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Development of Multiplex Nested PCR for Simultaneous Detection of Ectoparasitic Fungi *Laboulbeniopsis termitarius* and *Antennopsis gallica* on *Reticulitermes speratus* (Blattodea: Rhinotermitidae)

I. Guswenrivo,[1,2,3] S. P. Tseng,[1] C. C. Scotty Yang,[1] and T. Yoshimura[1]

[1]Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan, [2]Research and Development Unit for Biomaterials, Indonesian Institute of Sciences, Cibinong Science Center, Cibinong-Bogor 16911, Indonesia, and [3]Corresponding author, e-mail: ikhsan_guswenrivo@rish.kyoto-u.ac.jp

Abstract

Laboulbeniopsis termitarius (Thaxt) and *Antennopsis gallica* (Buchli and Heim) are two of the most common ectoparasitic fungi found on the body surface of termites. While visual observation under a dissecting microscope is a common method used to screen for such fungi, it generally requires a large number of termites and is thus very time consuming. In this study, we develop a fast, efficient protocol to detect fungal infection on the termite *Reticulitermes speratus* (Kolbe). Species-specific primers were designed based on sequence data and amplified using a number of universal fungus primer pairs that target partial sequences of the 18s rRNA gene of the two fungi. To detect these fungi in a robust yet economic manner, we then developed a multiplex nested polymerase chain reaction assay using species-specific primers. Results suggested that both fungi could be successfully detected, even in cases where *L. termitarius* was at low titer (e.g., a single thallus per termite). The new method described here is recommended for future surveys of these two fungi, as it is more sensitive, species specific, and faster than visual observation, and is likely to facilitate a better understanding of these fungi and their dynamics in host populations.

Key words: ectoparasitic fungi, multiplex nested PCR, nested PCR, termite

In the natural environment, subterranean termites live underground with relatively high humidity and are thus exposed to fungal pathogens that also prefer moist conditions. The relationships between termites and fungi can be generally divided into two categories: symbiotic mutualism and pathogenic relationships and have received tremendous research attention for more than 50 yr (Chouvenc et al. 2011). While numerous studies have studied such relationships, little research focused on ectoparasitic fungi (Lai et al. 1982, Zoberi 1995, Culliney and Grace 2000, Su and Scheffrahn 2000, Chouvenc et al. 2011). Ectoparasitic fungi are a group of fungi that live and attach on the cuticle layer of its host. The presence of these fungi may interrupt the activity and colony stability and even induce collapse of the host colony (Buchli 1952, Guswenrivo et al. 2018).

To date, 22 species of ectoparasitic fungi have been reported to be associated with termites, with *Laboulbeniopsis termitarius* (Thaxt) and *Antennopsis gallica* (Buchli and Heim) being the most common species (Blackwell and Rossi 1986). Hosts of the two fungal species include *Nasutitermes costalis* (Holmgren), *Reticulitermes flavipes* (Kollar), *Reticulitermes virginicus* (Banks), *Reticulitermes*

lucifungus sanonensis (Feytaud), *Ahmaditermes* sp., *Coptotermes crassus* (Snyder), and *Kalotermes flavicollis* (Fabr) (Blackwell and Rossi 1986). Geographic distribution of these two fungi ranges from tropical to temperate zones, and they are regarded as two of the most widespread species of all known ectoparasitic fungi (Thaxter 1920, Buchli 1952, Gouger and Kimbrough 1969, Blackwell and Rossi 1986, Myles et al. 1998). Recently, *L. termitarius* and *A. gallica* were also found on *Reticulitermes* spp. in Japan (Guswenrivo et al. 2017, 2018).

L. termitarius and *A. gallica* can be morphologically characterized by their unique shape and size. Their body sizes are small, barely longer than termite setae, and they often are found attached to the cuticular surface of termites. *L. termitarius* can be identified from three main body structures: the foot cell, stalk, and sporogonium. Meanwhile, *A. gallica* possesses a holdfast, conidiophores, and conidial head as its main body parts.

