the two different collection time showed a similar pattern, where termite numbers in the group of 1–5 were significantly higher than other thallus number groups. The time of termite collection, Sep (autumn) and May (spring), showed no significant effect on the infection strength per infected termite ($P < 0.05$).

2.4. Discussion

Since the original description by Thaxter in 1920, this fungus has been recorded in several places with different termite hosts and environmental conditions. The geographical distribution range of *L. termitarius* covers North to South America, Africa and Southeast Asia (Blackwell and Rossi 1986) with involves various host species: *R. flavipes* (Kollar) in Florida (Kimbrough and Gouger 1970; Blackwell and-

Table 2.1. Termite infected number by *Laboulbeniopsis termitarius* and the thallus number per infected termite.

Sampling locations	Colonies	Workers	Soldiers	Infected	Infected	Thallus Number				
				workers	soldiers	1 - 5	6 - 10	11 - 15	16 - 20	>20
Uji	C1	500	20	8	0	2	2	3	1	0
	C2	500	20	3	0	2	1	0	0	0
	C3	500	20	6	0	5	0	1	0	0
Kyoto	C1	500	20	15	0	5	2	3	4	0
Univ. Uji campus	C2	500	20	12	0	9	3	0	0	0
	C3	500	20	9	0	7	2	0	0	0
	C4	500	20	18	0	14	4	0	0	0
	C5	500	20	11	0	7	1	3	0	0

Kimbrough 1976), Georgia (Blackwell 1980), Michigan and Louisiana (Blackwell and Rossi 1986), *R. virginicus* Banks in Florida (Kimbrough and Gouger 1970), *R. lucifugus* Rossi in Italy (Rossi and Cesari Rossi 1977), *Leptomyxotermes doriae* Silvestri in Sierra Leone, *Ahmaditermes* sp. in Indo-China, *Nasutitermes* sp. in Indonesia, and *Coptotermes crassus* Snyder in French Guiana (Blackwell and Rossi 1986).

Discovering *L. termitarius* in Japan extends the geographical range to East Asia, and introduce a new host to the record, *R. speratus*. Blackwell (1980) observed thalli of *L. termitarius* growing on legs, mouthparts and antennae of *R. flavipes*. The present observation showed that *L. termitarius* grows on the leg, antenna, abdomen, head, and sometimes the thorax and mandible of *R. speratus*. In our study the most infested body part was antenna, followed by the abdomen, leg, head, and thorax. Our observation also clearly supports that the growth of *L. termitarius* is not limited to a specific part of termite body, but can occur on any part of the termite body surface.

The infection rate of *L. termitarius* was varied, and most of the reports have observed laboratory – as opposed to wild – colonies, and the infection rates were over 20% (Blackwell 1980, Kimbrough and Gouger 1970, Henk et al. 2003). Blackwell (1980) found 25–53% infection rates from 19 out of 21 *R. flavipes* collection, while Kimbrough and Gouger (1970) reported that less than 40% individuals from their samples were infected by *L. termitarius*. The most recent study of *L. termitarius* by Henk et al. (2003) showed the infection rate from the

collection of *R. flavipes* as 20%, but they did not mention the number of termite being observed. Regarding the number of thalli per individual, Kimbrough and Gouger (1970) reported 30–35 thalli of *L. termitarius* on *R. flavipes* individuals.

The lower infection rate suggests that at least several hundred termites are required for detection of this ectoparasitic fungus in at least in Kyoto. The infection rate of *L. termitarius* on *R. speratus* in this study were relatively low when compared with the previous studies on *R. flavipes* (Kimbrough and Gouger 1970, Blackwell 1980, Henk et al. 2003). A possible cause of both lower infection rate and smaller number of thalli on an individuals might be due to collecting termites in the high activity season under favorable environmental conditions (September: ave. 22.9°C, max. 27.5°C, min. 19.5°C, 70% RH; May: ave. 21.0°C, max. 27.0°C, min. 15.8°C, 59% RH; Japan Meteorological Agency, <http://www.jma.go.jp/jma/indexe.html>).

The activity of *R. speratus* is highest at temperatures of 25–30°C and decreases over 30°C and below 12°C (Kambara et al. 2017). Those data clearly support the high activity of the collected termites in this study. As a social insect, *R. speratus* engages in grooming or cleaning their bodies with nest-mates like other eusocial insects (Wilson 1971). The grooming activity has been reported to be beneficial to termites in conferring resistance and as an antifungal defense strategy (Boucias et al. 1996, Shimizu and Yamaji 2003, Yanagawa and Shimizu 2005). High grooming activity of the host insect might have resulted in the lower infection rate of colonies as well as the lower thallus number in individual termite.

A higher infection rate on both individuals or in termite colonies was reported for winter in the case of *Antennopsis gallica*, another ectoparasitic fungus of termites (Buchli 1952).

2.5. Summary

Laboulbeniopsis termitarius Thaxter, an ectoparasitic fungus of termites was collected from the body surface of *Reticulitermes speratus* (Kolbe) (Isoptera: Rhinotermitidae), collected from Uji, Kyoto Prefecture, Japan. This is the first record of this fungus from Japan. Three to eight termite workers from 500 individuals were infected in three colonies investigated. Several hundreds of termite are required for the survey of this ectoparasitic fungi in Japan. The temperature effect on relationship between the infection rate and the host activity.

Chapter **3**

**The first record of *Antennopsis gallica*
Buchli and Heim, an ectoparasitic fungus on
the termite *Reticulitermes speratus* (Kolbe)**

3.1. Introduction

In the proceeding chapter, Chapter 2, *Laboulbeniopsis termitarius* Thaxt was found in eight colonies from Uji, Japan. Several hundreds of termites were required to be observed in order to detect the occurrence of ectoparasitic fungi, due to its low infection rate. As mentioned in Chapter 1, 22 species from 9 genera of ectoparasites fungi have been reported on termites (Blackwell and Rossi 1986), and one of the most termite-specific genus is *Antennopsis*. In 1952 Buchli and Heim described *Antennopsis gallica* on *Reticulitermes lucifungus* Rossi in southern France, proposed the genus *Antennopsis*, and placed it in a new order, Gloeohaustoriales, of the class Hyphomycetes (Buchli 1952).

Presently the genus *Antennopsis* contains three species: *Antennopsis gallica* Heim and Buchli, *A. grassei* Buchli and *A. gayi* Buchli. Each species of *Antennopsis* is specific to a specific termite species. Buchli (1960b) discovered *A. grassei* on *Neotermes amplius* Sjost, *Neotermes desneuxi* Sjost and *Glyptotermes longiceps* Paulian from Madagascar. *Antennopsis gayi* was found on *Coptotermes elisae* Desneux and *C. obiratus* Hill from New Guinea (Buchli 1966). *Antennopsis grassei* and *A. gayi* occurred on termites in Madagascar, South America, Africa and Asia (Blackwell and Rossi 1986). *Antennopsis gallica* was found on *Kaloterms flavicolis* Fabricius, *R. virginicus* Banks and *R. flavipes* (Kollar) in Italy, USA, Canada, and Brazil (Buchli 1960b, Gouger and Kimbrough 1969, Blackwell 1980, Blackwell and Rossi 1986, Myles et al. 1998, Blackwell and Kimbrough, 1978). Arai et al. (2009) presented the *Antenopsis* sp. from *R.*

speratus colonies in Japan, but the identification of the species was not done yet. In the present chapter, Chapter 2, following the first discovery of *L. termitarius* in Japan, *Antennopsis* species associated with *R. speratus* (Kolbe) was targeted.

3.2. Materials and Methods

3.2.1. Sample preparation

Termites were collected from Uji City, Kyoto Prefecture, Japan (34°53'11.5" N 135°49'3.1" E) on September 5, 2015 as described in Chapter 2. Three colonies of termite were collected from dead tree branches on the forest ground, and were wrapped with paper and kept inside plastic bags, then brought back to the laboratory. The weather conditions during sampling were measured. Five hundred workers and twenty soldiers were chilled by a refrigerator and examined for infection by ectoparasitic fungus by observing them individually under a dissecting microscope (S8APO, Leica, Wetzlar, Germany).

3.2.2. Morphological identification

The infected termites were separated and kept at 4°C prior to identification. Mounting samples were prepared for morphological identification by the method of Dring (1971). A clean slide glass was prepared with 100 µL of lacto phenol (phenol 10 g, lactic acid 10 g, glycerin 20 g and distilled water 10 g) solution at the center. An infected body part of *R. speratus* was dissected using an entomological pin (5ST 11252-00, Dumont, Heidelberg, Germany) and mounted

with lactophenol solution. A cover glass was placed on the lacto phenol solution, and ringed with nail varnish. The infected body parts of the termite were cut, dissected, and treated with the same way. The fungus was observed using a light microscope (BX51, Olympus, Tokyo) and a digital microscope (VHX-5000, Keyence, Osaka, Japan) for the identification process. Infection rates of 500 workers and 20 soldiers were counted by counting the numbers of thalli per individual termite, and recording the infected body parts.

3.3. Results

3.3.1. Morphological identification

An *Antennopsis* species was found from all the 3 colonies of *R. speratus* (Fig. 3.1). It showed three major thallus parts: namely, a holdfast (H), conidiophores (C) and conidial head (Ch) (Fig. 3.2). The holdfast (H) has an ellipse shape 21.49 μm in length and 8.90 μm in width on average. As the part that attaches to the termite cuticle (Tc), it consists of four cells with an average cell size of 5.17 μm . One or two conidiophores (C) were commonly observed from the two-center holdfast cells, and they were 104.12 μm in length and 5.10 μm in width. They comprised 18–27 cells with strong constriction at the base. The conidial head (Ch) was 23.18 μm in length by 8.92 μm width on average, comprising 3–10 cells, and showing an oval shape at maturity. Spherical spores ca. 3–4 μm in diameter were released from the conidial head. These morphological characteristics clearly suggested that the fungus was *A. gallica*,

although only two spores were observed. A specimen of *A. gallica* was deposited at the National Museum of Nature and Science, Japan with registration number TNS-F-54006.

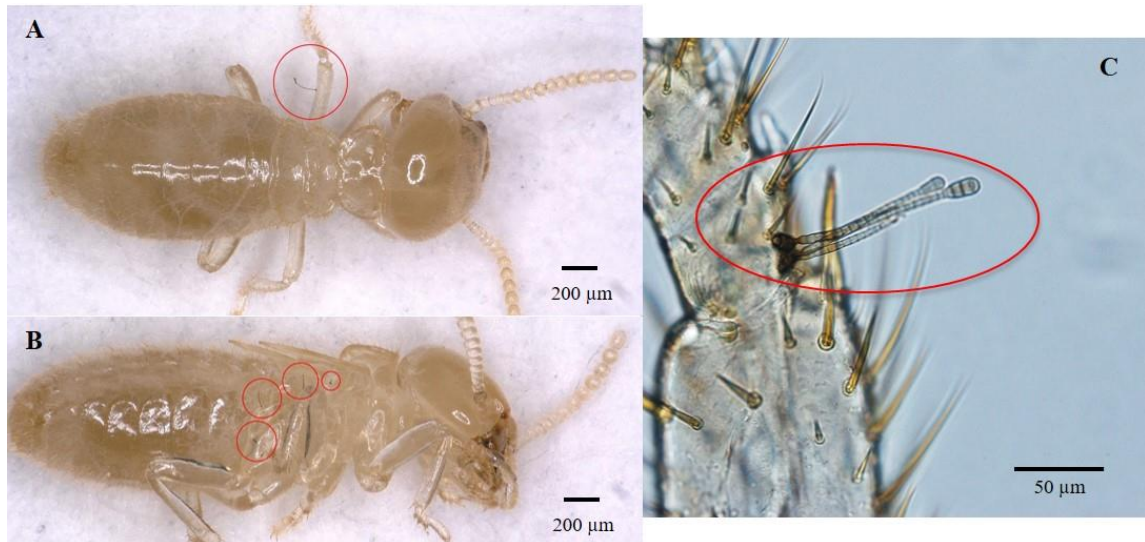


Fig. 3.1. **A** and **B**: Workers of *Reticulitermes speratus* infected by *Antennopsis gallica* Heim and Buchli. **A** thallus growing on the leg (**A**) and thorax (**B**) (red circle) of a *R. speratus*. **C**: Thallus of *A. gallica* on termite leg.

