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Symbiosis of a termite and a sclerotium-forming fungus: Sclerotia mimic termite eggs

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Brown balls, of a similar size but different shape to termite eggs, were found frequently in the piles of eggs of the subterranean termite *Reticulitermes speratus*. rDNA analysis identified the ball as the sclerotia of the fungus, *Fibularhizoctonia* sp. nov, which is phylogenetically closest to decay fungi, *Athelia* spp. Laboratory observation showed that the workers gathered the eggs and the sclerotia indiscriminately, even if they were widely scattered in a Petri dish, and piled them up in a specific place for egg care. We compared the morphology of the eggs with that of sclerotia of *Fibularhizoctonia* spp. and *Athelia* spp. in relation to egg carrying behaviour, and found that the workers could only carry the *Fibularhizoctonia* spp. sclerotia whose diameters were similar to the short diameter of the eggs. We also conducted a bioassay using termite eggs and dummy eggs (glass beads and sea sand) of two diameter-classes, coated with or without the egg-derived chemicals. The workers recognized the eggs based on a combination of the size, shape, and chemical cues. All the results suggested that the sclerotia mimic the eggs both morphologically and chemically. Finally, we found that the workers suppressed germination of sclerotia, and termite egg survival increased in the presence of sclerotia only if they were tended by the workers. If the workers were removed experimentally, the sclerotia germinated and grew by exploiting termite eggs. These results suggest that the sclerotia protect termite eggs from putative pathogens.

Key words: egg recognition; sclerotium; subterranean termite; symbiosis, termite–fungus interaction.

INTRODUCTION

A wide variety of interactions between insects and fungi have been described, with the total dependence of insects on fungi as food at one end of the spectrum, and total dependence of fungi on insects at the other, and incorporating many shades of interdependence in between (Wilding *et al*. 1989). Although it is often accepted that mutualisms are derived evolutionarily from initially antagonistic interactions between species, we actually know little about the ecological bases for mutualisms (Thompson 1982). In this paper, we report a novel termite–fungus interaction as material for understanding evolutionary backgrounds that favor the origination and maintenance of mutualisms.

Colonies of the subterranean termite *Reticulitermes speratus* are usually founded by a pair of primary reproductives. With the growth of the colony, supplementary reproductives are added. The reproductives move together and lay eggs in different places in the nests. When workers recognize the scattered eggs, they bring them together and heap them up in order to tend them. In nests of *R. speratus*, workers may make several piles of eggs in this way. Brown balls (tentatively named 'termite balls') that are similar in size but different in shape and colour compared to the termite eggs, are frequently found within these piles of eggs (Fig. 1a). Termite balls have been observed by some termitologists in Japan, but they remain

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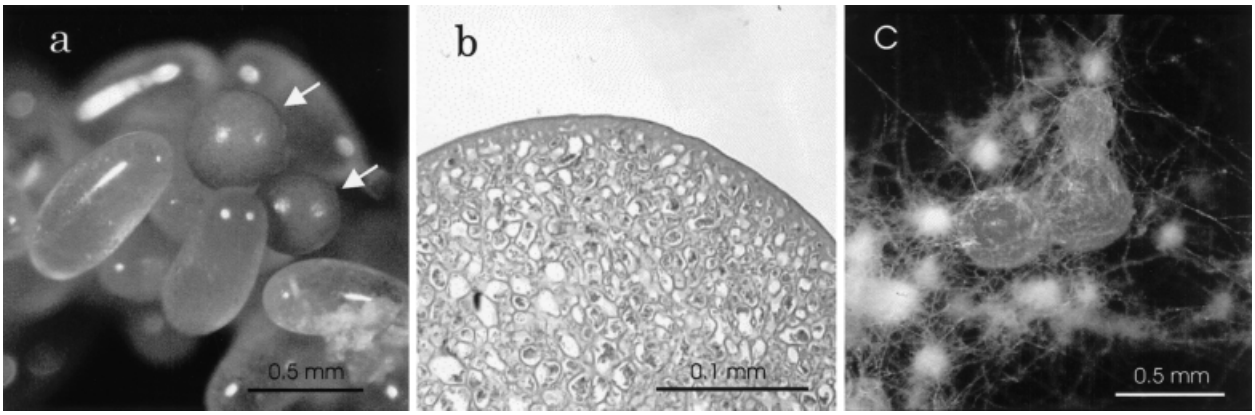

Fig. 1. The 'termite balls'. (a) The termite balls in the piles of eggs (termite balls indicated by arrows). The termite balls have spherical shape, although the termite eggs have an oval shape; (b) the cross section of a termite ball; (c) the production of termite balls on potato dextrose agar.

unidentified, however, the 'presumed nutritive egg', noted by Iwata *et al*. (1999), seems to be the same thing.