Ectoparasitic fungi can reduce the lifespan of termites and eventually eliminate the colony (Buchli 1952; Guswenrivo, unpublished data). However, the effects of ectoparasitic fungi on termite remain

unclear, possibly due to the inability to culture them under laboratory conditions. Furthermore, detection of *L. termitarius* and *A. gallica* has generally been performed under a light microscope (Thaxter 1920; Buchli 1952; Gouger and Kimbrough 1969; Myles et al. 1998; Guswenrivo et al. 2017, 2018), a method in which several hundred termites are typically required for a robust verification of the infection status of a focal colony (Guswenrivo et al. 2017). Therefore, an assay that requires minimum number of termites to detect a fungal infection is advantageous.

In recent years, numerous DNA-based methods have been developed to detect fungal infection on plants and insects. Polymerase chain reaction (PCR) is a promising method because of its simplicity, specificity, and sensitivity (Luo and Mitchell 2002). PCR-based methods targeting specific gene regions have been used to identify mycotoxigenic fungi (Kocsubé and Varga 2017) and also to assess the molecular variations in multiple entomopathogenic fungi (Cobb and Clarkson 1993). It also has been applied to examine and detect phytopathogenic fungi in plants, and to facilitate the detection of other pathogens in plants (Henson and French 1993, Martin et al. 2000).

In this study, we aimed to develop a fast and efficient PCR-based assay to detect the termite-associated ectoparasitic fungi *L. termitarius* and *A. gallica* on the cuticular surface of the termite *R. speratus*. As termite colonies are often infected by multiple ectoparasitic fungi, including the two species tested (Blackwell 1980; Guswenrivo, unpublished data), a multiplex PCR assay was further designed to allow simultaneous detection of *L. termitarius* and *A. gallica*.

Materials and Methods

Sample Collection and Fungal Identification
Multiple colonies of the termite *R. speratus* were collected from Hokkaido and kept at 4°C prior to the subsequent observations and experiments. The presence of *L. termitarius* and *A. gallica* was first assessed using a dissecting microscope (S8AP0, Leica, Wetzlar, Germany). Thalli of each fungus, if observed, were removed from the termite using an entomological pin and mounted following the method described by 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*.

DNA Extraction
Total genomic DNA was extracted from *R. speratus* using the Gentra Puregene Cell and Tissue Kit (Qiagen, Hilden, Germany). 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) One termite worker with differential fungus infection strengths (as defined by the number of thalli per infected specimen, Table 1); and 2) samples with mixed infected and noninfected termites at different ratios (referred to as infection rate,

Table 1. Research design to determine fungal detection sensitivity

Laboulbeniopsis termitarius		*Antennopsis gallica*	
Infection strength	Infection rate	Infection strength	Infection rate
3 thalli	1:1 (50%)	5–10 thalli	1:5 (16.7%)
5 thalli	1:5 (16.7%)	20–30 thalli	1:10 (9.1%)
7 thalli	1:10 (9.1%)	50–60 thalli	1:15 (6.25%)

Table 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.

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 2), as part of the PCR optimization process.

Standard Nested PCR
As our standard PCRs often resulted in either low-intensity amplification or nonspecific amplification (data not shown), a standard nested PCR assay was developed to ensure specificity. The first step in the PCR reaction was to combine 25-μl Emerald Amp MAX PCR master mix (Takara, Japan), 2 μl of DNA, 0.2 μM each of the forward and reverse primers, and sterilized distilled water up to 50 μl. The reaction for the second-step PCR was identical, except for the primers (Table 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).

Test of Sensitivity and Specificity
The first two DNA preparations with different levels of fungal infection 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 noninfected termites was included as a negative control. The reaction mixture was cycled according to the PCR protocol described previously.