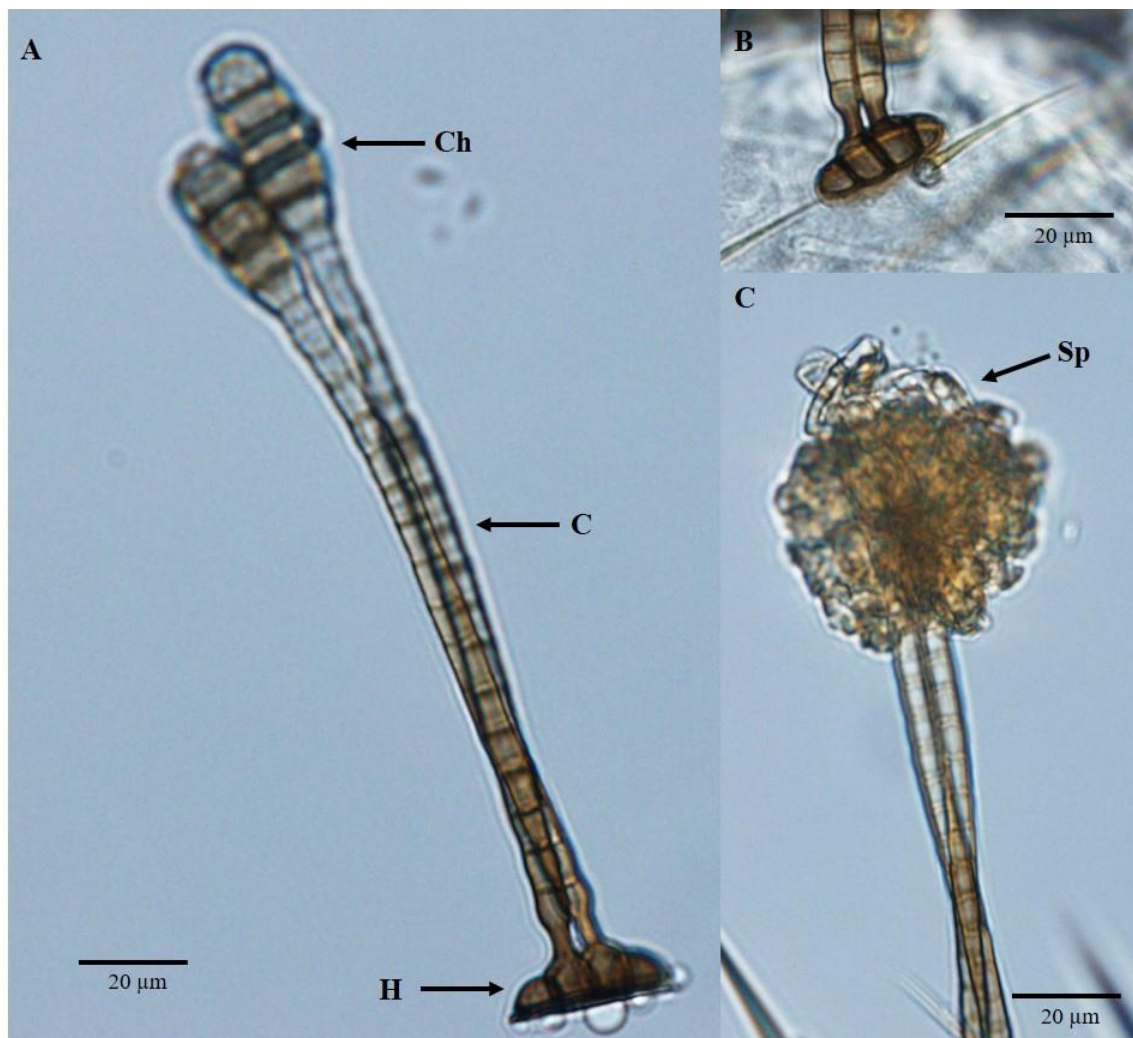


Fig. 3.2. **A:** Two thalli of *Antennopsis gallica* from the holdfast with an immature conidial head. **B:** A foot cell/holdfast of *A. gallica* attach to termite cuticle. **C:** A bunch of spore after releasing from conidial head. Holdfast (H), conidiophore (C), and conidial head (Ch).

3.3.2. Infection rate and strength

The infection rate of *A. gallica* in 3 colonies of *R. speratus* from Uji City was stable in 500 workers (17.8–25.0%) (Table 3.1). For soldiers, a 0–10% infection rate (0–2 individuals) was observed for all 3 colonies (Table 3.1). On the other

hand, number of thallus per infected termite were recorded varies from 3 up to 50 per individual termite worker (Table 3.2). The thalli numbers were classified into 5 groups: 1–5, 6–10, 11–15, 16–20 and ≥ 21 thalli/worker. The number of thalli per termite showed a similar pattern in the 3 colonies. The majority of infected workers had 1–5 thalli, followed by 6–10 and/or 11–15; workers with 21 or more thalli showed the lowest rate in all the colonies. Of the 307 infected workers from 3 colonies of *R. speratus*, the legs showed the highest thallus number followed by antennae, abdomen, head capsule, mandible and thorax (Table 3.3).

Table 3.1. The infection rate of *Antennopsis gallica* in workers and soldiers of 3 colonies of *Reticulitermes speratus*.

Colony number	Observed termite number		Infected termite (%)	
	Workers	Soldiers	Workers	Soldiers
C1	500	20	93 (18.6)	0
C2	500	20	125 (25.0)	2 (10)
C3	500	20	89 (17.8)	2 (10)

3.4. Discussion

The morphology of *A. gallica* in the present study was similar to that first described by Buchli on the fungus obtained from *R. lucifungus* in France (Buchli 1952). He described it as an extraordinary fungus with 3 major body structures, namely conidial head, conidiophores and holdfast (Buchli 1960a). From his description, *A. gallica* has a 23- μ m-long ellipsoid holdfast and a 100- to 280- μ m-long conidiophore on average. In 1969, Gouger and Kimbrough reported the existence of *A. gallica* on *R. virginicus* and *R. flavipes* in and around Gainesville, Florida, USA. They measured the size of the holdfast (24–27 μ m in length, 8–9 μ m width, and 6–6.5 μ m height), the length of conidiophores (100–500 μ m) and illustrated the fungus development stage in detail.

Table 3.2. The number of *Antennopsis gallica* thalli recorded from the body of workers of *Reticulitermes speratus*.

Colony	Number of thalli per worker					Average
Number	1–5	6–10	11–15	16–20	≥ 21	
C1	46	24	18	3	2	7.7
C2	53	21	37	10	4	7.0
C3	59	23	5	2	0	7.1

The distribution of *A. gallica* covers a wide geographical range with different termite species as hosts: *Antenopsis gallica* was found on *R. lucifungus*

santonensis later synonymized with *R. flavipes* (Austin et al. 2005) in France (Heim 1951), on *R. virginicus* and *R. flavipes* in Florida (Gouger and Kimbrough 1969), Georgia (Blackwell 1980), Wisconsin and Michigan (Blackwell and Rossi 1986), on *Reticulitermes* sp. in Louisiana (Blackwell and Rossi 1986), on *Kaloterms flavicollis* in Yugoslavia (Buchli 1969), on *R. lucifungus* in Italy (Rossi and Cesari Rossi 1977) and on *R. flavipes* in Canada (Myles et al. 1998). The new record of *A. gallica* in Japan with a different host termite, *R. speratus*, further expands the known geographical range of the fungus. A country-wide survey is required to gain more information on its distribution in Japan. Also, in order to clarify the precise morphological development including infection process and lifecycle of *A. gallica*, artificial cultivation of the fungus is indispensable.

Table 3.3. Distribution of the fungus *Antennopsis gallica* in body parts of 307 workers of *Reticulitermes speratus*.

Colony Number	Location of fungi infection					
	Head capsule	Antenna	Thorax	Mandible	Legs	Abdomen
C1	77	135	39	45	337	83
C2	79	145	45	58	452	96
C3	63	120	31	50	288	75

Myles et al. (1998) and Buchli (1952) reported that nymphoids and soldiers were the castes of *R. flavipes* most heavily infected by *A. gallica*, but they did not

show any comparable data on infection rates. Their results conflict with our observation with *R. speratus* that the workers had a higher infection rate than that of soldiers. Our results might be explained by the high activity of workers compared to soldiers. Workers are the major caste in termite colonies, and they do most of the duties in the colony such as foraging, nursing and grooming. When they forage outside the nest, they have more opportunities to come into contact with fungi and bring them back to the nest.

Workers of *R. speratus* also engage in grooming activities, and they groom and clean themselves and nestmates including soldiers (Wilson 1971). This social behavior facilitates the transmission of ectoparasitic fungi even though grooming also has the benefit in termites of conferring resistance and is an antifungal defense strategy (Boucias et al. 1996, Shimizu and Yamaji 2003, Yanagawa and Shimizu 2005 and 2007). Further study of *R. speratus* colonies from other parts of Japan is required to discuss the caste-dependent infection trends. The number of thalli on a single infected termite has a reported range of 1–35 (Gouger and Kimbrough 1969, Blackwell 1980, Blackwell and Kimbrough 1978, Kimbrough and Gouger 1970). Another report mentioned that each individual termite can be infested with up to 150 thalli (Blackwell and Kimbrough 1976). The highest number of fungal thalli was counted by Myles et al. (1998) on *R. flavipes*: 479 thalli on one infected termite.

Termites were collected in September, when the monthly average temperature was 22.9°C with 70% relative humidity (RH). Generally, fungi can

grow in a wide range of temperatures and RH, but their growth is affected by environmental conditions. The observation of *A. gallica* on *R. speratus* showed a lower infection rate in comparison with those of most records of ectoparasitic fungi on termites. Buchli (1952) reported that he took his sample during winter and found higher infection rates both per individual and per colony. The relatively low infection rates in the present study may have a relation with the season. Further seasonal studies are needed to determine the effect of season on infection rate.

Due to difficulties in observing *A. gallica* in a living and moving termite, host insects were examined after being killed. Therefore, the observation of effects of *A. gallica* on termite behavior such as movement, feeding activity, and grooming was not available. In the present investigation, the visual observation of infected termites showed no differences of body size and color from those of uninfected individuals. Myles et al. (1998) also observed that there was no difference in size and color of infected *R. flavipes* termites compared to infection-free individuals.

Thalli of *A. gallica* did not restrict themselves to specific body sections of *R. speratus*. The fungal thalli of *A. gallica* were found on every body part including head capsule, antennae, mandible, thorax, legs, and abdomen. According to the report by Myles et al. on *R. flavipes* in Canada (1998), *A. gallica* were found on many parts of the body, with the abdomen being the most infected area, followed by the thorax and the head. These results strongly suggest that *A. gallica* does not show a preference for the particular body part of termites on which to grow.

To clarify the biology of *Antennopsis gallica*, more data are required. The latest report regarding *A. gallica* was published in Canada as a new biodiversity report (Myles et al. 1998). Unfortunately, there are no comparable data regarding environmental factors and infection rates. Country-wide and seasonal surveys will be the next targets. In addition, the artificial cultivation of *A. gallica* is indispensable to investigate the behavioral effect of *A. gallica* on termites and to evaluate the possibility of using this widely distributed ectoparasitic fungal group as a biocontrol agent.

3.5. Summary

The ectoparasitic fungus *Antennopsis gallica* Buchli and Heim was found from three colonies of *Reticulitermes speratus* (Kolbe) collected from Uji, Kyoto Prefecture. This is the first record in Japan of this species. The infection rate was 17.8–25.0% in workers and 0–10% in soldiers. On average, five thalli were found per individual. The fungus grew on any surface of the termites and showed no preference for particular body parts.

Chapter **4**

Surveillance of ectoparasitic fungi *Laboulbeniopsis termitarius* Thaxt and *Antennopsis gallica* Buchli and Heim on subterranean termite *Reticulitermes* spp. in Japan

4.1. Introduction

Laboulbeniopsis termitarius and *Antennopsis gallica* from termite colonies in Uji, Japan were found (Chapters 2 and 3, respectively). *Antennopsis gallica* has been found from *Reticulitermes speratus* (Kolbe) as the first record in Japan (Guswenrivo et al. 2017), the most widely distributed common species, and one of the most economically important wood pest insects in Japan. However, there has been no national survey on the distribution of ectoparasitic fungi in Japan so far. As described on Chapters 2 and 3, the infection rate and infection level of *L. termitarius* and *A. gallica* were affected by the environmental factor. Japan is an island country with varied environmental conditions, from the southern part (Okinawa prefecture) to northern part (Hokkaido prefecture). Therefore, the distribution and abundance of these fungi was stood out to discuss the environmental factors affecting to the growth and distribution of the fungi.

On the other hand, culturing the fungi in the laboratory is indispensable in order to understand the effect of ectoparasitic fungi on termite activity. In this chapter, Chapter 4, the distribution and abundance of 2 species of ectoparasitic fungi, *L. termitarius*. and *A. gallica* in Japan was surveyed together with trials on isolation and cultivation of the fungi in the laboratory.

4.2. Materials and Methods

4.2.1. Sampling and preparation for observation

Colonies of *Reticulitermes* spp. were collected from 17 locations in Japan: Hokkaido 3 colonies, Akita 4 colonies, Niigata 5 colonies, Miyagi 5 colonies, Ibaraki 5 colonies, Toyama 5 colonies, Gifu 3 colonies, Aichi 2 colonies, Shizuoka 2 colonies, Kyoto 3 colonies, Wakayama 4 colonies, Okayama 3 colonies, Tokushima 4 colonies, Yamaguchi 5 colonies, Oita 4 colonies, Kagoshima 3 colonies and Okinawa 3 colonies. Sampling locations, season and temperatures are summarized in Table 4.1. The colonies of termite were collected from dead tree branches on the forest ground, and were wrapped with paper and kept inside plastic bags, then brought back to the laboratory. The weather conditions during sampling were measured. Five hundred workers and twenty soldiers were chilled by a refrigerator and examined for infection by ectoparasitic fungus by observing them individually under a dissecting microscope (S8APO, Leica, Wetzlar, Germany). The numbers of thalli per individual termite, and the infected body parts were recorded.