We investigated the relationship between the termite, *R. speratus*, and sclerotium-forming fungi. First, we identified termite balls by the analysis of DNA sequences of the internal transcribed spacers (ITS) and the 5.8 S ribosomal RNA gene of the nuclear ribosomal repeat unit. Second, we conducted a wide-area sampling in western Japan to examine whether all the termite nests contain termite balls in the piles of eggs, regardless of locality, termite subspecies and the species of nesting wood. Third, we examined how termite workers recognize the eggs by a bioassay using glass beads and sea sand as dummy eggs, in order to discover whether termite balls are morphological and/or chemical mimics of termite eggs. Finally, we investigated the influence of termites on sclerotial germination, and the influence of sclerotia on termite egg survivorship, in order to reveal the cost and/or benefit of the termite–fungus relationship.

METHODS

Identification of termite balls

Fungal isolation

Termite balls were obtained from the piles of termite eggs in nests of *Reticulitermes speratus* during August 1998 at Takaragaike, a northern suburb of Kyoto, in western Japan. They were soaked in a solution of sodium hypochlorite (approximately 1% active chlorine) for 5 min, then rinsed with sterilized distilled water several times. The surface-sterilized termite balls were inoculated on potato dextrose agar (PDA; Difco Laboratory, Detroit, MI, USA) and incubated at 25°C for 3 weeks. One of the sclerotia, which was newly developed from the termite ball on the colony, was re-isolated and cultured on the new PDA plate and designated TMB.

DNA preparation, polymerase chain reaction amplification, DNA sequencing and phylogenetic analysis

The TMB isolate was inoculated into 3 ml potato dextrose broth and incubated in an orbital shaker (150 r.p.m.) at 25°C for 3 d. The culture was harvested by centrifugation and re-suspended in 500 µl of DNA extraction buffer [50 mmol Tris-HCl pH 8.0, 100 mmol NaCl, 125 mmol trisodium edetate, 2% (w/v) sodium dodecanoylsarcosinate, 1% (v/v) 2-mercaptoethanol] in a 1.5 ml microcentrifuge tube. The tube was heated in boiling water for 5 min to extract DNA. The genomic DNA was partially purified by several steps of extractions with phenol and chroloform, then precipitated by ethanol, in standard DNA manipulation protocols (Sambrook *et al*. 1989). The precipitated DNA pellet was dissolved in 20 µl TE buffer. PCR was performed in a total volume of 50 µl, containing 1 µl of template DNA solution, 10 pmol of each primer (ITS4: 5′-TCCTCCGCT

TATTGATATGC-3′ and ITS5: 5′-GGAAG TAAAAGTCGTAACAAGG-3′; White *et al*. 1990), 50 µmol of each dNTP, 5 µl of 10× PCR buffer and 1.5 IU of Taq DNA polymerase (Takara Shuzo, Kyoto, Japan). The thermal cycler (TP3000; Takara) was programmed as follows: initial denaturation, 2 min at 95°C; then 35 cycles for 0.5 min at 95°C, 1 min at 53°C, 1.5 min at 72°C; and final extension, 5 min at 72°C. After electrophoresis in LMP agarose (Sea Plaque GTG, FMC, Rockland, ME, USA), an amplified product was excised from the gel. The DNA fragment was briefly processed by T4 DNA polymerase to form blunt ends and cloned into pZErO-2 vector (Invitorogen, Irvine, CA, USA). DNA sequencing conditions were described in Oda *et al*. (1999). Sequences were aligned using the CLUSTALW multiple alignment program (version 1.8) (Thompson *et al*. 1994). The alignment was completed by the 'Slow/Accurate' method with the parameters as follows: Gap Open Penalty, 15.00; Gap Extension Penalty, 4. The sequence alignment has been submitted to TreeBASE, a relational database of phylogenic information (http://www.harbaria.harvard.edu/treebase/). The aligned sequences were analyzed by the neighbor-joining method (Saitou & Nei 1987), using NEIGHBOUR in the PHYLIP package (version 3.5c) (Felsenstein 1993). The distance matrix was calculated using DNADIST with Kimura's two-parameter method and the topology was tested with 500 bootstrap trials. The phylogram was visualized by TREEVIEWPPC (version 1.6) software obtained from the following URL, http://taxonomy.zoology. gla.ac.uk/rod/treeview.html.