Multiplex Nested PCR
To detect the two fungi simultaneously, a multiplex nested PCR assay was developed. The first-step PCR mixture was set up by mixing 25-μl Emerald Amp MAX PCR master mix (Takara, Japan), 2 μl of DNA (from the third set of DNA preparation), 1 μl each of forward and reverse primer, and sterilized distilled water up to 50 μl. 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 1) 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 1). 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).

Table 2. Primers designed in this study for detecting *Laboulbeniopsis termitarius* and *Antennopsis gallica*

Fungal species	Primer name	Sequence	Length	Product (bp)
Specific primers for *L. termitarius*	Lter18s-speF1	TAATCTCGACGTAAGAAGGGATGT	24	477
	Lter18s-speR1	GACCCAGCCAGACCAGTACA	20	
	Lter18s-speF2[a]	TATGGCCTTTGGCTGACGC	19	596
	Lter18s-speR2[a]	CTCTGACCATTGAATACTGATGC	23	
	Lter18s-speF3	CGACATGGGGAGGTAGTGAC	20	300
	Lter18s-speR3	GCATATGCCTGCTTTGAACA	20	
	Lter18s-speF4[b]	TCACATGCTTTTGACGGGTA	20	225
	Lter18s-speR4[b]	CACCAGACTTGCCCTTCAGT	20	
Specific primers for *A. gallica*	Agal18s-speF1	GACTCGGGGAGGTAGTGACA	20	194
	Agal18s-speR1[b]	GCCCAAGGTTCAACTACGAG	20	
	Agal18s-speF2[a]	CGATGCGAAGGTCTTGTCTT	20	458
	Agal18s-speR2[a]	CCTGCCTGGAGCACTCTAAT	20	
	Agal18s-speF3[b]	AACGGGTAACGGAGGGTTAG	20	282
	Agal18s-speR3	AACTACGAGCTTTTTAACCAC	21	

[a]Used in the first-step PCR.

[b]Used in the second-step PCR.

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 the 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.

Results

Primer Selection

From all possible specific primer combinations used for *L. termitarius*, primer pairs Lter18s-speF2/Lter18s-speR2 and Lter18s-speF4/Lter18s-speR4 succeeded in amplifying PCR products of 596 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 and 293 bp, respectively, and showed high specificity to *A. gallica*. We concluded that the optimal primer pair combinations for fungal 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*).

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

three thalli in an individual termite) and infection rate (as low as 6.25%; Supp Figs. 1 and 2 [online only]). Results of the multiplex nested PCR revealed the presence of three fragments with various yet expected sizes corresponding to the three amplification targeting 225 bp for *L. termitatius*, 293 bp for *A. gallica*, and 786 bp for a partial mtDNA COII region of termite (Fig. 1). No sign of preferential amplification was observed, even though higher infection strength of *A. gallica* was represented in the DNA template.

Sequencing

The sequences of *L. termitatius* and *A. gallica* were successfully recovered in this study. We found the sequence of *L. termitarius* was grouped in the Laboulbeniomycetes clade with a high bootstrap support value of 100% (Fig. 2) and showed a high sequence similarity (97.7% similarity after excluding 37-bp gaps) to a reference sequence available from GenBank (AY212810, *L. termitatius* isolated from *R. flavipes* in Louisiana; Henk et al. 2003). The *A. gallica* sequence in this study was clustered within *Ascomycota*, with a high bootstrap support value of 100% (Fig. 2). The results of the sequence comparison showed 98% identity with *Graphium euwallaceae* in the class Sordariomycetes (Fig. 2).

Discussion

It has been suggested that neither *L. termitarius* nor *A. gallica* can be cultured under laboratory conditions (Henk et al. 2003; Guswenrivo, unpublished data). These two fungal species have mainly been detected using visual examination based on several key morphological characters, which is a generally time-consuming, labor-intensive process that requires knowledge of fungal taxonomy. Furthermore, previous studies have shown that a robust detection of ectoparasitic fungi on termites normally requires examination of 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, 2018). 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*.