4.2.2. Morphological identification

The infected termites were separated and kept at 4°C prior to identification. Mounting samples were prepared for morphological identification. Fungus thalli were removed from the termite body using an entomological pin (Dumont 5ST

11252-00, British Columbia, Canada) and embedded in lacto phenol solution (phenol 10g, lactic acid 10g, glycerin 20g and distilled water 10g). The fungus was observed using a light microscope (BX51, Olympus, Tokyo, Japan) and a digital microscope (VHX-5000, Keyence, Osaka, Japan) for the identification process. Standard keys were used to identify the fungi and consulted to experts for confirmation, Thaxter (1920) and Kimbrough and Gouger (1970) for *L. termitarius*, and Buchli (1960) and Gouger and Kimbrough (1969) for *A. gallica*.

4.2.3. Isolation and laboratory cultivation of the fungi

Infected termites were separated, and the fungus thallus was removed aseptically and placed on media. Eight different media (7 commercial and 1 artificial) were prepared: water agar (WA), potato dextrose agar (PDA), L-broth agar (LBA), molisch agar (MA), brain heart infusion (BHI) oatmeal agar (OA), cornmeal agar (CA) and termite agar (TA). Termite agar (TA) medium was prepared by mixing 2% of termite (wet weight) and 2% agar in distilled water. Two ppm streptomycin was added in all the media as an anti-biotic agent to control bacteria. Inoculation was conducted with two ways: a) Fungi were directly inoculated on the surface of the media, and b) fungi were inoculated on sterile termite alate wing on the surface of the media. The fungi were incubated in the Deterioration Organism Laboratory (DOL), Research Institute for Sustainable Humanosphere (RISH), Kyoto University under the dark condition with $26\pm 1^{\circ}\text{C}$

and 60±5% relative humidity. The growth of fungi was observed every 2 days for the first two weeks, and every week up to 3 months.

4.2.4. Statistical analysis

The data from each observation was statistically analyzed. The analysis of variance was used to evaluate the significant difference between factors and levels. A two-factor analysis of variance (ANOVA) was used to test the influence of sampling locations, and infected body part of termite into number of thallus. The comparison of the means was done by using Tukey HSD post hoc test to identify which groups were significantly different from other groups at 95% confidence level.

4.3. Results

4.3.1. Distribution of ectoparasitic fungi

A Total of 63 *Reticulitermes* spp. colonies were collected from 17 locations over Japan. The samples were collected in different season: 1 sample on winter (Hokkaido), 5 samples on spring (Ibaraki, Gifu, Yamaguchi, Oita, and Kagoshima), 4 samples on summer (Akita, Toyama, Tokushima, and Okinawa), and 7 samples on autumn (Niigata, Sendai, Nagoya, Shizuoka, Kyoto, Wakayama, and Okayama) (Table 4.1). Termite colonies collected from Hokkaido and Okinawa have been kept in the lab over 6 months before observation. The results showed that *Reticulitermes* spp. in Japan were widely infected by *L. termitarius* and *A.*

gallica. Both species were found most of the sampling locations (Fig. 4.1). *Laboulbeniopsis termitarius* was widely distributed (Hokkaido, Akita, Niigata, Miyagi, Ibaraki, Toyama, Gifu, Aichi, Shizuoka, Kyoto, Wakayama, Okayama, Yamaguchi, Oita, Kagoshima and Okinawa) around Japan, while *A. gallica* was not found in termite colonies in Akita, Gifu, Shizuoka, Yamaguchi and Kagoshima. Termite colonies were infected by one or two species of ectoparasitic fungi at the same time.

Table 4.1. Season and temperature of sampling locations at collection period (source: Japan Meteorological Agency, <http://www.jma.go.jp/jma/indexe.html>).

No.	Sampling location		Season	Sampling date	Temperature of the sampling date		
	Prefecture	City/Town			average	Max	Min
1	Hokkaido	Sapporo	Winter	20 Dec 2014	-1.3	3.7	-4.3
2	Akita	Noshiro	Summer	17-18 June 2015	19.9	24.4	15.9
3	Niigata	Niigata	Autumn	15 Oct 2015	15.8	19.9	11.5
4	Miyagi	Sendai	Autumn	16 Oct 2015	15.5	20.4	11.0
5	Ibaraki	Shirosato	Spring	25 May 2015	18.8	24.3	13.8
6	Toyama	Imizu	Summer	23 Aug 2015	26.7	30.6	23.3
7	Gifu	Ena	Spring	11 May 2015	21.3	27.2	16.2
8	Aichi	Nagoya	Autumn	15 Sept 2015	23.1	27.5	19.8
9	Shizuoka	Shizuoka	Autumn	4 Sept 2015	23.3	27	20.4
10	Kyoto	Uji	Autumn	5 Sept 2015	22.9	27.5	19.5
11	Wakayama	Kozagawa	Autumn	15 Sept 2015	23.4	27	20.3
12	Okayama	Maniwa	Autumn	8 Sept 2015	19.0	24.4	15.1
13	Tokushima	Tokushima	Summer	2 June 2015	22.4	26.1	19.3
14	Yamaguchi	Yamaguchi	Spring	25-30 May 2015	19.8	26.4	13.9
15	Oita	Bungotakada	Spring	20-23 May 2015	20.1	25.0	15.5
16	Kagoshima	Hioki	Spring	21 Apr 2015	18.8	23.1	15.2
17	Okinawa	Miyako	Summer	13 Aug 2014	28.7	31.4	26.7



Fig. 4.1. The distribution of ectoparasitic fungi *L. termitarius* and *A. gallica* on *Reticulitermes* spp. from 17 locations: (1) Hokkaido, (2) Akita, (3) Niigata, (4) Miyagi, (5) Ibaraki, (6) Toyama, (7) Gifu, (8) Aichi, (9) Shizuoka, (10) Kyoto, (11) Wakayama, (12) Okayama, (13) Tokushima, (14) Yamaguchi, (15) Oita, (16) Kagoshima and (17) Okinawa.
 * Only *L. termitarius* was found in the sampling location
 ** Both *L. termitarius* and *A. gallica* were detected in the location.

4.3.2. *Laboulbeniopsis termitarius* Thaxt.

The morphological observation showed that the average length of *L. termitarius* was 98.90 μm , which was small and hardly seen by naked eyes. This fungus had three main body structures namely: foot cell (F), stalk (St) and sporogonium (S) (Fig. 4.2). The foot cell had 26.20 μm long on average, and was characterized from the doughy brown colour and had convex shape with dark black flat base attached to termite cuticle.

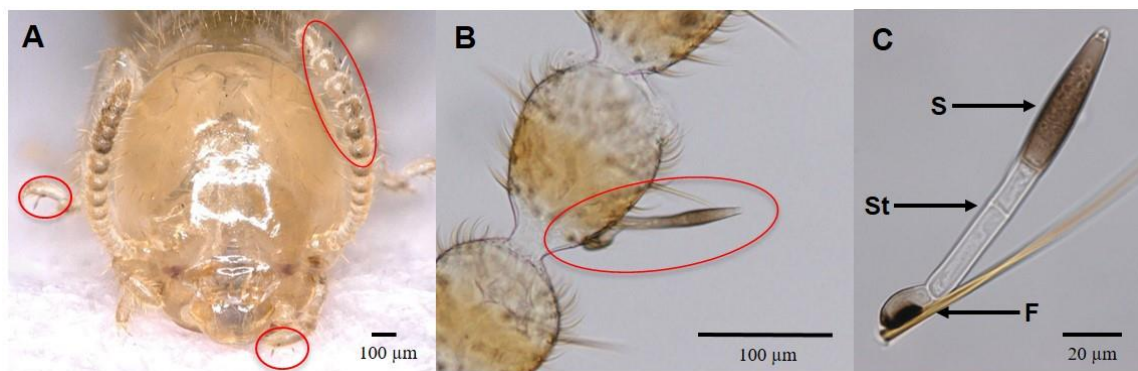


Fig. 4.2. **A:** *Reticulitermes speratus* infected by *Laboulbeniopsis termitarius* (red circle). **B:** Thallus of *L. termitarius* attach to termite antenna (red circle). **C:** *Laboulbeniopsis termitarius* construct with 3 major body structures: foot cell (F), stalk (St) and sporogonium (S).

*refer to Chapter 2 for detail morphological identification of *Laboulbeniopsis termitarius*.

The foot cell adjoining with the stalk had two cells, narrower and elongated tube shape. The length of stalk was 45.66 μm on average. The sporogonium was 52.53 μm in length on average, link on the top of the thalli, had an elongated shape, slightly bulging with apical ring. The fungal spore

came out from the apical ring when it reached to the maturity stage. The morphological identification of the fungi from 17 locations has high similarity with the first previous report, as well as the description on Chapter 2.

4.3.3. Antennopsis gallica Buchli and Heim

Similar with *L. termitarius*, the morphological observation of *A. gallica* on *Reticulitermes* spp. from 17 locations in Japan showed the high similarity with the first report by Heim and Buchli in 1951 and the fungi description on Chapter 3. It was 139.28 μm in length on average, and was slightly longer than *L. termitarius*. Similar with *L. termitarius*, *A. gallica* had three main body parts: holdfast (H), conidiophores (C) and conidial head (Ch) (Fig. 4.3). The holdfast (H) attached to termite cuticle was 20.46 μm in length on average with ellipse shape, and consisted of 4 cells. One or two conidiophores (C) were grown from the two centre cells of the holdfast, and was 105.14 μm in length on average, and comprised of 18–27 cells. The conidial head (Ch) was 22.81 μm in length on average, comprising 3–10 cells, showing an oval shape at maturity and spores released from the conidial head.

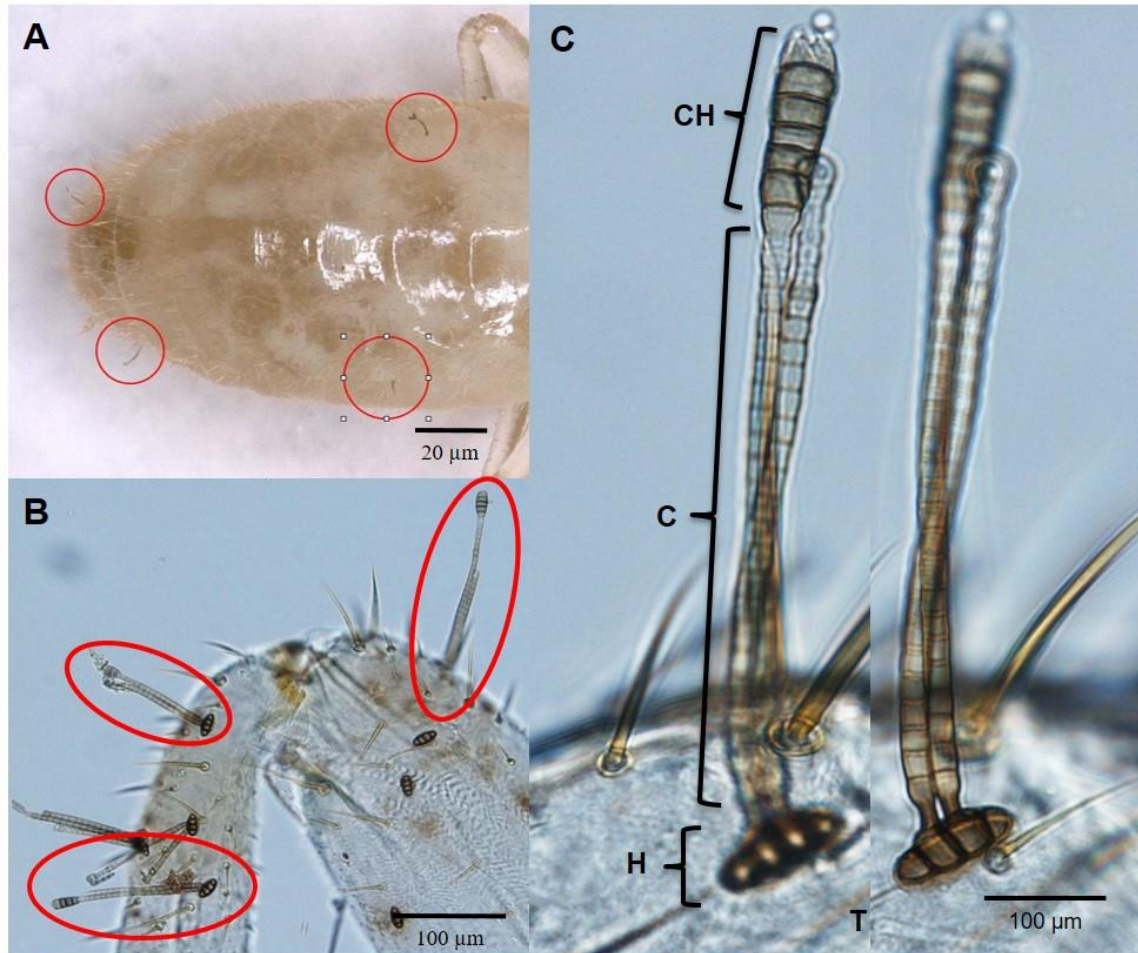


Fig. 4.3. **A:** *Antennopsis gallica* growth on *Reticulitermes speratus* abdomen (red circle). **B:** Thallus of *A. gallica* grew on *R. speratus* leg (red circle). **C:** Full body structure of *A. gallica* consist of holdfast (H), conidiophores (C) and conidial head (Ch) attach to termite cuticle (T).