Sclerotial comparison

To know whether termite balls mimic termite eggs, we first compared their morphology with that of the sclerotia of closely related fungi, *Athelia arachnoidea* and four isolates of *Athelia epiphylla*, identified by the present phylogenetic analysis. These two species are decomposers of the dead leaves of deciduous trees, and are also known to cause an economically damaging rot in carrot and apple fruit in cold storage (Adams 1996). Four plates were incubated with a 5 mm diameter mycelial plug taken from the margin of a 20-day-old colony of each isolate, and placed in the laboratory bench at 25°C. After a 20 d incubation period, the number of sclerotia per colony was counted. Measurements of sclerotial diameter were made from 15 sclerotia (if fewer sclerotia were formed, all were measured). The data from the replicate plates were averaged.

The sizes of sclerotia and termite eggs were also compared, although eggs are oval and sclerotia are spherical. Because termite workers always grasp the short side of eggs when they carry them, the short diameter of the eggs was compared to the sclerotial diameters using an ANOVA followed by Scheffe's test. To compare the variation in diameters having different means, the coefficient of variation (CV) was applied, which is the standard deviation expressed as a percentage of the mean. The variance of a log transformed value is known to be almost equal to the square of the CV of the original measurements. Thus, the analysis of log transformed diameters using *F*-test denotes the comparison of the CV of the diameters (Kuno 1986).

Sampling conducted over a wide area

In western Japan, we examined whether all termite nests contained termite balls in the piles of eggs, regardless of locality, termite subspecies, and the species of nesting wood. We investigated a total of 50 nests of *R. speratus* including three subspecies (*speratus, leptolabralis*, and *kyushuensis*) (Morimoto 1968). The sampling locality was as follows; two *speratus* nests in Nagano (36°14′ N, 138°2′ E), two *speratus* nests in Gifu (35°24′ N, 136°44′ E), one *speratus* nest in Shiga (35°20′ N, 136°18′ E), 28 speratus nests in Kyoto (35°6′ N, 135°39′ E), nine *laptplabralis* nests in Okayama (34°40′ N, 133°56′ E), four *kyushuensis* nests in Hiroshima (34°24′ N, 132°25′ E), two *laptplabralis* nests in Kochi (33°40′ N, 133°40′ E), one *kyushuensis* nest in Ehime (34°0′ N, 133°5′ E), and two *kyushuensis* nests in Ooita (33°20′ N, 131°39′ E). This was carried out from June to October in 1998 and 1999. The termite-colonized wood was dissected and the piles of eggs scattered within the nest were collected with an aspirator. The eggs and sclerotia were preserved in 70% ethanol and brought to the laboratory. We counted the eggs and sclerotia collected from each nest under a stereoscopic microscope and calculated the percentage of sclerotium (the number of sclerotia × 100/the number of eggs and sclerotia).

Experiment 1: Egg-carrying test

Dummy egg-carrying test

We conducted dummy egg-carrying tests using dummy eggs made of glass beads or sea sand, in order to evaluate how termites recognize eggs. The 0.4 mm glass beads (0.097 ± 0.0018 mg, mean ± SE) were approximately the same size as termite eggs and termite balls, and had a spherical shape. The 0.6 mm glass beads(0.309 ± 0.0055 mg) have a spherical shape, but are larger than termite eggs and termite balls. The 40 mesh sea-sand (0.069 ± 0.0021 mg, Wako Pure Chemical Industries, Osaka, Japan) consists of the same material as the glass beads, but does not have a spherical shape.

Termite eggs used in this experiment were obtained from the field colony of *R. speratus* at Takaragaike, Kyoto. The glass beads and sea sand were washed twice with ethyl ether to remove contaminants before the experiment. We prepared various types of dummy eggs and applied egg wax to their surface as follows: approximately 6800 eggs (320 mg) were added to 2.0 ml ethyl ether and washed by an ultrasonic bath cleaner for 30 s; then the eggs were removed from the solution with a filter funnel. In order to apply the egg wax to the surface of the glass beads, 0.5 ml of the solution (1700 egg/ml) was added to approximately 140 objects (i.e. 13.6 mg of the 0.4 mm glass beads, 43.4 mg of the 0.6 mm glass beads, and 9.7 mg of the 40 mesh sea sand), and then ethyl ether was dried up. Thus we obtained objects with the equivalent of 12.2 eggs worth of wax on their surfaces. Of the 20-fold diluted solution, 0.5 ml (85 egg/ml) was added to approximately 140 of the 0.4 mm glass beads (13.6 mg), in order to apply the equivalent of 0.6 eggs worth of wax on their surfaces. We also prepared some of the eggs by washing twice with ethyl ether to remove their surface wax.