The nested PCR assay developed in this study succeeded in detecting *L. termitarius* and *A. gallica* by using the designed specific primers. Primer specificity was assessed by aligning sequences of *L.*
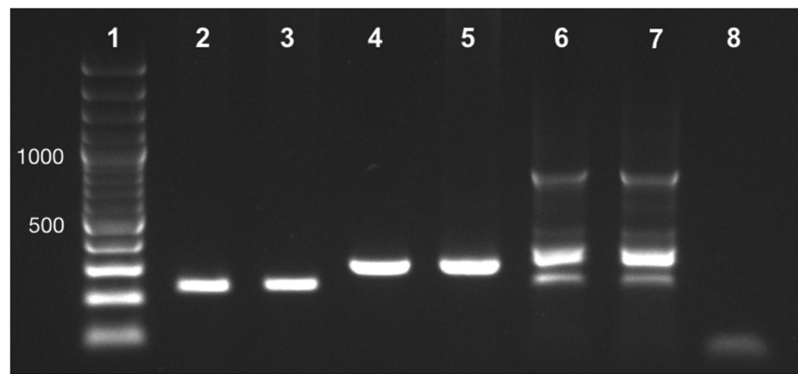
Fig. 1. Detection of *Laboulbeniopsis termitarius* and *Antennopsis gallica* using standard nested PCR and multiplex nested PCR, visualized on 2% agarose gels. Column 1, molecular weight markers expressed as base pairs; columns 2–3, standard nested PCR conducted on a termite infected with 7 thalli of *L. termitarius*; columns 4–5, standard nested PCR conducted on a termite infected with 20 thalli of *A. gallica*; columns 6–7, multiplex nested PCR conducted on the DNA mixtures of two termites with one infected with 7 thalli of *L. termitarius* and the other infected with 20 thalli of *A. gallica*.

termitarius and *A. gallica* generated from our specific primers with those of closely related fungi obtained from GenBank. The results indicated that the regions where the specific primers for *L. termitarius* reside differ from those of other closely related fungi (Weir and Blackwell 2001, Schoch et al. 2009, Bratton 2018) by 4–15 bp, suggesting that cross-amplification is unlikely. For *A. gallica*, while our primers are conserved across the closely related fungi (e.g., 1 bp difference from *G. euwallaceae*), none of these fungi has been reported to be associated with termite (Weir and Blackwell 2001, De Kesel and Haelewaters 2014, Haelewaters et al. 2015, Bratton 2018). Coupled with the fact that all these fungi have distinct life history strategies and do not overlap with termite in habitat, the primers for *A. gallica* developed in this study should be considered robust and specific.

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 United States (Kimbrough and Gouger 1970, Blackwell 1980), whereas the infection rate could be as low as 10% in sampled colonies in Japan (Guswenrivo, unpublished data). 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., three thalli per termite and 9.1%, respectively, Supp Fig. 1 [online only]). Furthermore, additional PCR runs were also conducted using template DNA extracted from a single termite infected with single thallus, and the results are as robust as that from three thalli (data not shown), suggesting the capability of this assay to detect a field infection of *L. termitarius* in the termite *R. speratus*, where a single thallus per individual is apparently common for this fungal species.

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 to 150 across several locations in the United States (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 to 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%; Supp Fig. 2 [online only]).

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 with 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 CoII) 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 the number of *A. gallica* thalli usually outnumbers that of *L. termitarius* in field conditions.