*refer to Chapter 3 for detail morphological identification of *Antennopsis gallica*.

4.3.4. Infection rate and strength

Laboulbeniopsis termitarius was distributed widely in Japan. Worker termites collected from all the 17 location in Japan were infected with *L. termitarius*. On the other hand, no soldier was observed for the fungal infection. The infection

rates of the fungus varied from each location: 0.10–16.10% on average (Table 4.2). The statistically higher infection rates were obtained in Ibaraki (16.10%), Hokkaido (14.13%) and Okinawa (13.93%) colonies, and the Ibaraki colonies showed the statistically highest rate. Statistical analysis showed that sampling location gave significance effect onto fungal infection rate ($P>0.05$).

The infection strength characterized by the thallus number grown per termite was classified into 5 groups: 1–5; 6–10; 11–15; 16–20; and >20. The results showed that the infection strength of *L. termitarius* was relatively low, and the major groups were 1–5 and 6–10 in all the sampling locations (Fig. 4.4A). As shown in Fig. 4.5A, in *L. termitarius*, the higher infection rates gave the higher thallus number per termite with the linear correlation ($R^2 = 0.9665$). The fungus was found in every body parts of termites: from antenna, mandible, thorax, abdomen and legs. Legs and abdomen were significantly preferable for *L. termitarius* to grow followed by abdomen, antennae, thorax, head and mandible (Table 4.3).

The infection rates of *A. gallica* on *Reticulitermes* spp. varied much wider than those of *L. termitarius*: 0 – 66.40% on average (Table 4.2). Out of 17 sampling locations, Hokkaido gave a significantly higher infection rate. The most frequency groups of thalli number per termite was similar with *L. termitarius*: 1-5 (Fig. 4.4B). Differ with *L. termitarius*, the correlation between infection rate and thallus number on *A. gallica* had polynomial trendline ($R^2 = 0.9812$) (Fig. 4.5B). Some locations showed high infection rates such as Hokkaido (66.40% on

average), Kyoto (20.47% on average) and Okinawa (15.73% on average). Similar with the results of *L. termitarius*, *A. gallica* was found in every body parts of termites. The significantly higher strength was observed in abdomen and legs, followed by antennae, head, thorax and mandible (Table 4.3).

Table 4.2. The infection rates of *Laboulbeniopsis termitarius* and *Antennopsis gallica* on *Reticulitermes* spp. in Japan

No.	Location	Season	No. of colonies	<i>Laboulbeniopsis termitarius</i>		<i>Antennopsis gallica</i>	
				Infected colonies	Infection rate (%)***	Infected colonies	Infection rate (%)***
1	Hokkaido	Winter**	3	3/3	14.13 (cd*)	3/3	66.40 (b)
2	Akita	Summer	4	4/4	7.04 (bc)	0/4	2.16 (a)
3	Niigata	Autumn	5	5/5	5.87 (ab)	5/5	0.00 (a)
4	Sendai	Autumn	5	3/5	4.40 (ab)	2/5	0.00 (a)
5	Ibaraki	Spring	5	3/5	16.10 (d)	1/5	1.60 (a)
6	Toyama	Summer	5	3/5	0.13 (a)	3/5	0.00 (a)
7	Gifu	Spring	3	3/3	3.55 (a)	0/3	0.00 (a)
8	Nagoya	Autumn	2	2/2	0.64 (a)	2/2	4.72 (a)
9	Shizuoka	Autumn	2	1/2	0.10 (a)	0/2	0.55 (a)
10	Kyoto	Autumn	3	3/3	0.20 (a)	3/3	15.73 (a)
11	Wakayama	Autumn	4	1/4	5.04 (a)	1/4	5.76 (a)
12	Okayama	Autumn	3	3/3	0.40 (a)	3/3	1.04 (a)
13	Tokushima	Summer	4	1/4	0.80 (a)	1/4	11.10 (a)
14	Yamaguchi	Spring	5	5/5	0.30 (a)	0/5	0.00 (a)
15	Oita	Spring	4	4/4	1.13 (a)	1/4	20.47 (a)
16	Kagoshima	Spring	3	1/3	0.10 (a)	0/3	0.70 (a)
17	Okinawa	Summer**	3	1/3	13.93 (cd)	1/3	12.60 (a)

*small letters show the significant difference of infection rates between sampling locations ($P>0.05$).

**termite colonies have been reared in the lab over 6 months before observation

***infection rate is an average from colonies being observed for each location.

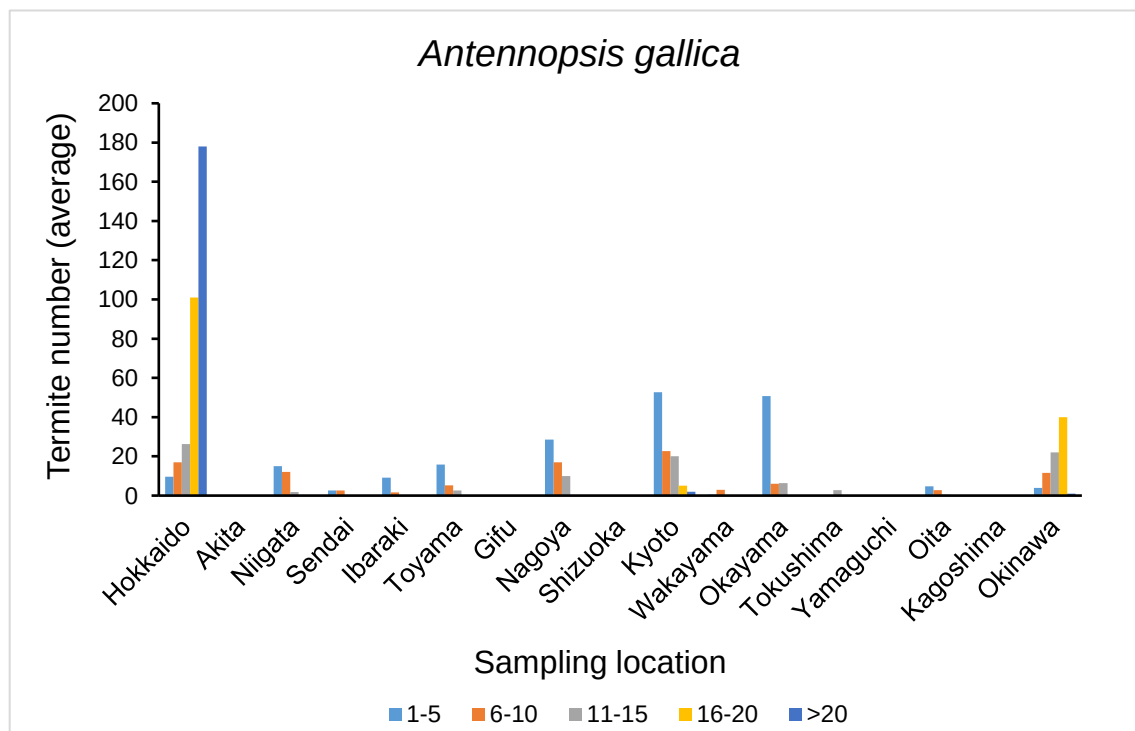
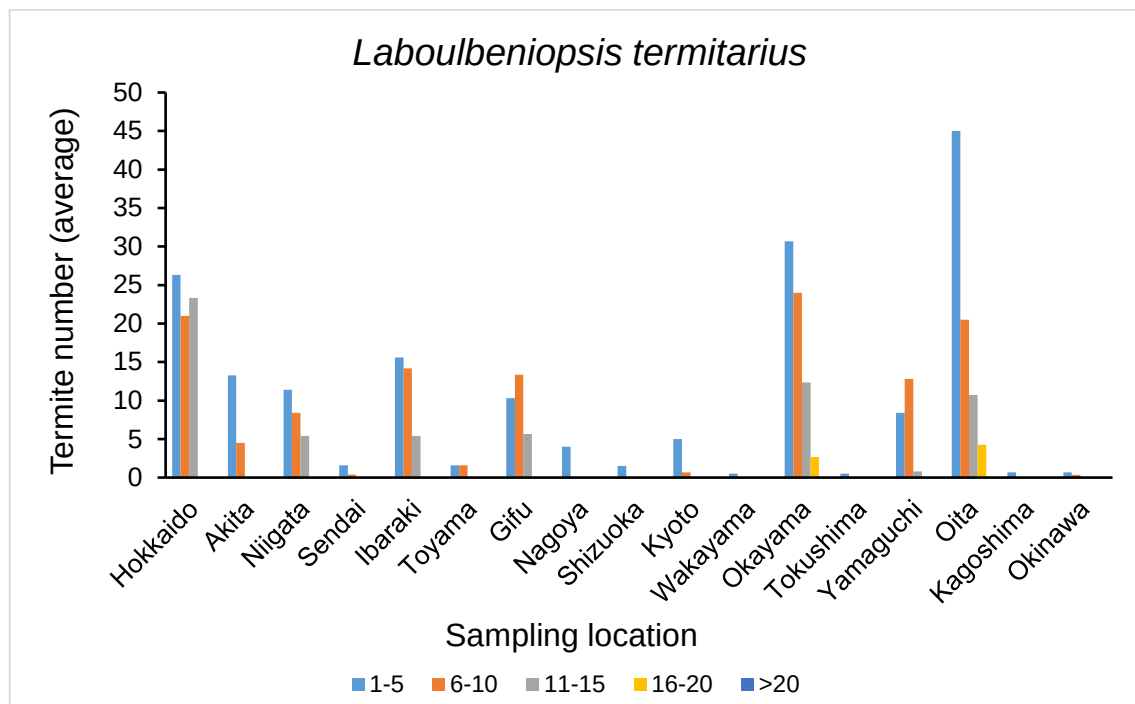


Fig 4.4. The infection strength of ectoparasitic fungi *Laboulbeniopsis termitarius* and *Antennopsis gallica* on termites from 17 sampling locations. Thallus number were groups into 5: 1–5, 6–10, 11–15, 16–20, and >20.

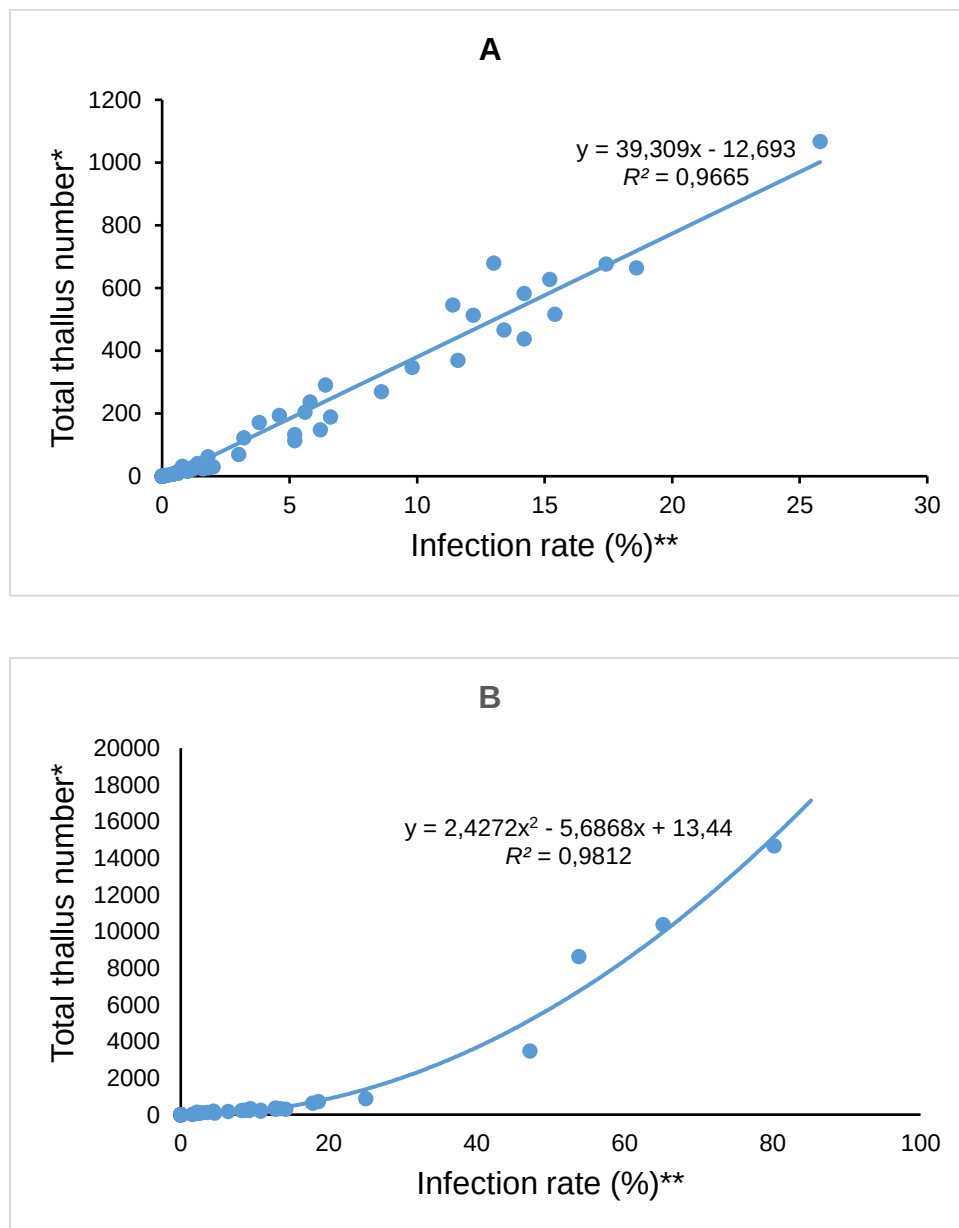


Fig. 4.5. The relationship between infection rate and the thallus number per individual of *Laboulbeniopsis termitarius* (A) and *Antennopsis gallica* (B).