We tested whether they were recognized as eggs by termites as follows: we arranged 20 eggs and 20 dummy objects randomly on a Petri dish (90 mm diameter) on moist filter paper and released 30 workers into the dish. The dishes were maintained at 25°C in dark conditions. Fifteen hours later, we investigated the piles of eggs made by the workers, and counted the eggs and the dummy objects. The pile-up rates were determined by counting the number of dummy objects within the piles of eggs as a fraction of 20. This experiment was replicated five times for each treatment. The pile-up rates were arcsine transformed before statistical analysis in order to stabilize variances (Sokal & Rohlf 1995). We analyzed the arcsine transformed data using ANOVA, followed by Scheffe's test. The transformed means and standard errors were sine transformed and are shown in Table 2.

Sclerotia-carrying test

To test whether sclerotia are recognized as eggs by termites, the same protocol as in the dummy egg-carrying test was used for sclerotia. We arranged 20 eggs and 20 sclerotia randomly on a Petri dish and released 30 workers into the dish. Fifteen hours later, the pile-up rate of the sclerotia was determined by counting the number of sclerotia within the piles of eggs as a fraction of 20. We tested five types of sclerotia treated as follows:

1 Collected 1; obtained from the piles of eggs in a termite nest collected in the field.
2 Washed; washed twice with ethyl ether to remove the chemical on their surface.
3 Re-coated; washed twice, then the chemical re-applied.
4 Collected 2; obtained from the nest, but out of the piles of eggs.
5 Cultured; obtained from a 20-day-old fungal colony cultured on PDA.

Statistical analysis was conducted as it was in the dummy egg-carrying test. The arcsine transformed data was sine transformed and are shown in Table 2.

Experiment 2: The influence of termites on sclerotial germination

The piled eggs and sclerotia were observed to be manipulated by worker termites, and would therefore be smeared with saliva. The saliva of workers has been reported as inhibiting fungal germination (Thomas 1987c). We examined the effect of termite workers on sclerotial germination. Fifty eggs, 50 sclerotia and 30 workers were placed in a Petri dish (90 mm diameter) on moist filter paper, and maintained at 25°C. The workers piled up the eggs and sclerotia within 15 h. All workers were then removed from three of the six Petri dishes as a control. The Petri dishes were kept at 25°C for

2 weeks. The sclerotia in each dish were then examined under a stereoscopic microscope. Percentage germination was determined by counting the number of sclerotia with visible germ tubes as a fraction of the total number of sclerotia in each Petri dish.

Experiment 3: The influence of sclerotia on termite eggs

We tested whether sclerotia in the piles of eggs has an effect on egg survivorship by comparing the survival rates of eggs maintained with and without sclerotia. Four colonies used for this experiment were collected at Takaragaike, Kyoto. Fifty eggs, 10 sclerotia and 50 workers obtained from a single colony were placed into a Petri dish (90 mm diameter). A non-woven, non-shrinking cloth made from wood pulp was stuck on the bottom of the Petri dish and filter paper was laid over it, and then moistened with distilled water. Without the cloth, termites were liable to become trapped by the surface-tension of the drops of water. The termites fed on the moist filter paper and non-woven cloth. The Petri dishes were maintained at 25°C for 2 weeks, then the survival rates of eggs were investigated by counting living eggs and larva as a fraction of the initial number of eggs. The control consisted of 50 eggs and 50 workers without sclerotia. Both treatments were repeated seven times for each colony (i.e. $2 \times 7 \times 4$). We analyzed arcsine transformed survival rates using ANOVA. The transformed means and standard errors were sine transformed and are shown in Table 3.