The taxonomic status of *L. termitarius* and *A. gallica*, since discovered, has been determined largely based on morphology. Henk et al. (2003) placed *L. termitarius* in Laboulbeniomycetes based not only on morphological but also on molecular evidence. The sequence data in this study represent a new dataset in supporting linear superposed cells as an informative trait linking the Laboulbeniomycetes. 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 Laboulbeniomycetes (Fig. 2).
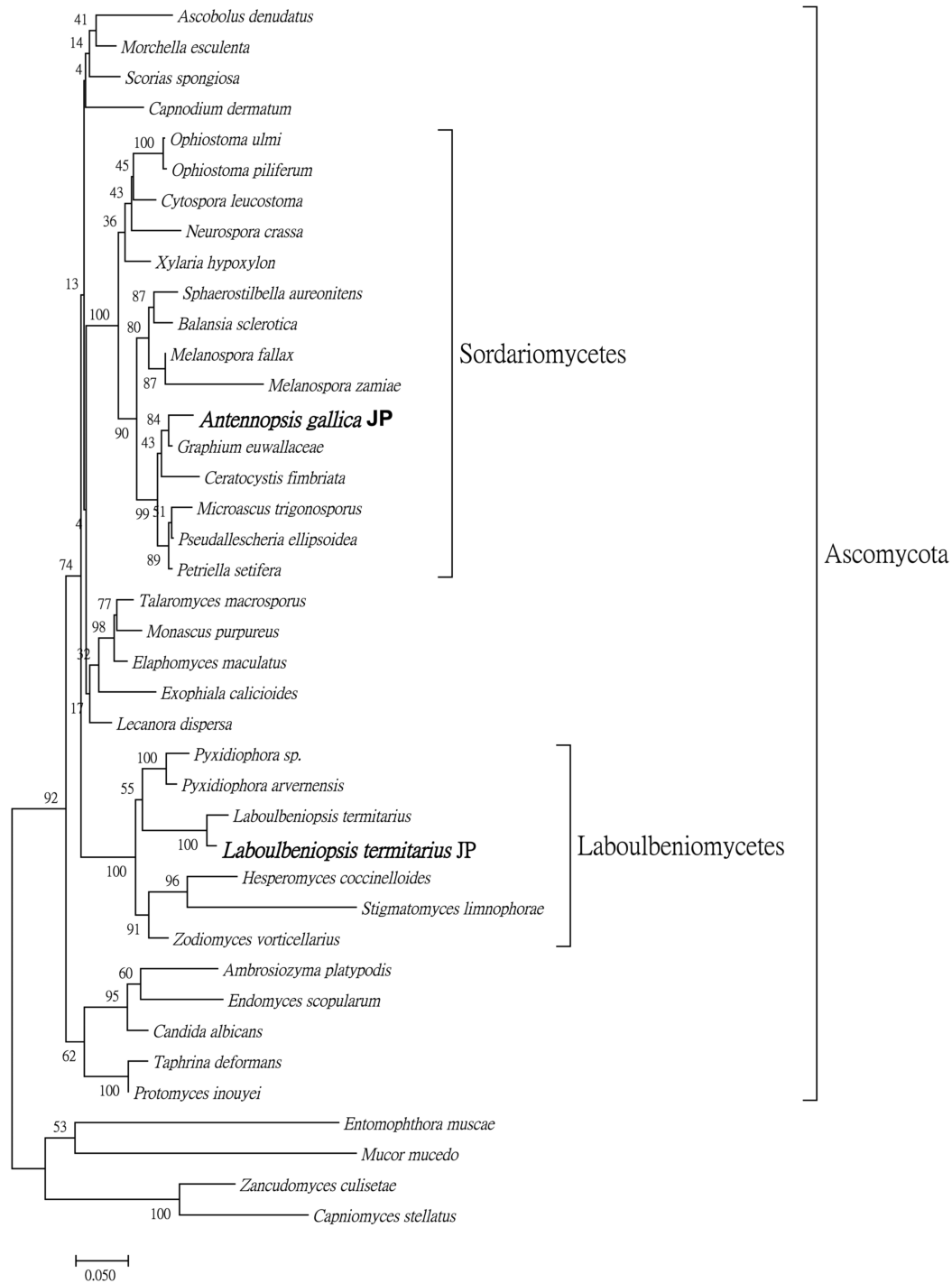
Fig. 2. A maximum likelihood phylogenetic tree for partial 18S rRNA gene sequences of *Laboulbeniopsis termitarius* and *Antennopsis gallica*, together with those of closely related fungal species. Numbers above the nodes represent bootstrap value. Note that *Laboulbeniopsis termitarius* JP and *Antennopsis gallica* JP (in bold) represent the sequences generated in this study.

