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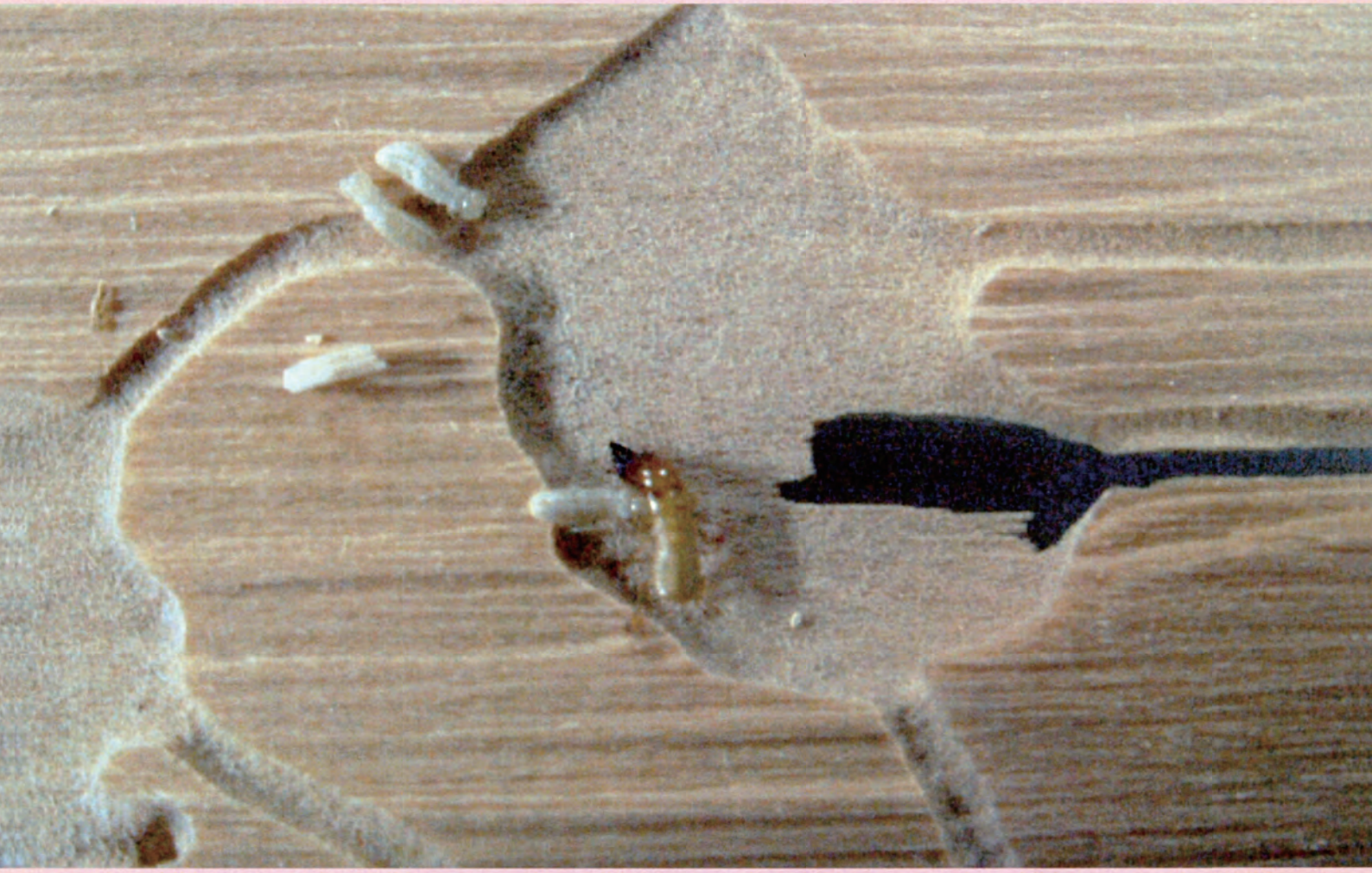