RESULTS

Identification of termite balls

The termite balls were spherical or nearly spherical (0.33–0.34 mm in diameter) and ranged from light brown to brown in colour. The cross-section of the termite ball showed that internal tissue was pseudoparenchymatous and the sclerotium consisted of one type of tissue with slightly pigmented outer cells (Fig. 1b). A few days after incubation on PDA, the termite ball germinated, giving rise to a fungal colony. The colony was white, with some hyphal strands. Primary conductive hyphae were 3–6 µm wide, bearing clamp connections at widely spaced septa. Secondary and derived hyphae were narrower, 1.5–3.0 µm wide, usually lacking clamp connections. Sclerotia identical to the termite balls were produced near the margins of the colony and hyphal strands. Teleomorphic organs were not found on the PDA culture. These observations implied that the termite ball was a sclerotium of a corticioid basidiomyceteous fungus. The 5.8S rRNA gene and two internal transcribed spacers were amplified from the culture of TMB. The amplified fragment was 674 bp in length (Accession No. AB032423 in the DDBJ/EMBL/GenBank nucleotide sequence database). The results of a BLAST search (http://www.blast.genome.ad.jp/) showed that the fragment was similar to several Basidiomyceteous fungi, especially, to the corticioid fungi, *Athelia* spp. and their anamorphs *Fibularhizoctonia* spp. (data not shown). The DNA data was also analyzed to infer phylogenic relationships via neighbor-joining (Fig. 2). Within this tree, the termite ball fungus formed an exclusive group with *A. epiphylla* (anamorph: *Fibularhizoctonia centrifuga*) and *A. neuhoffii*. The genetic distances between the TMB and *A. epiphylla*, and between the TMB and *A. neuhoffii* were 0.02291, 0.01970, respectively. The results strongly suggested that the termite ball fungus is one species of *Athelia*. However, we have not observed its teleomorph on cultures or in nature. We tentatively classified it as a species of *Fibularhizoctonia* based on its anamorphic characters.

Sclerotial comparison

The TMB produced many more sclerotia than the other isolates (Table 1). The sclerotia of the TMB (i.e. the termite balls) had a texture as smooth as termite eggs (Fig. 1c), while those of the other isolates had rougher textures. The sclerotia of the TMB were significantly smaller than those of the other isolates, and their diameter was the same as the short diameter of termite eggs (Table 1). In the TMB, the level of variation in sclerotial diameters was significantly smaller than in other isolates (Table 1). thus, these results show that termite balls are morphologicaly similar to termite eggs.

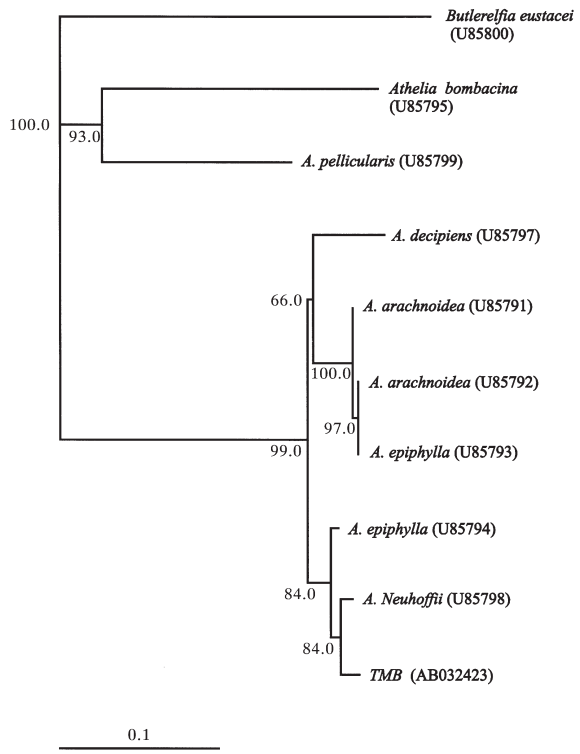

Fig. 2. Phylogenetic position of the termite-ball fungus (TMB) based on nucleotide sequences of the internal transcribed spacers region. The tree was constructed by the neighbor-joining method. The value on nodes are the percentage confidence levels from 500 replicate bootstrap samplings. The distance corresponding to 10 base changes per 100 nucleotide positions is indicated by a bar. Numbers in paretheses are accession no. in the DDBJ/EMBL/GenBank nucleotide sequence databases.

Sampling conducted over a wide area

Sclerotia were found in the nests of all three termite subspecies (*speratus, leptolabralis* and *kyushuensis*). The percentage of colonies possessing sclerotia differed significantly between the tree species that the termites harvested ($P < 0.01$, Fisher's exact test). All 40 colonies nesting in rotten pine wood (*Pinus densiflora*) had sclerotia in their piles of termite eggs, although only 4 out of the 10 colonies nesting in rotten Japanese cedar (*Cryptomeria japonica*) had sclerotia present. This was probably because fungistatic substances in fresh cedar wood can inhibit the growth of this fungus (e.g. Kirk & Cowling 1984). The colonies nesting in ceder wood that was relatively more rotten had many sclerotia present in the piles of eggs (Fig. 3).