*Total thallus number from total infected termite per termite colony.

**Infection rate per colony.

Table 4.3. Thallus number of *Laboulbeniosis termitarius* and *Antennopsis gallica* on termite body parts

No.	Termite Body part	<i>Laboulbeniosis</i> <i>termitarius</i>	<i>Antennopsis</i> <i>gallica</i>
1	Head	8 ± 12* (a)	60 ± 146,4 (ab ^{**})
2	Antenna	29 ± 43 (bc)	102 ± 250,8 (ab)
3	Thorax	13 ± 25 (ab)	60 ± 183,2 (ab)
4	Mandible	3 ± 10 (a)	18 ± 57,59 (a)
5	Legs	76 ± 101 (d)	129 ± 284,9 (b)
6	Abdomen	30 ± 49 (c)	469 ± 1820 (c)

*Average thallus number followed by standard deviation

**Small letters indicate the significant difference

4.3.5. Isolation and laboratory cultivation of the fungi

Trials for isolation and cultivation of *L. termitarius* and *A. gallica* were conducted in eight artificial media: water agar (WA), potato dextrose agar (PDA), L-broth agar (LBA), molisch agar (MA), brain heart infusion (BHI) oatmeal agar (OA), cornmeal agar (CA) and termite agar (TA). The observation showed that all the media with two different inoculation processes gave negative results. Direct

inoculation was contaminated faster than indirect inoculation and the both fungi did not give any sign of growing.

4.4. Discussion

Reticulitermes spp. are the most important wood pests in Japan, and most widely distributed from the northern part of Hokkaido to the southern part of the Ryukyu Islands, Okinawa. They cause damages in houses, books, furniture and even plantation trees. As subterranean termites, *Reticulitermes* spp. generally live and build their nests in the fallen logs and branches. They maintain the nest temperature stable and keep the humid environment. These conditions are favorable for fungi to grow and disperse their spores. In fact, there are some fungal groups closely associated with termite nests such as *Termitomyces* spp. (Rouland-Lefèvre 2000). Moreover, termite activity may invite and bring some fungi spore back into the colonies. This lead to the finding of fungi inside termite nest even those fungi are in dormant condition (Guswenrivo et al. 2013).

On the other hand, there are numbers of ectoparasitic fungi that infest termites. Some species connect the basal cell with termite cuticle, and others are restricted with haustoria system and only one (*Cordycepioideus* sp.) is mycelioid (Blackwell and Rossi 1986). Fungal genera *Laboulbenia*, *Dimeromyces*, *Cordycepioideus*, *Laboulbeniopsis*, *Coreomycetopsis*, *Amphoromorpha*, *Hormiscioideus*, *Antennopsis*, *Termitaria* and *Mattirolla* were found on termite body surface from 8 termite families (Blackwell and Rossi 1986). Blackwell and

Rossi (1986) reviewed the distribution of termite ectoparasitic fungi, and stated that among all of these species *Laboulbeniopsis termitarius*, *Antennopsis gallica* and *Termitaria* sp. were most common ectoparasitic fungi from temperate to tropical regions.

Laboulbeniopsis termitarius was reported from many places with different termite species as its hosts: Florida (Kimbrough and Gouger 1970, Blackwell and Kimbrough 1976), Georgia (Blackwell 1980), Michigan and Louisiana (Blackwell and Rossi 1986) on *R. flavipes*, Florida on *R. virginicus* Banks (Kimbrough and Gouger 1970), Italy on *R. lucifugus* Rossi (Rossi and Cesari Rossi 1977), Sierra Leone on *Leptomyxotermes doriae* Silvestri, Indo-China on *Ahmaditermes* sp., Indonesia on *Nasutitermes* sp., and French Guiana on *Coptotermes crassus* Snyder (Blackwell and Rossi 1986). While *A. gallica* was found on *Kaloterms flavicollis* Fabricius, *R. virginicus* Banks and *R. flavipes* (Kollar) in Italy, USA, Canada, and Brazil (Buchli 1960b, Gouger and Kimbrough 1969, Blackwell 1980, Blackwell and Rossi 1986, Myles et al. 1998, Blackwell and Kimbrough 1978). Discovering *L. termitarius* and *A. gallica* in Japan has extended the geographical distribution with *Reticulitermes* spp. as their host.

The infection strength of ectoparasitic fungi can be measured by the infection rate and thallus number grown on each termite. Blackwell (1980) observed that 20–53% of *R. flavipes* was infected by *L. termitarius*, and Kimbrough and Gouger (1970) found less than 40% individuals of *R. flavipes* was infected by *L. termitarius*. In this study, the infection rates per colony and thalli

numbers per termite of *L. termitarius* and *A. gallica* varied with sampling locations. This might be affected by environmental conditions as well as termite activities. Buchli (1952) noticed that the infection rate of *A. gallica* was high on termites collected in winter and the colony was gradually died. The similar pattern was observed in the present result on Hokkaido colonies, showing high infection rates for both fungi (*L. tarmitarius*: 14.13%, *A. gallica*: 66.40%) after being kept for 6 months prior to examination. It might be due to both the low level of termite activity during winter and rearing in the laboratory in a small container. Kambara et al. (2017) reported that the activity of *R. speratus* is high at 25–30°C and it would be decreased over 30°C and below 12°C. The infection rates of the Okinawa colonies collected in summer (average air temperature: 28.7°C) and reared in the laboratory for almost one year were also high (*L. tarmitarius*: 13.93%, *A. gallica*: 12.60%). In other colonies collected during summer (Akita, Toyama and Tokushima), the infection rates—significantly varied. The colonies collected in spring from Kyoto, Ibaraki, and Oita showed high infection rates of *A. gallica* (Kyoto and Oita) and *L. termitarius* (Ibaraki). These results might suggest that both environmental factors such as temperature and rearing in the laboratory have effects on the infection rate by the fungi. In order to conclude this speculation, further study should be carried out by observing termite colonies from the same location with different seasons.

Grooming is one of the most important termite activities affecting fungal growth. Grooming is the activity that termites clean up themselves and nest-mates.

The grooming activity will reduce the infection rate among termites themselves. This social behavioral facilitates the transmission of ectoparasitic fungi even though grooming also has the benefit in termites for conferring resistance and is an antifungal defense strategy (Boucias et al. 1996, Shimizu and Yamaji 2003, Yanagawa and Shimizu, 2005 and 2007).

Laboulbeniopsis termitarius and *A. gallica* grew over any body parts of termite. It was supported by the results of our previous report that *A. gallica* thalli did not have any specific termite body parts to grow, though thallus numbers varied depending on each body part (Guswenrivo et al. 2017). Legs, antennae and abdomen were three body parts with higher thallus numbers of *L. termitarius* and *A. gallica*. Legs contact with soil, the sources of fungi, so that the thallus numbers would be higher. The antennae were found highly infected too, due to the antennae's function. Termite antennae have a number of functions such as sensing of touch, taste, odours (including pheromones), heat and vibration (Bignel et al. 2011), which may lead to fungal infection. This was proved by a previous study by Bao and Yendol (1971). They observed that antennae and legs are the first body parts of termite infected by *Beauveria bassiana* after 10 hour of inoculation followed by thorax and abdomen (Bao and Yendol 1971).

The infection strength of entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* was evaluated by the conidial number grown on its host (Wraight et al. 1998 and 2000). The highest number of fungal conidia would be in line with the infection strength and turn out the highest mortality of the host

(Wraight et al. 1998 and 2000). Differing from entomopathogenic fungi, the infection strength of ectoparasitic fungi *L. termitarius* and *A. gallica* is likely to be considerable by the number of thalli grown on each infected termite. The highest thallus number would increase the possibility of the fungi to germinate and infected other termite in a colony. This assumption was supported by our observation that the highest infection rate of ectoparasitic fungi positively correlate with thallus number of *L. termitarius* and *A. gallica* (Fig. 4.5). As shown in Fig. 4.5, the manipulation and spreading manners of *L. tarmitarius* and *A. gallica* are likely to be different. In *L. tarmitarius* the thallus number of the individual was increased with the colony infection rate linearly, meaning that the fungus manipulates and spreads by the simple manner. But for *A. gallica* there might be a threshold line of the infection rate for manipulating and spreading, and over the line the fungus will have a faster infection.

Buchli (1952) mentioned that infected termite colony by *A. gallica* would gradually die by the time, but effects of ectoparasitic fungi on termite activity remain unclear. Isolation and culturing of the ectoparasitic fungi must be an only possible way to understand the effect of ectoparasitic fungi on termites. Generally, ectoparasitic fungi grow on body surface of their hosts are believed to take the nutrient through absorption or via contact with living tissues (Benjamin 1971, Tavares 1985). Ectoparasitic laboulbenialean fungi stick to host cuticle and produce some penetration structures (Benjamin 1971, Tavares 1985). *Laboulbeniopsis termitarius* was reported to secrete some chemicals to stick with

the host through basal cell, while *A. gallica* constructed a haustorium in order to absorb nutrition from termite cell wall (Blackwell and Rossi 1986). Incubation condition and media contents will affect on growth of fungi. Fungi have wide range of temperature tolerance, but the optimal temperature range for fungi to grow is 20–30°C with high moisture or free water (Wraight et al. 2007).

The media preference has to be synchronized with the nutrient needs by the fungi to grow. Several media such as cornmeal, malt extract, potato dextrose agar, maltose and yeast extract have been used to isolate for most entomopathogenic Hyphomycetes (Goettel 1984, Wraight et al. 2007). However, these media did not support the growth of *L. termitarius* and *A. gallica* in the lab conditions. Further research has to be conducted for culturing *L. termitarius* and *A. gallica*. Chitin-based or cuticular lipid-based media might be the options for culturing ectoparasitic fungi *L. termitarius* and *A. gallica*.

4.5. Summary

Distribution of *L. termitarius* and *A. gallica* in *Reticulitermes* spp. colonies in Japan was observed. Meanwhile, the infection rate and strength of *L. termitarius* and *A. gallica* were discussed with references to effects of environmental factors at the collections sites. In total of 63 colonies of *Reticulitermes* spp. were collected from seventeen locations (from Hokkaido Prefecture to Okinawa Prefecture) in Japan. Five hundred workers and twenty soldiers from each colony were examined individually to see the infection of *L.*

termitarius and *A. gallica*. The survey showed that *L. termitarius* distributed in whole Japan and *A. gallica* had a little bit restricted distribution. The infection rate of workers of *Reticulitermes* spp. varied among all locations: 0.10 – 16.10% for *L. termitarius* and 0 – 66.40% for *A. gallica*. No infected soldiers was observed. The negative relationship between temperature and infection rate was speculated in both the fungi. Rearing the colonies in the laboratory might result in the spreading of the fungi in the colonies. The both fungi grew on any body parts of the termites. The trials for isolation and cultivation of *Laboulbeniopsis termitarius* and *A. gallica* with eight media did not succeed under the laboratory conditions.

Chapter 5

Development of multiplex nested PCR for simultaneous detection of ectoparasitic fungi *Laboulbeniopsis termitarius* Thaxt and *Antennopsis gallica* Buchli and Heim on *Reticulitermes speratus* (Kolbe)

5.1. Introduction

Laboulbeniopsis termitarius Thaxt and *Antennopsis gallica* Buchli and Heim are two of the most commonly found ectoparasitic fungi on termite body surface. On the first part (Chapters 2–4), the distribution and prevalence of the two fungi are described by using visual observation under dissecting microscope. However, it is time consuming and required a high number of termites for observation.

In the second part, Chapter 5, a fast, efficient assay was developed to detect infection of the ectoparasitic fungi on the termite *Reticulitermes speratus* (Kolbe). Nowadays, DNA-based methods are the common methods used to detect fungal infection. The polymerase chain reaction (PCR) was used, and species-specific primers were designed to detect ectoparasitic fungi. The primers were designed based on sequence data amplified using a number of universal fungus primer pairs that amplified partial sequences of 18s rDNA gene of the two fungi. To detect the two fungi in a robust yet economic manner, a multiplex nested PCR assay, using species-specific primers, was then developed in this chapter.

5.2. Materials and Methods

5.2.1. Sample preparation

Multiple colonies of the termite *R. speratus* (Kolbe) were collected from Hokkaido and kept at 4 °C prior to the subsequent observations and experiments.