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 *G. 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).

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

References Cited

Blackwell, M. 1980. New records of termite-infesting fungi. J. Invertebr. Pathol. 35: 101–104.

Blackwell, M., and J. W. Kimbrough. 1976. Ultrastructure of the termite-associated fungus *Laboulbeniopsis termitarius*. Mycologia 68: 541–550.

Blackwell, M., and J. W. Kimbrough. 1978. *Hormiscioideus filamentous* gen. et. sp. nov., a termite-infesting fungus from Brazil. Mycologia 70: 1275–1280.

Blackwell, M., and W. Rossi. 1986. Biogeography of fungal ectoparasites of termites. Mycotaxon 25: 581–601.

Bratton, J. H. 2018. *Zodiomyces verticellarius*, a parasite of water beetles, new to Britain from Anglesey. Field Mycol. 19: 26–27.

Bronzoni, R. V., M. L. Moreli, A. C. R. Cruz, and L. T. M. Figueiredo. 2004. Multiplex nested PCR for Brazilian *Alphavirus* diagnosis. Trans. R. Soc. Trop. Med. Hyg. 98: 456–461.

Buchli, H. H. R. 1952. *Antennopsis gallica*, a new parasite on termites. Trans. IXth Int. Congr. Entomol. 1: 519–524.

Buchli, H. H. R. 1960. Une nouvelle espéce de champignon parasite du genre *Antennopsis* Heim sur les termite de Madagascar. C. R. Hebd. Séances Acad. Science 250: 3365–3367.

Chouvenc, T., N. Y. Su, and J. K. Grace. 2011. Fifty years of attempted biological control of termites—Analysis of a failure. Biol. Control 59: 69–82.

Clair, D., J. Larrue, G. Aubert, J. Gillet, G. Cloquemin, and E. Boudon-Padieu. 2003. A multiplex nested-PCR assay for sensitive and simultaneous detection and direct identification of phytoplasma in the Elm yellows group and Stolbur group and its use in survey of grapevine yellows in France. Vitis 43: 151–157.

Cobb, B. D., and J. M. Clarkson. 1993. Detection of molecular variation in the insect pathogenic fungus *Metarhizium* using RAPD-PCR. FEMS Mycrobiol. Lett. 112: 319–324.

Culliney, T. W., and J. K. Grace. 2000. Prospects for the biological control of subterranean termite (Isoptera: Rhinotermitidae), with special reference to *Coptotermes formosanus*. Bull. Entomol. Res. 90: 9–21.

De Kesel, A., and D. Haelewaters. 2014. *Laboulbenia slackensis* and *L. littoralis* sp. nov. (*Ascomycota*, *Laboulbeniales*), two sibling species as a result of ecological speciation. Mycologia 106: 407–414.

Dring, D. M. 1971. Techniques for microscopic preparation, pp. 95–98. *In* C. Booth (ed.), Methods in microbiology. Academic Press, New York, NY.

Elnifro, E. M., A. M. Ashshi, R. J. Cooper, and P. E. Klapper. 2000. Multiplex PCR: optimization and application in diagnostic virology. Clin. Microbiol. Rev. 13: 559–570.

Gargas, A., and J. W. Taylor. 1992. Polymerase chain reaction (PCR) primers for amplifying and sequencing 18S rDNA from lichenized fungi. Mycologia 84: 589–592.