Experiment 1: Egg-carrying test

Only half of the termite eggs washed with ether were piled up, whereas almost all the normal eggs were carried and piled up by workers (Table 2). The 0.4 mm glass beads coated with egg wax were piled up much more than beads without the egg wax ($P < 0.000$, Scheffe's test). Neither 0.6 mm glass beads nor sea sand was piled up, even if they had been coated with egg wax. These results suggest that the termites recognize their egg by chemical cues and physical cues, that is, by the spherical shape and the size.

More than 90% of the sclerotia (collected from the piles of eggs in a termite nest) were piled up

Table 1 Comparison of the characteristics of termite balls with other closely related sclerotium-forming fungi and termite eggs. Sclerotial characteristics were measured after 20 days of incubation

	No. sclerotia ± SE*	Diameter (mm) ± SE†	Coefficient of varience‡	*n*
Termite egg	—	0.356 ± 0.004^{a}	8.8^{a}	60
TMB	2482.0 ± 416.0^{a}	0.337 ± 0.006^{a}	13.1^{b}	60
Athelia archnoidea	31.8 ± 7.7^{b}	0.745 ± 0.019^{b}	19.6^{cd}	55
Athelia epiphylla				
IFO 5253	93.0 ± 54.3^{b}	1.030 ± 0.048^{b}	25.7^{c}	30
IFO 6142	12.5 ± 4.8^{b}	2.139 ± 0.093^{c}	26.2^{c}	36
IFO 6184	27.8 ± 8.2^{b}	2.240 ± 0.049^{c}	17.1^{d}	60
IFO 6646	8.8 ± 5.2^{b}	2.958 ± 0.144^{d}	23.9^{cd}	24

There were significant differences between different superscript letters of the alphabet the 0.05 level. *Data are the average of four replications of each isolate, significance was determined by Sheffe's test; †diameters of sclerotia and short diameters of termite eggs, significance was determined by Scheffe's test; ‡coefficient of variation of the diameters, the log transformed diameters were compared using an *F*-test.

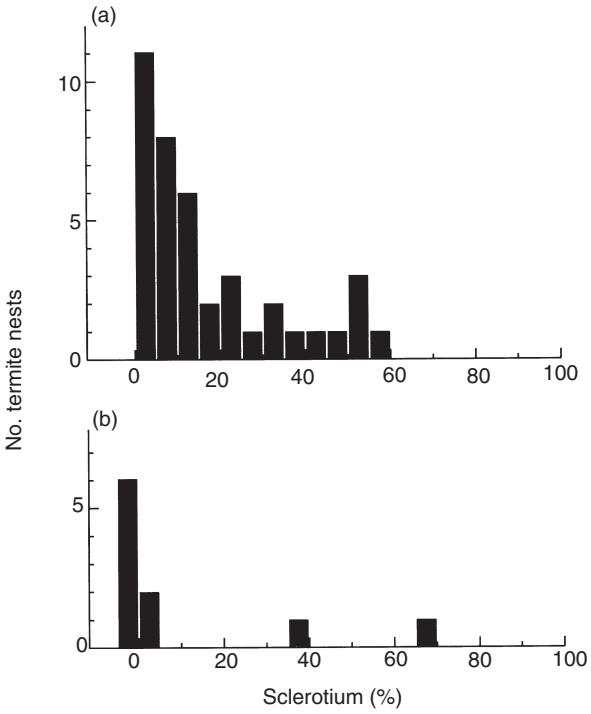

Fig. 3. Frequency distribution of the percentage of termite balls in the piles of eggs. Data were pooled according to the wood species, (a) *Pinus densiflora* (n=38) and (b) *Cryptomeria japonica* (n=10).

with the eggs (Table 2), but sclerotia that had been washed with ether were rarely piled up, and the washed sclerotia recovered their pile-up rates if they were coated with egg wax again. These results indicate that the sclerotia have chemicals on their surfaces that are used in termite egg recognition. Approximatel;y 40% of sclerotia obtained from the nest, but not in the piles of eggs (therefore, never having been manipulated by termites), were piled up, and only 6% of the sclerotia collected from the PDA culture were piled up.