The presence of *L. termitarius* and *A. gallica* were first assessed using a dissecting microscope (S8AP0, Leica, Wetzlar, Germany). Thalli of each fungus were removed from the termite using an entomological pin, and mounted following the method described in Dring (1971). Morphological identification of the ectoparasitic fungi was carried out to confirm fungal species identity based on previous studies: Thaxter (1920), and Kimbrough and Gouger (1970) for *L. termitarius*; Buchli (1960) and Gouger and Kimbrough (1969) for *A. gallica*.

5.2.2. DNA extraction

Total genomic DNA was extracted from *R. speratus* using the Gentra Puregene Cell and Tissue Kit (Qiagen, USA). To evaluate the sensitivity of PCR assays on detecting *L. termitarius* and *A. gallica* under various conditions, DNA was extracted from two sample preparations: 1) termites with differential fungus infection strengths (as defined by the number of thalli per infected specimen, Table 5.1); and 2) samples with mixed infected and non-infected termites at different ratios (referred to as infection rate, Table 5.1; note that each infected termite possesses similar infection strength). A third set of DNA samples was prepared to test the efficiency of the multiplex nested PCR assay, in which we mixed DNA extracted from a termite infected with 7 thalli of *L. termitarius* with that of a termite infected with 20 thalli of *A. gallica*, to simulate an asymmetrical infection of the two fungi in the termite samples.

5.2.3. Primer design

The specific primers for *L. termitarius* were designed based on the partial sequence of the small-subunit 18S rRNA gene of *L. termitarius* obtained from GenBank (accession number AY212810), whereas the specific primers for *A. gallica* were designed based on the sequencing results we generated using universal primers NS17 (Gargas and Taylor 1992) and NS4 (White et al. 1990) that target a partial 18S rRNA gene region of the fungus. In total, four pairs of specific primers were designed for *L. termitarius*, and three for *A. gallica* in this study (Table 5.2), as part of the PCR optimization process.

5.2.4. Development of PCR assay

As our standard PCRs often resulted in either low-intensity amplification or non-specific amplification (data not shown), a standard nested PCR assay was developed to ensure the specificity. The first-step PCR reaction was set up by mixing 25- μ L Emerald Amp MAX PCR master mix (Takara, Japan), 2 μ L of DNA, 0.2 μ M each of the forward and reverse primers (see **Multiplex nested PCR** for primer details), and sterilized distilled water up to 50 μ L. The reaction for the second-step PCR was identical, except for the primers (Table 5.2), with PCR product from the first PCR as the DNA template. The PCR cycling conditions included an initial denaturation step at 94 °C (3 min) followed by 35 cycles at 94 °C (30 s), 50 °C (30 s), and 72 °C (40 s), with a final extension phase at 72 °C (5 min).

5.2.5. Sensitivity and specificity tests

The first two DNA preparations with differential fungus infection strengths and infection rates were used as a template for the standard nested PCR assay to test sensitivity and specificity. The primer pair TL2J3037 and TKN3785 (Simon et al. 1994) targeting the mtDNA COII region of *R. speratus* was included in each reaction as an internal control to ensure the quality of DNA extractions. DNA from non-infected termites was included as a negative control. The reaction mixture was cycled according to the PCR protocol described above.

Table 5.1. Designed specific primers for detecting an ectoparasitic fungi *Laboulbeniopsis termitarius* Thaxt and *Antennopsis gallica* Buchli and Heim.

Primers Name		Sequence	Length	Product (bp)
Specific primers for <i>Laboulbeniopsis termitarius</i>	Lter18s-speF1	TAATCTCGACGTAAGAAGGGATGT	24	
	Lter18s-speR1	GACCCAGCCAGACCAGTACA	20	477
	Lter18s-speF2 ^a	TATGGCCTTTGGCTGACGC	19	
	Lter18s-speR2 ^a	CTCTGACCATTGAATACTGATGC	23	596
	Lter18s-speF3	CGACATGGGGAGGTAGTGAC	20	
	Lter18s-speR3	GCATATGCCTGCTTTGAACA	20	300
	Lter18s-speF4 ^b	TCACATGCTTTTGACGGGTA	20	
	Lter18s-speR4 ^b	CACCAGACTTGCCCTTCAGT	20	225
Specific primers for <i>Antennopsis gallica</i>	Agal18s-speF1	GACTCGGGGAGGTAGTGACA	20	
	Agal18s-speR1 ^b	GCCCAAGGTTCAACTACGAG	20	194
	Agal18s-speF2 ^a	CGATGCGAAGGTCTTGTCTT	20	
	Agal18s-speR2 ^a	CCTGCCTGGAGCACTCTAAT	20	458
	Agal18s-speF3 ^b	AACGGGTAACGGAGGGTTAG	20	
	Agal18s-speR3	AACTACGAGCTTTTAAACCAC	21	282

^aUsed in the first step PCR

^bUsed in the second step PCR

Table 5.2. Research design for sensitivity test of fungal detection on termite body.

<i>Laboulbeniopsis termitarius</i>		<i>Antennopsis gallica</i>	
Infection strength	Infection rate	Infection strength	Infection rate
3 thallus	1:1 (50%)	Low	1:5 (16.7%)
5 thallus	1:5 (16.7%)	Medium	1:10 (9.1%)
7 thallus	1:10 (9.1%)	High	1:15 (6.25%)

5.2.6. Multiplex nested PCR

Termites, including *R. speratus*, are generally observed to be infected with multiple ectoparasitic fungus species (Blackwell 1980). To simultaneously detect the two fungi, a multiplex nested-PCR assay was developed. The first-step PCR mixture was set up by mixing 25- μ L Emerald Amp MAX PCR master mix (Takara, Japan), 2 μ L of DNA (from the third set of DNA preparation), 1 μ L each of forward and reverse primer, and sterilized distilled water up to 50 μ L. Primers in the first-step PCR reaction included the fungus-specific primers that amplify the partial 18S rRNA gene (Lter18s-speF2^a and Lter18s-speR2 for *L. termitarius*, and Agal18s-speF2 and Agal18s-speR2 for *A. gallica*; see Table 5.1 and **Results** for more details) and termite-specific primers that amplify the mtDNA COII gene at a ratio of 8:1:1 (*L. termitarius* : *A. gallica* : termite mtDNA). The products generated in the first-step PCR were used as a template for the second-step PCR. Primers included Lter18s-speF4/Lter18s-speR4 and Agal18s-speF3/Agal18s-speR1 for *L.*

termitarius and *A. gallica*, respectively (see Table 5.1 and **Results** for more details). The PCR conditions included an initial denaturation step at 94 °C (3 min), followed by 35 cycles at 94 °C (30 s), 50 °C (30 s), and 72 °C (90 s), with a final extension phase at 72 °C (5 min).

5.2.7. Sequencing and phylogenetic analysis

The standard nested PCR products were purified using a FastGene Gel/PCR Extraction Kit (Nippon Generics Co. Ltd, Japan), and sequenced in both directions with DNA Sequencing Core, Kyoto University (Kyoto, Japan) using ABI 3130XL genetic analyzer. Sequence data from both directions were assembled and checked with Sequencher 4.9 (Gene Codes). Alignment of the generated sequences was carried out using MUSCLE as implemented in MEGA 6 using the default settings (Tamura et al. 2013). Maximum likelihood phylogenetic analysis was conducted using an online program, PhyML 3.0 (<http://www.atgc-montpellier.fr/phyml/>; Guindon et al. 2010). The substitution model TN93 + G was selected automatically using PhyML's Smart Model Selection (SMS) (Lefort et al. 2017) under the Aikake Information Criterion. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 100 replicates.

The taxa included in the analyses, along with their Genbank accession numbers, were *Ascobolus denudatus* AF121076, *Morchella esculenta* U42642, *Scorias spongiosa* AF006726, *Capnodium dermatum* AF006724, *Ophiostoma ulmi* M83261, *Ophiostoma piliferum* U20377, *Cytospora leucostoma* M83259,

Neurospora crassa X04971, *Xylaria hypoxylon* U20378, *Sphaerostilbella aureonitens* U32415, *Balansia sclerotica* U32399, *Melanospora fallax* U47842, *Melanospora zamiae* U78356, *Graphium euwallaceae* KF542902, *Ceratocystis fimbriata* U43777, *Microascus trigonosporus* L36987, *Pseudallescheria ellipsoidea* M89782, *Petriella setifera* U32421, *Talaromyces macrosporus* M83262, *Monascus purpureus* M83260, *Elaphomyces maculatus* U45440, *Exophiala calicioides* AB007655, *Lecanora dispersa* L37535, *Pyxidiophora* sp. IMI-1989 AF313769, *Pyxidiophora arvernensis* FJ176839, *Laboulbeniopsis termitarius* AY212810, *Hesperomyces coccinelloides* AF407575, *Stigmatomyces limnophorae* AF407576, *Zodiomyces vorticellarius* AF407577, *Ambrosiozyma platypodis* L36984, *Endomyces scopularum* AF267229, *Candida albicans* M60302, *Taphrina deformans* U20376, *Protomyces inouyei* D11377, *Entomophthora muscae* D29948, *Mucor mucedo* X89434, *Zancudomyces culisetae* D29950, and *Capniomyces stellatus* AF007531.

5.3. Results

5.3.1. Primer selection

From all possible specific primer combinations of *L. termitarius*, primer pairs Lter18s-speF2/ Lter18s-speR2 and Lter18s-speF4/Lter18s-speR4 succeeded in amplifying PCR products of 596 bp and 225 bp, respectively, from *L. termitarius* DNA. Primer pairs Agal18s-speF2/Agal18s-speR2 and Agal18s-speF3/Agal18s-speR1 resulted in successful amplifications of products of 458 bp

and 293 bp, respectively, that showed high specificity to *A. gallica*. We concluded the optimal primer pairs for fungus detection were Lter18s-speF2/Lter18s-speR2 (the first-step PCR of *L. termitarius*) and Lter18s-speF4/ Lter18s-sp R4 (the second-step PCR of *L. termitarius*), Agal18s-speF2/Agal18s-speR2 (the first-step PCR of *A. gallica*) and Agal18s-speF3/Agal18s-speR1 (the second-step PCR of *A. gallica*).

5.3.2. Sensitivity of standard nested-PCR and multiplex nested PCR

We tested the sensitivity of the standard nested PCR assay using the two DNA preparations with differential infection strength and infection rates as templates. The results showed that the standard nested PCR assay was able to amplify and detect each of the two fungi from DNA extracted from samples with low infection strength (as low as 3 thalli in an individual termite) and infection rate (as low as 6.25%) (Figs. 5.1 and 5.2). Results of the multiplex nested PCR revealed the presence of three fragments with various yet expected sizes corresponding to the three amplification targets (225 bp for *L. termitarius*, 293 bp for *A. gallica*, and 786 bp for a partial mtDNA COII region of termite, Fig. 5.3). No sign of preferential amplification was observed, even though higher infection strength of *A. gallica* was represented in the DNA template.