Gouger, R. J., and J. W. Kimbrough. 1969. *Antennopsis gallica* Heim and Buchli (Hyphomycetes: Gloeohaustoriales), an entomogenous fungus on subterranean termites in Florida. J. Invertebr. Pathol. 13: 223–228.

Guindon, S., J. F. Dufayard, V. Levort, M. Anisimova, W. Hordijk, and O. Gascuel. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assesing the performance of PhyML 3.0. Syst. Biol. 59: 307–321.

Guswenrivo, I., H. Sato, I. Fujimoto, and T. Yoshimura. 2017. The first record of *Antennopsis gallica* Buchli and Heim, an ectoparasitic fungus on the termite *Reticulitermes speratus* (Kolbe) in Japan. Jpn. J. Environ. Entomol. 28: 71–77.

Guswenrivo, I., H. Sato, I. Fujimoto, and T. Yoshimura. 2018. First record of the termite ectoparasite *Laboulbeniopsis termitarius* Thaxter in Japan. Mycoscience. (in press).

Haelewaters, D., M. Gorczak, W. P. Pfliegles, A. Tartally, M. Tischer, M. Wrzosek, and D. H. Pfister. 2014. Bringing *Laboulbeniales* into the 21st century: enhanced techniques for extraction and PCR amplification of DNA from minute ectoparasitic fungi. IMA Fungus 6: 363–372.

Hamelin, R. C., P. Bèrubè, M. Gignac, and M. Bourassa. 1996. Identification of root rot fungi in nursey seedlings by nested multiplex PCR. Appl. Environ. Microbiol. 62: 4026–4031.

Henk, D. A., A. Weir, and M. Blackwell. 2003. *Laboulbeniopsis termitarius*, an ectoparasite of termite newly recognized as a member of Laboulbeniomycetes. Mycologia 95: 561–564.

Henson, J. M., and R. French. 1993. The polymerase chain reaction and plant disease diagnosis. Annu. Rev. Phytopathol. 31: 81–109.

Hojo, M., T. Miura, K. Maekawa, and R. Iwata. 2001. *Termitaria* species (Termitariales, Deuteromycetes) found on Japanese termites (Isoptera). Sociobiology 38: 1–12.

Kalle, E., M. Kubista, and C. Rensing. 2014. Multi-template polymerase chain reaction. Biomol. Detect. Quantif. 2: 11–29.

Kimbrough, J. W., and R. J. Gouger. 1970. Structure and development of the fungus *Laboulbeniopsis termitarius*. J. Invertebr. Pathol. 16: 205–213.

Kocsubé, S., and J. Varga. 2017. Targeting conserved genes in *Aspergillus* species, pp. 131–141. In A. Moretti and A. Susca (eds.), Mycotoxigenic fungi: methods and protocols. Springer Nature, New York, NY.

Lai, P. Y., M. Tamashiro, and J. K. Fujit. 1982. Pathogenicity of six strains of entomogenous fungi to *Coptotermes formosanus*. J. Invertebr. Pathol. 39: 1–5.

Lam, W. Y., A. C. Yeung, J. W. Tang, M. Ip, E. W. C. Chan, M. Hhui, and P. K. S. Chan. 2007. Rapid multiplex nested PCR for detection of respiratory viruses. J. Clin. Microbiol. 45: 3631–3640.

Lefort, V., J.-E. Jean, and O. Gascuel. 2017. SMS: smart model selection in PhyML. Mol. Biol. Evol. 34: 2422–2424.

Luo, G., and T. G. Mitchell. 2002. Rapid identification of pathogenic fungi directly from cultures by using multiplex PCR. J. Clin. Microbiol. 40: 2860–2865.

Lynch, C. C., M. Twizeyimana, J. S. Mayorquin, D. H. Wang, F. Na, M. Kayim, M. T. Kasson, P. Q. Thu, C. Bateman, P. Rugman-Jones, et al. 2016. Identification, pathogenicity and abundance of *Paracremonium pembeum* sp. nov. and *Graphium euwallaceae* sp. nov. two newly discovered mycangial associates of the polyphagous shot hole borer (*Euwallacea* sp.) in California. Mycologia 108: 313–329.