Experiment 2: Influence of termites on sclerotial germination

Percentage germination of sclerotia maintained by termite workers was significantly lower than for sclerotia maintained without workers ($P < 0.001$, Fisher's exact test). Within 2 weeks, none of the 50 sclerotia germinated in the presence of workers, while 46 out of 50 sclerotia germinated in the absence of workers, over-growing termite eggs. This result suggests that termite workers somehow inhibit sclerotial germination. However, workers did not completely inhibit the long-term germination of sclerotia, because a few germinated. In fact the fungus grew on the termite excreta at the corner of the Petri dish and on the underside of the filter paper where termites could not reach. The number of sclerotia approximately doubled after 2 months.

Table 2 Comparison of the pile-up rates for termite eggs, dummy eggs and (b) sclerotia

Materials	Treatments*	Pile-up rates ± SE†
Termite egg	None	0.998 ± 0.043^{a}
(0.36 mm)	Washed	0.542 ± 0.104^{b}
Glass beads	0 (eq)	0.080 ± 0.049^{c}
(0.4 mm)	0.6 (eq)	0.251 ± 0.049^{bc}
	12 (eq)	0.471 ± 0.117^{b}
Glass beads	0 (eq)	0^{c}
(0.6 mm)	12 (eq)	0^{c}
Sea sand	0 (eq)	0^{c}
(40 mesh)	12 (eq)	0^{c}
Sclerotia	Collected 1	0.9610 ± 0.075^{a}
	Washed	0.040 ± 0.040^{b}
	Re-coated	0.970 ± 0.102^{a}
	Collected 2	0.421 ± 0.028^{c}
	Cultured	0.060 ± 0.029^{b}

There are significant differences between different superscript letters of the alphabet at the 0.05 level. *eq, the equivalent of the egg chemical applied on each dummy material; Collected 1, obtained from the piles of eggs in a termite nest; Collected 2, obtained from a termite nest, but out of the piles of eggs; †data are the average of five replicates of each treatment.

Experiment 3: The influence of sclerotia on the termite eggs

The survival rates of eggs maintained with sclerotia were significantly higher than those maintained without sclerotia (Table 3; $F = 6.857$, $P < 0.05$, ANOVA). This tendency held for all four colonies, although mean survival rates of eggs were different from colony to colony ($F = 21.748$, $P < 0.0001$, ANOVA).

DISCUSSION

There is a large number of studies regarding the interactions between fungi and soil-dwelling termites (Amburgey 1979); especially in higher

Table 3 The influence of sclerotia on the survivorship of termite eggs (mean ± SE, data from an average of seven replications per treatment)

Colony	Without sclerotia	With sclerotia
A	0.880 ± 0.096	0.914 ± 0.032
B	0.573 ± 0.141	0.732 ± 0.120
C	0.777 ± 0.069	0.839 ± 0.053
D	0.344 ± 0.067	0.553 ± 0.027

Source of variation	d.f.	*F*	*P*
Colony	3	21.748	0.000
Sclerotia	1	6.857	0.012
Colony × sclerotia	3	0.409	0.747
Error	48		

termites – the mutualistic association between *Termitomyces* and Macrotermitinae is well known (Wood & Thomas 1989). The *Reticulitermes–Fibularhizoctonia* relationship, where the fungus mimicks the termite eggs, had not been described until the current study. Zoberi & Grace (1990) isolated 40 species of fungi in Canada in a survey of the mycoflora associated with *Reticulitermes flavipes*, which is closely related to *Reticulitermes speratus*, but they did not find any sclerotium-forming fungi. In the *Termitomyces*–Macrotermitinae relationship, the termite cannot survive without the fungus combs because of a strong mutual dependency (Wood & Thomas 1989). The wide-area sampling showed that the *Reticulitermes–Fibularhizoctonia* relationship was quite prevalent in western Japan. All of the 40 colonies that were collected from pine wood had sclerotia present in the piles of eggs. One interpretation of this high rate is that alates carry spores of the fungus during swarming. If the spores disperse by taking advantage of the flight of alates, the fungus benefits greatly from termites. An investigation into the transportation of spores by alates could test this possibility. Another interpretation is that fungi that form termite balls are ubiquitous and sclerotia are opportunistically brought to termite nests by workers. We consider the latter possibility unlikely because we have never found the sclerotia in pine wood that has not been colonized by termites.