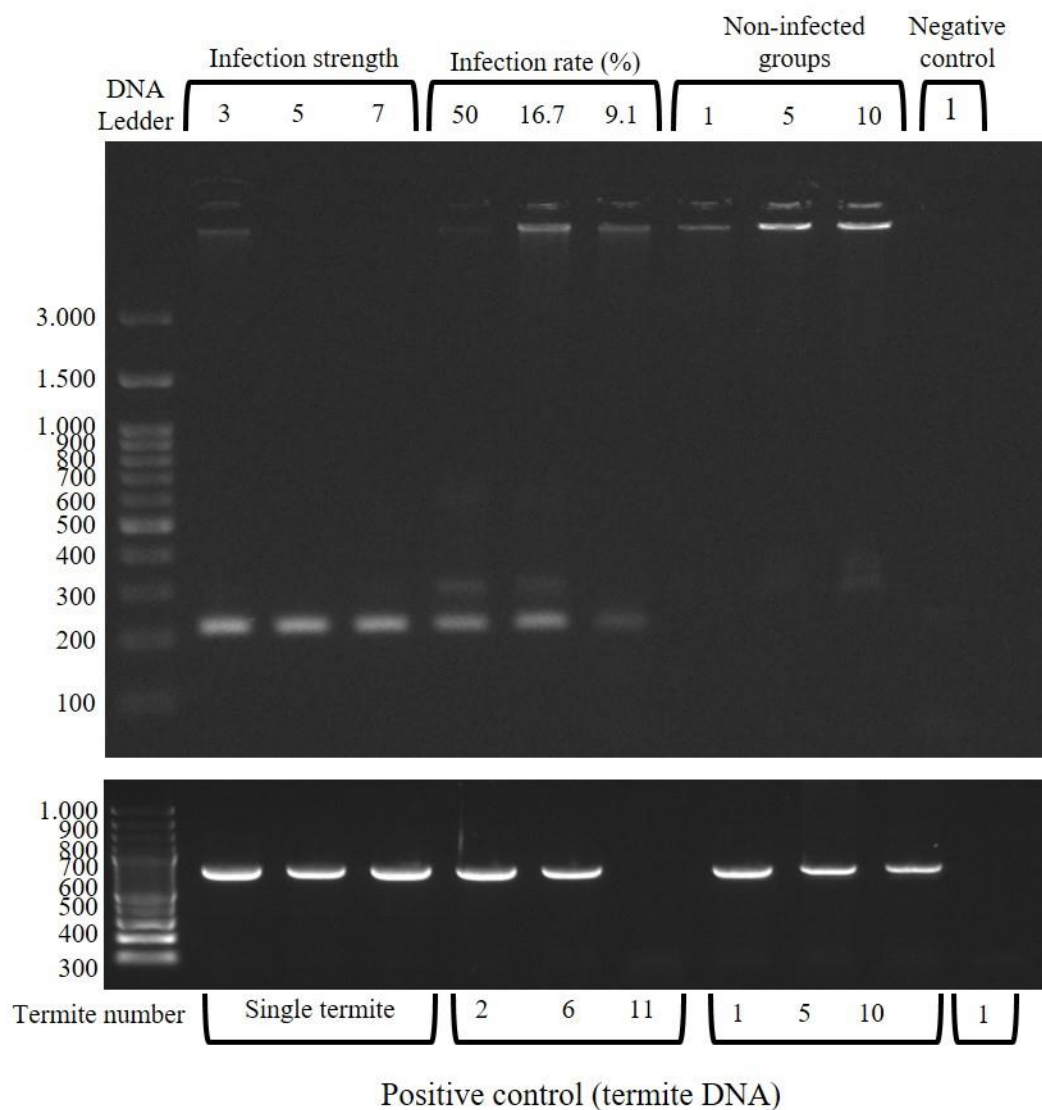


Fig. 5.1. Detection of *Laboulbeniopsis termitarius* with different infection strength and infection rate on infected termite. Number in infection strength, 3. 5. And 7, represent number of thallus per termite. The infection rate is the ratio between infected and total of infected and non-infected termite ($1/2 \times 100\% = 50\%$; $1/6 \times 100\% = 16.7\%$; and $1/11 \times 100\% = 9.1\%$). Number in Non-infected group represent the number of termite being extracted. The nested PCR products were separated with a 2% agarose gel and the size of the marker in base pairs.

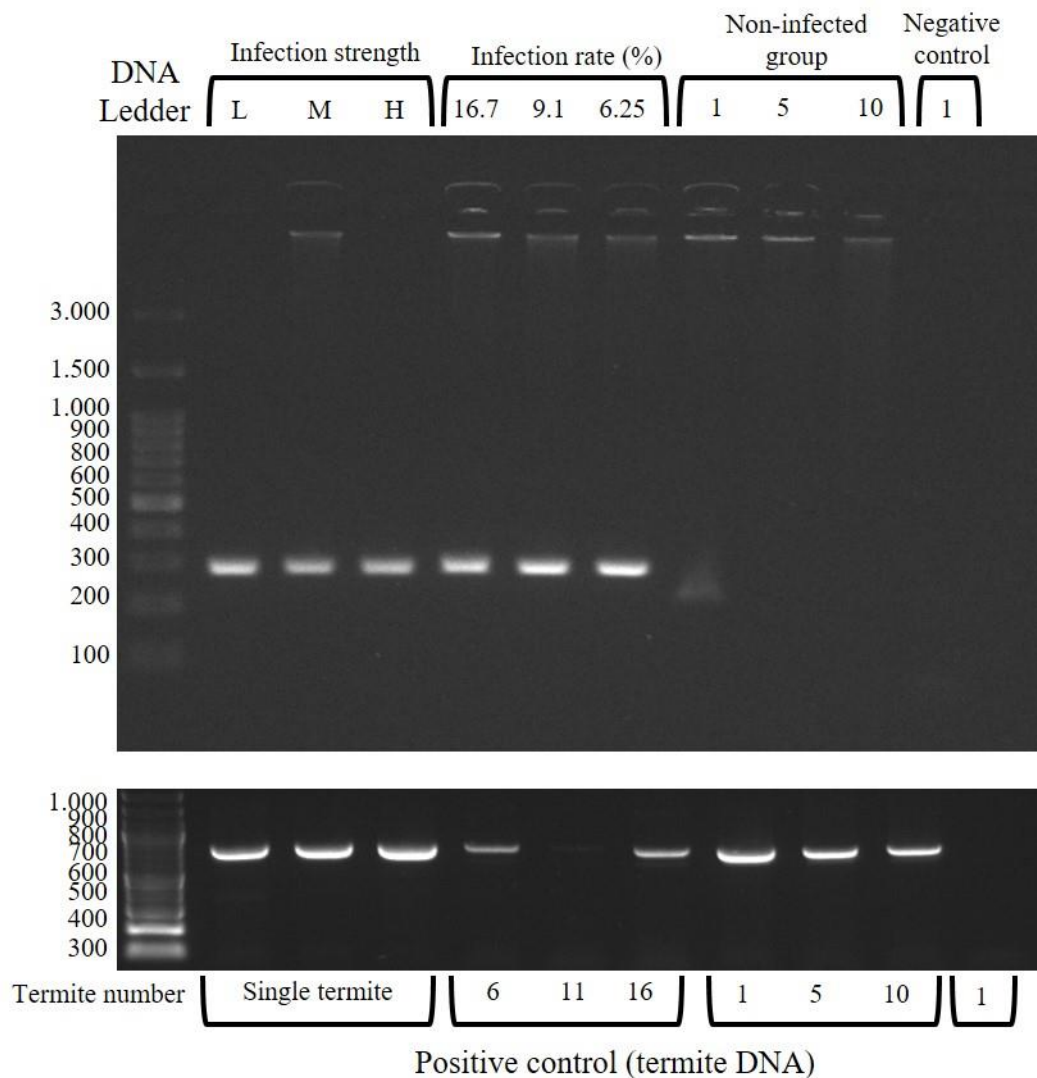


Fig. 5.2. Detection of *Antennopsis gallica* with different infection strength and infection rate on infected termite. Infection strength: L= low; M=medium; and H= high infection strength. The infection rate is the ratio between infected and total of infected and non-infected termite ($1/6 \times 100\% = 16.7\%$; $1/11 \times 100\% = 9.1\%$; and $1/16 \times 100\% = 6.25\%$). Number in Non-infected group represent the number of termite being extracted. The nested PCR products were separated with a 2% agarose gel and the size of the marker in base pairs.

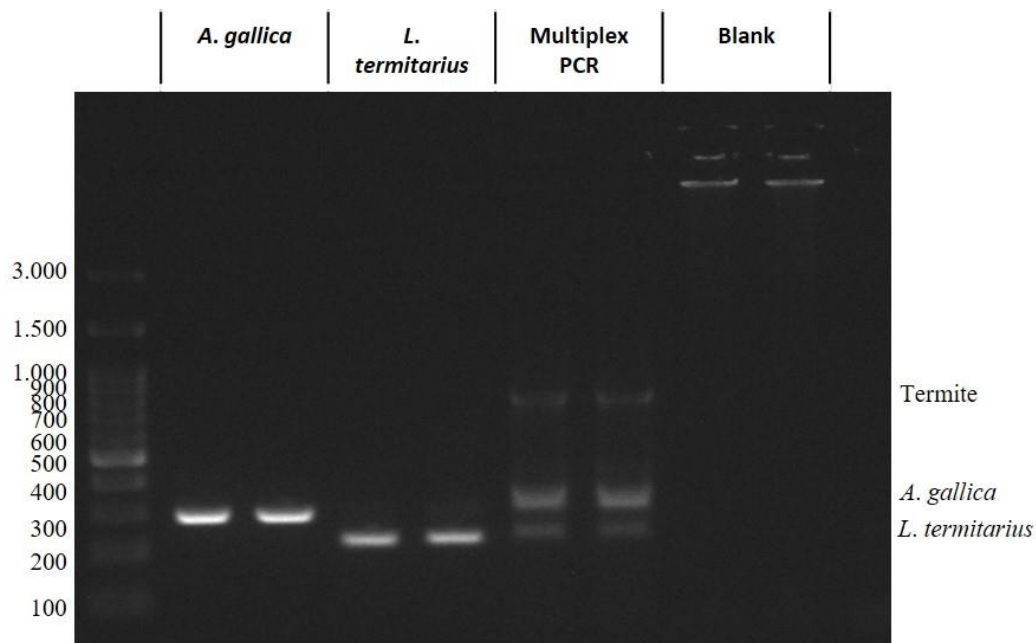


Fig. 5.3. Multiplex PCR products for detecting *L. termitarius* and *A. gallica* were separated in a 2% agarose gel, the size of the marker in base pairs is shown on the left.

5.3.3. Sequencing

The sequences of *L. termitarius* and *A. gallica* were successfully recovered in this study. We found the sequence of *L. termitarius* was grouped in the Laboulbeniomyces clade with a high bootstrap support value of 100% (Fig. 5.4), and showed a high sequence similarity (97.7% similarity after excluding 37-bp gaps) to a reference sequence available from Genbank (AY212810, *L. termitarius* isolated from *R. flavipes* in Louisiana, USA; Henk et al. 2003). The *A. gallica* sequence in this study was clustered within *Ascomycota*, with a high bootstrap support value of 100% (Fig. 5.4). The results of the sequence comparison showed 98% identity with *Graphium euwallaceae* in the class Sordariomycetes (Fig. 5.4).

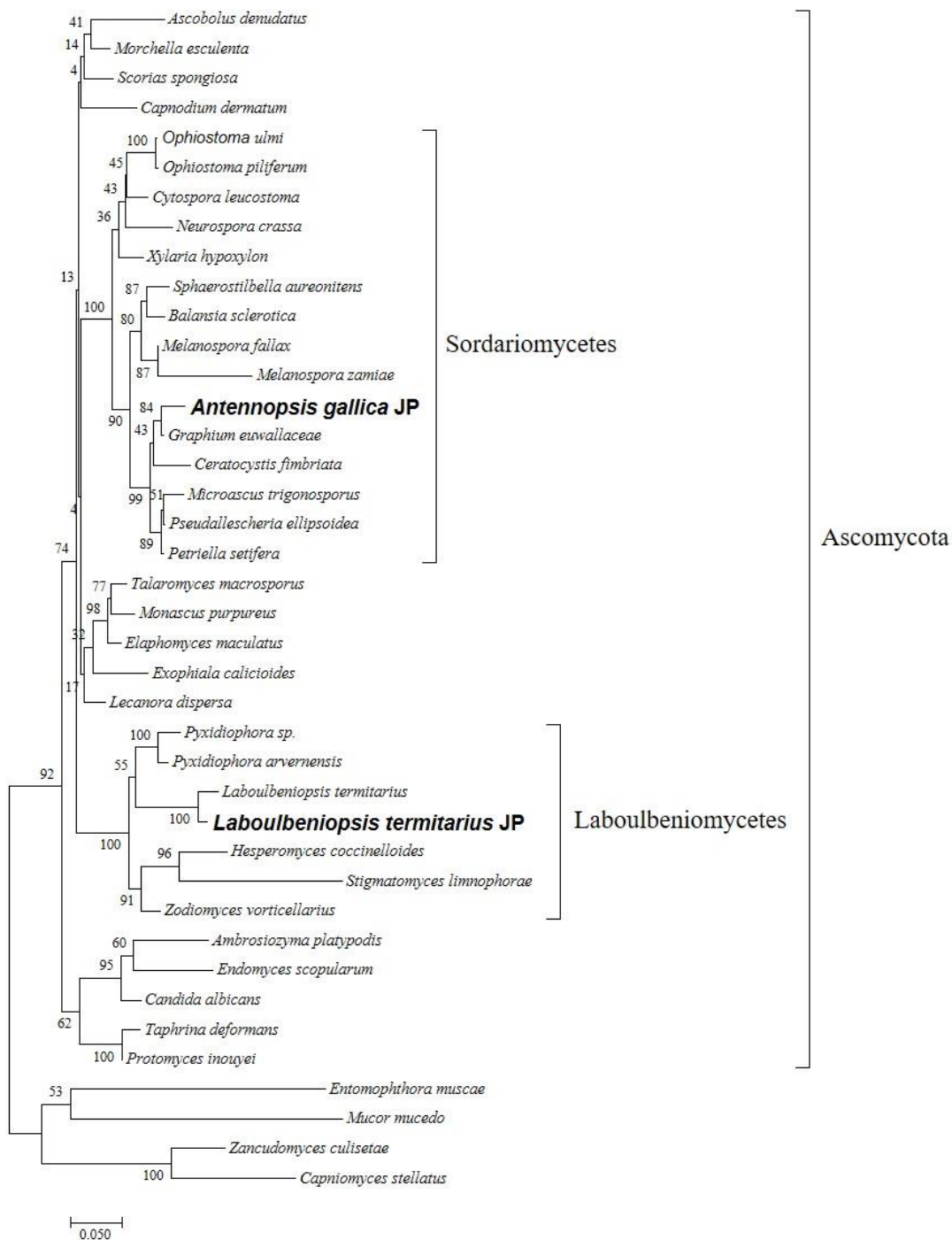


Fig. 5.4. Polygram of the most similarity and closely related tree. Numbers above the nodes represent bootstrap support. *Laboulbeniopsis termitarius* Japan specimen and *Antennopsis gallica* is shown in bold.