Maharachchikumbura, S. S., K. D. Hyde, E. G. Jones, E. H. C. McKenzie, S. K. Huang, M. A. Abdel-wahab, D. A. Daragama, M. Dayarathne, M. J. D'souza, I. D. Goonasekara, et al. 2015. Towards a natural classification and backbone tree for Sordariomycets. Fungal Divers. 72: 199–301.

Martin, R. R., D. James, and C. A. Lévesque. 2000. Impact of molecular diagnostic technologies on plant disease management. Annu. Rev. Phytopathol. 38: 207–239.

Myles, T. G., B. H. Strack, and B. Forschler. 1998. Distribution and abundance of *Antennopsis gallica* (Hyphomycetes: Gleohaustoriales), an ectoparasitic fungus, on the eastern subterranean termite in Canada. J. Invertebr. Pathol. 72: 132–137.

Park, L., L. Ten, S. Y. Lee, C. G. Back, J. J. Lee, H. B. Lee, and H. Y. Jung. 2017. New recorded species in three genera of the Sordariomycetes in Korea. Mycobiology 45: 64–72.

Roberts, D. W. 1989. World picture of biological control of insects by fungi. Mem. Inst. Oswaldo Cruz 84: 89–100.

Samuels, G. J., and M. Blackwell. 2001. Pyrenomycetes-fungi with perithecia, pp. 221–255. In D. J. McLaughlin, E. G. McLaughlin and P. A. Lemke (eds.), The Mycota VII part A. Springer, Berlin, Germany.

Schoch, C. L., G. H. Sung, F. López-Giráldez, J. P. Townsend, J. Miadlikowska, V. Hofstetter, and C. Gueidan. 2009. The Ascomycota tree of life; a phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. Syst. Biol. 58: 224–239.

Seifert, K. A., and W. Gams. 2001. The taxonomy of anamorphic fungi, pp. 307–347. In D. J. McLaughlin, E. G. McLaughlin and P. A. Lemke (eds.), The Mycota VII part A. Springer, Berlin, Germany.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, P. Flook, and H. Liu. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87: 651–701.

Su, N. Y., and R. H. Scheffrahn. 2000. Termites as pests of buildings, pp. 437–453. In T. Abe, D. E. Bignell and M. Higashi (eds.), Termites: evolution, sociality, symbioses, ecology. Kluwer Academic Publishers, Dordrecht, Netherland.

Tamura, K., G. Stecher, D. Peterson, A. Filipshki, and S. Kumar. 2013. Mega 6: molecular evolutionary genetic analysis version 6.0. Mol. Biol. Evol. 30: 2725–2729.

Thaxter, R. 1920. On certain peculiar fungi-parasites of living insects. Bot. Gaz. 58: 235–253.

Weir, A., and M. Blackwell. 2001. Molecular data support the *Laboulbeniales* as a separate class of *Ascomycota*, *Laboulbeniomycetes*. Mycol. Res. 105: 1182–1190.

White, T. J., T. Bruns, S. B. Lee, and T. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetic, pp. 315–352. In M. A. Innis, D. H. Gelfand, J. J. Snisky and T. J. White (eds.), White PCR protocols: a guide to methods and applications. Academic Press Inc., San Diego, CA.

Zhang, N., L. A. Castlebury, A. N. Miller, S. M. Hunhndorf, C. L. Schoch, K. A. Seifert, A. Y. Rossman, J. D. Rogers, J. K. B. Volkmann-Kohlmeyer, and G. H. Sung. 2006. An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. Mycologia 98: 1076–1087.

Zoberi, M. H. 1995. *Metarhizium anisopliae* a fungal pathogen of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Mycologia 87: 354–359.