We found that termites recognize their eggs by a combination of chemical cues and the shape and size of the eggs. The dummy eggs having non-spherical shapes or different sizes compared to termite eggs were not recognized as eggs, even if they were coated with the chemical. By contrast, the workers carried a few dummy eggs without the chemical if they were spherical in shape and similar in size to the eggs. Therefore, we believe that this termite–fungus interaction began when termites mistook the sclerotia of the ancestral fungus (which had a similar size to their eggs) for their eggs, and brought them to the piles of eggs. This caused stabilizing selection by termites on the size of the sclerotia, and may explain why the amount of variation in the diameters of the termite balls was smaller than in the sclerotia of the other species. We tested the pile-up rates of sclerotia that had never come into contact with eggs, to ascertain whether the fungus itself produced an egg-recognition chemical or absorbed it from the termite colonies. Forty-two percent of the sclerotia obtained from the nest and out of the piles of eggs were carried, while those obtained from the PDA culture were rarely carried. This suggests that the sclerotia do not produce an egg-recognition chemical by themselves but gain from the termites. The identification of any egg-recognition chemical would be required in order to ascertain this.

Experiment 2 showed that the sclerotia in the piles of termite eggs could not germinate in the presence of workers, probably because the saliva of the workers inhibited sclerotial germination. In the 'foodstore' of *Macrotermes bellicosus*, which consists of comminuted food mixed with saliva, spores of a wide variety of fungi are present, but very few germinate when incubated (Thomas 1987c). Additionally, dampwood termites line nest chambers and galleries with fecal pellets, which has fungistatic effect (Rosengaus *et al*. 1998), protecting nests from naturally occurring fungi. However, the termites did not completely inhibit sclerotial germination and fungal growth in the long term. Termites removed a few of the sclerotia, which shrank and became deformed, from the piles of eggs. Such sclerotia germinated and grew on termite excretions mainly on the underside of the filter paper where termites could not reach. Transportation of sclerotia by termites is probably important for the *Fibularhizoctonia* sp. nov. because the sclerotia can not germinate until they are

transported from their native place (K. Matsuura, unpubl. data). In *Reticulitermes* termites, colonies migrate to neighboring woods when the quality of nest-wood for food and shelter is low. Perhaps *Fibularhizoctonia* sp. nov. can be transported by termites and can gain a competitor-free habitat more quickly than other fungi by mimicking termite eggs. Earlier access to a competi-tor-free habitat may be of great benefit to *Fibularhizoctonia* sp. nov. because this fungus grows much more slowly than other naturally occurring fungi such as *Aspergillus* and *Penicillium* (K. Matsuura unpublished data). Further research on the life history of this fungus would clarify whether a net benefit is achieved from this interaction.

Without the inhibition of the workers, the sclerotia in the piles of eggs germinated and absorbed the eggs around them. Interestingly, the results from experiment 3 suggested that sclerotia enhance egg survival if their germination is inhibited by workers. One interpretation of this result is that bacteriostatic and/or fungistatic substances in the sclerotia might protect termite eggs from pathogens. The evidence is that this fungus forms an antagonistic zone against some other fungi. Recognizing the potential importance of the fungal sclerotium as a survival structure in the life cycle, Wicklow and Cole (1982) predicted that there should be greater fungal investment in sclerotial chemical defenses. Indeed, the sclerotia of *Aspergillus flavus* contain additional toxic metabolites not produced by fungal hyphae. Thus, termites can protect their eggs from pathogens with their own saliva, and the fungus can also produce the antifungal agent. However, an interaction may still be beneficial for both sides because each antifungal and antibacterial compound can not have a entirely protective effect against all fungi and bacteria. It is possible that the antifungal and antibacterial compounds found in sclerotia and in termites complement each other. It is also possible that *R. speratus* gains the advantage of eating wood that has been previously decayed by *Fibularhizoctonia* sp. nov. because *Fibularhizoctonia* (an anamorph of *Athelia*) is known as a decay fungus (Adams 1996). Decay fungi may render poor foods palatable or good foods unpalatable, depending on the fungus, the termite and the host food (Waller & LaFage 1985).

It may be possible to put a time scale on the evolution of the *Reticulitermes–Fibularhizoctoni* symbiosis by comparing a phylogeny of the termites with a phylogeny of the fungus. Further information about the distribution of the *Fibularhizoctonia* sp. nov. that produce termite balls on a global scale is required in order to elucidate the evolutionary background of the *Reticulitermes–Fibularhizoctonia* relationship.

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