5.4. Discussion

It has been suggested that neither *L. termitarius* nor *A. gallica* are fungi that can be cultured under laboratory conditions (Henk et al. 2003; Guswenrivo unpublished data). Moreover, these two fungus species have mainly been detected using visual examination based on several key morphological characters, which is a generally time-consuming, labour-intensive process that may require prior knowledge of fungal taxonomy. Furthermore, previous studies have shown that a robust detection of ectoparasitic fungi on termite normally requires viewing hundreds of termites (Gouger and Kimbrough 1969; Kimbrough and Gouger 1970; Blackwell and Kimbrough 1978; Blackwell 1980; Myles et al. 1998; Guswenrivo et al. 2017; Guswenrivo unpublished data). To facilitate screening efficiency, our study established a new, highly sensitive, and species-specific multiplex PCR assay for the rapid detection of *L. termitarius* and *A. gallica* from the termite *R. speratus*.

Previous survey efforts revealed that the intracolony infection rates of *L. termitarius* in colonies of *R. flavipes* and *R. virginicus* termites varied across different sites in the USA (Kimbrough and Gouger 1970; Blackwell 1980), whereas the infection rate could be as low as 10% in sampled colonies in Japan (Guswenrivo, unpublished data). While a single thallus per individual was commonly observed, 30-35 thalli might be found on an individual termite in a colony with a high infection rate (Kimbrough and Gouger 1970; Blackwell 1980). Our standard nested PCR assay, however, remained effective even for samples

characterized by a low fungal infection strength and low infection rate for *L. termitarius* (e.g., 3 thalli per termite and 9.1%, respectively, Fig. 5.1), suggesting the ability of this assay to detect a field infection of *L. termitarius* in the termite *R. speratus*, and possibly other termite species if a focal fungus species is characterized by a similar infection pattern.

Both infection strength and intracolony infection rate of *A. gallica* are generally higher than those of *L. termitarius*. The number of thalli for *A. gallica* on a single infected termite has been reported to range from 1–150 across several locations in the USA (Gouger and Kimbrough 1969; Kimbrough and Gouger 1970; Blackwell and Kimbrough 1976; Blackwell 1980). The highest number of *A. gallica* on *R. flavipes* was observed by Myles et al. (1998), where a total of 479 thalli were detected on a single infected termite in Canada. In Kyoto, the intracolony infection rates of *A. gallica* in colonies of *R. speratus* range from 17.8–25.0% (Guswenrivo et al. 2017). Despite the much lower infection rate found in the populations in Kyoto, we argue that the robustness of our assay remains viable, as it is capable of detecting the presence of *A. gallica* at both low infection strength (<15 thalli) and low infection rate (6.25%, Fig. 5.1).

Extensive research has reported using multiplex nested PCR for the detection of viruses, bacteria, and fungi (Hamelin 1996; Clair 2003; Bronzoni 2004; Lam et al. 2007). Moreover, Lam et al. (2007) suggested that the multiplex nested PCR assay significantly improved the diagnostic yield in terms of overall sensitivity, and that multiple infections did not reduce the sensitivity of the assay.

Consistent with Blackwell (1980), most of the termite colonies collected in this study were found to be infected with multiple species of ectoparasitic fungi, including *L. termitarius*, *A. gallica*, and *Termitaria* sp. Since detection of *Termitaris* sp. can be performed with the naked eye, due to its greater size and apparent infection-associated color change (Hojo et al. 2001), the multiplex nested PCR assay in this study focused on detection of the former two fungi. Considering the nature of infection patterns of the two, it was possible that an overly high amount of DNA of one target compared to the other, might lead to the occurrence of preferential amplification (Elnifro et al. 2000). We did not, however, observe any sign of preferential amplification in the results generated by our multiplex nested PCR assays.

It is possible that the results of the multiplex nested PCR simply reflected that the higher primer volume ratio of *L. termitarius* over the other two (*A. gallica* and mtDNA Coll) was able to compensate for the lower amount of *L. termitarius* DNA represented in the sample. This possibility is further supported by multiple studies reviewed in Kalle et al. (2014) showing that modification of primer concentrations in reaction solutions may improve the performance of a multi-template PCR. We therefore conclude that amplification bias is limited using the multiplex nested PCR developed in this study, even though number of *A. gallica* thalli usually outnumber that of *L. termitarius* in field conditions.

Since they were first discovered, the taxonomic status of *L. termitarius* and *A. gallica* have been determined based on morphology. Henk et al. (2003) placed

L. termitarius in Laboulbeniomyces based not only on morphological but also on molecular evidence. The sequence data in this study supports linear superposed cells as an informative trait linking the Laboulbeniomyces. Sequences of *L. termitarius* generated here were most similar to the reference sequences from Genbank, and generally shared a high sequence similarity with other species in the Laboulbeniomyces (Fig. 5.4).

On the other hand, the partial 18S rRNA gene sequences of *A. gallica* generated in this study were placed in the class Sordariomycetes, and showed the closest affinity with *Graphium euwallaceae* (98%), a mycangial fungus associated with a polyphagous shot hole borer (*Euwallacea* sp.) (Lynch et al. 2016). Despite the molecular similarity between *A. gallica* and *G. euwallaceae*, their morphology and life histories are markedly distinct. For example, *G. euwallaceae* is considered a fungal symbiont of *Euwallacea* sp., and can be found not only from the head of *Euwallacea* sp. but also the gallery walls of the borer's host plants (Lynch et al. 2016). Such a pattern, coupled with previous studies, is consistent with the fact that morphologically distinct fungi in the class Sordariomycetes have been frequently found to share similar sequence identity (Samuels and Blackwell 2001; Seifert and Gams 2001; Zhang et al. 2006; Park et al. 2017).

5.5. Summary

Ectoparasitic fungi *L. termitarius* and *A. gallica* can be detecting by using a nested PCR method with specific primers. The method can be use to detect the low infection strength, 3 thallus per termite, and infection rate, ration 1 over 15. The multiplex n-PCR is recommended for future survey of the two fungi as this method is more sensitive, species-specific and time-saving compared to the visual observation.

Chapter **6**

General Conclusion

Ectoparasitic fungi attach to and grow on the body surface of their hosts. Twenty-two species of termite ectoparasitic fungi have been reported, with the two most common species being *Laboulbeniopsis termitarius* Thaxt and *Antennopsis gallica* Buchli and Heim. The two species are described in Chapter 2 and 3, respectively, as the first records of termite-ectoparasitic fungi in Japan (*L. termitarius* TNS-F-54007 and *A. gallica* TNS-F-54006). To these findings we add information on the geographical distributions of these species.

The distribution of *L. termitarius* and *A. gallica* in *Reticulitermes* spp. colonies in Japan is reported in Chapter 4. *Laboulbeniopsis termitarius* was found to be distributed throughout Japan whereas *A. gallica* had somewhat more restricted distribution. The infection rate of workers of *Reticulitermes* spp. varied among all locations: 0.10 – 16.10% for *L. termitarius* and 0 – 66.40% for *A. gallica*. No infected soldiers were observed. A negative relationship between temperature and infection rate was speculated in both fungi. Rearing the colonies in the laboratory might result in the spreading of the fungi in the colonies. Both fungi grew on any body part of the termites. Several hundred termites were required for the survey of this ectoparasitic fungi in Japan, due to the low infection rates of both fungi.

The negative results in trials for isolation and cultivation of *L. termitarius* and *A. gallica* with eight media convinced that the fungi are unable to grow on the laboratory culture media we used. Both fungi species are hardly cultivated in the laboratory conditions, leading to the lack of information on the teleomorphs' status. Due to this characteristic, it is still unclear whether the spore is a sexual or asexual phase.

While visual observation under a dissecting microscope has been the common method to screen for the presence of fungi on termites, a high number of termites is generally required and thus the process is extremely time

consuming. Therefore, a fast, efficient assay was developed to detect infection of the fungi on the termite *Reticulitermes speratus* (Kolbe). Species-specific primers were designed based on sequence data amplified using a number of universal fungus primer pairs that amplify partial sequences of the 18s rDNA gene of the two fungi. To detect the two fungi in a robust yet economic manner, a multiplex nested PCR assay using species-specific primers was then developed. The results suggested that both fungi were successfully detected even though in some cases *L. termitarius* was at a very low titer (ex. a single thallus per termite). The newly developed assay in this study is recommended for future surveys of the two fungi as this method is a more sensitive, species-specific and time-saving compared to microscopic observation, and may facilitate an understanding of the two fungi and their dynamics in the host populations.

The information obtained in this study will help us to understand more about termite ectoparasitic fungi. However, the infection process and the effect of the fungi on termite behavior still needs to be discovered. Therefore, in order to develop ectoparasitic fungi as a biological control agent, further studies are needed to get a deeper understanding and answering the questions of how the fungi infect the termite, how the fungi-infected termites are impaired, why the fungi-infected termites have a shorter lifespan, the effects on the termite of fungi infection, the processes and conditions of fungal infection of termites how the fungi infected termite.

Acknowledgments

The author would like to express his deepest gratitude and sincere appreciation to Professor Tsuyoshi Yoshimura (Laboratory of Innovative Humano-habitability, Research Institute for Sustainable Humanosphere, Kyoto University) for his invaluable enlightenment and guidance, kind encouragement, advices and constructive discussions during academic activities, reviewing and commenting this thesis.

The author would also like to express his deepest gratitude and sincere appreciation to Dr. Hiroki Sato (Department of Forest Entomology, Forestry and Forest Products Research Institute Japan) for conducting a short training, scientific advises, kind motivation and favor, and critical writing and reading of the publications.

The author would also like to thank Professor Yoichi Honda (Laboratory of Forest Biochemistry, Division of Environmental Science and Technology, Graduate School of Agriculture) and Professor Junji Sugiyama (Laboratory of Biomass Morphogenesis and Information, Research Institute for Sustainable Humanosphere), Kyoto University for their invaluable opinions, suggestions and cherished knowledge in reviewing and finalizing my dissertation.

The author wishes and special thanks goes to Junior Associate Professor Chin–Chen Yang (Laboratory of Ecosystem Management and Conservation Ecology, Research Institute for Sustainable Humanosphere, Kyoto University) for providing research facilities, scientific advises, kind motivation and favor, and critical writing and reading of the publications.

The appreciation goes to Professor Junji Sugiyama, Associate Professor Tomoya Imai and Professor Hiroyuki Yano of the Research Institute for Sustainable Humanosphere (RISH), Kyoto University for the use of the lab equipment during this study. I also thank to Dr. Toru Miura (Hokkaido University), Dr. Yasuji Kurimoto (Akita Prefectural University), Dr. Kengo Mikame (Niigata University), Dr. Wakako Ohmura (Forestry and Forest Products Research Institute), Mr. Hiroshi Kurishaki (Toyama Prefectural Agriculture, Forestry and Fisheries Research Center), Mr. Takeshi Katada (Kaneko Construction Co.), Mr. Shigeru Hashimoto (Tokushima Agriculture, Forestry and Fisheries Technology Support Center), Mr. Masao Yamashima (Alpine Enterprise Co.) and Prof. Yoko Takematsu (Yamaguchi University) for providing termite colonies.

The author would like to express sincere gratitude to the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) for providing the scholarship; as well as Research Center for Biomaterials, Indonesian Institute of Sciences (LIPI) for giving valuable supports during overseas years.

The author like to express his great appreciation to Senior Lecturer Toshimitsu Hata and Assistant Professor Aya Yanagawa (Laboratory of Innovative Humano-habitability, Research Institute for Sustainable Humanosphere, Kyoto University) for valuable advices, comments and discussions.

Special thanks to Ms. Shu-ping Tseng for her helpful assistance, scientific and valuable advices comments and discussion. The author would like to express his special thanks to Mr. Akio Adachi and Dr. Izumi Fujimoto (Laboratory of

Innovative Humano-habitability, Research Institute for Sustainable Humanosphere, Kyoto University) for their helpful assistance in preparing the experiments. The author would also like to express his special thanks to fellow lab mates in Laboratory of Innovative Humano-habitability, Research Institute for Sustainable Humanosphere, Kyoto University: Dr. Ono Kazuko, Mrs. Kaori Sunagawa, Mrs. Kyoko Inoue, Mr. Didi Tarmadi, Mrs. Munadian, Mr. Kazushi Nakai and Mr. Bramantyo Wikantyoso; my former lab mates: Dr. Kok-Boon Neoh, Dr. Lee-Jin Bong, Dr. Khoirul Himmi Setiawan, Dr. Titik Kartika, and Dr. Baekyong Choi, for for valuable advices, comments and discussions.

The very special thanks to Japanese teacher, Mrs. Akemi Taniguchi for her great kindness during staying in Japan. Heartiest gratitude is due to the author beloved parents, wife (Nadiyah Mohamed), son (Ikhsan Naufal Abqari – Naufal Kun), and all family for their continuous moral support and encouragements.

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List of Publication

Ikhsan Guswenrivo, Hiroki Sato, Izumi Fujimoto, Tsuyoshi Yoshimura. 2017.

The first record of *Antennopsis gallica* Buchli and Heim and ectoparasitic fungus on the termite *Reticulitermes speratus* (Kolbe) in Japan. Japan Journal of Environmental Entomology and Zoology 28(2): 71–77.

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