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Contents

Control of Formosan Subterranean Termite, *Coptotermes formosanus* Shiraki, Infesting Historic Buildings

by

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Abstract

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is the most important structural pest in the world. In Hawaii, the total damage caused by this termite was estimated at US$15 to 20 million dollars a year. *C. formosanus* feeds on anything containing cellulose, but due to lack of cellulase, cannot digest cellulose without the assistance of symbiotic protozoa harbored in the hindgut. In Taiwan, despite most of the buildings were built with concrete or bricks, 64% of the structures, particularly their interior decoration, were made of wooden materials. Historic buildings, however, were primarily made of wood. *C. formosanus* have caused considerable damage to many of the historic buildings. Recently, Taiwan Government, particularly Council for Cultural Affairs, has made a great effort in preserving, protecting and restoring historic monuments throughout the island. Much effort and resources have been allocated for these purposes for such widely known historic monuments as the Presidential Building, Shanshia Chu-Shih Temple, and Lukang Chao-Tien Temple. Historic monuments are categorized into three classes (I, II, and III) set by the national, provincial or local standards on the basis of their age and historical significance. In the process of protecting and restoring the historic monuments, damage was often found to be associated with natural aging, fungal rots, or insect pests or combinations of the three. Of the insect pests, powder-post beetles, drywood termite (*Cryptotermes brevipes*) and *C. formosanus* were found to cause much of the damage to the monuments, particularly those caused by *C. formosanus,* resulting in considerable economic losses in chemical treatment and repairing. Attempts have been made to control these pests by employing conventional drilling and chemical injection method, pest baiting and elimination with insect growth regulators, fumigation, and biological control method using fungi and nematodes. Although the first three methods were commonly used in controlling and eradicating *C. formosanus* colonies, a commercial product combining baits and insect growth regulators was the method of choice for protection of historic monuments. The challenge to a termite researcher is to find an effective, ecologically sound method for termite control or eradication.

Effect of 40-Months' Storage of Treated Sandy Loam on the Transfer of Fipronil from Exposed Workers to Unexposed Workers of *Coptotermes formosanus* (Isoptera: Rhinotermitidae)

by

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Abstract

Undifferentiated termite larvae (workers) of *Coptotermes formosanus* Shiraki were first exposed to 40-month-old fipronil-treated sandy loam [0.5, 1.0 and 5.0 ppm (m/m) fipronil in the sandy loam] for one hour to obtain fipronil-exposed workers. The exposed workers were then mixed with unexposed workers at mixing ratios of 1:1 and 1:10 (exposed vs. unexposed) to examine the transfer of fipronil from exposed to unexposed workers for up to 4 weeks at 28 ± 2°C and >80% relative humidity in the dark. At a mixing ratio of 1:1, mortality of exposed workers increased by approximately 307% after 40 months of storage. On the other hand, the fewer unexposed workers died at 1:1 ratio after storage of treated sandy loam. Mortality rates of exposed and unexposed workers at a mixing ratio of 1:10 were very similar to those at 1:1. Since mortality rates generally went down after 18-months' storage and this was partly supported by the decrease of fipronil recovered from the treated sandy loam in the previous research, the present results seem to make it difficult to understand the effect of storage. It is assumed that relatively low reproductivity of such termite bioassay might account for the inconsistency of the results. The comparison of these results, therefore, should be carefully done.

Key words: fipronil, nonrepellent termiticide, transfer of toxicant, *Coptotermes formosanus,* reproductivity of termite bioassay

Introduction

Termite management market is always searching for a novel approach to meet environmentally soundness. The application of nonrepellent soil termiticides such as fipronil looks promising (Tsunoda 2005) and some research efforts have been made (*e. g.* Ibrahim *et al.* 2003, Shelton & Grace 2003, Remmen & Su. 2005a, 2005b, Tsunoda 2006a)

Assuming that the treated soil is stored for a while until purchased or applied after the soil is treated with a selected termiticide, the current experiment was planned to stimulate such circumstances: fipronil-treated sandy loam was stored in dry and dark conditions (without any direct sunlight effect) for 40 months prior to bioassay with

Formosan subterranean termites *Coptotermes formosanus* Shiraki. The effect of mixing ratios of fipronil-exposed versus unexposed workers was determined when workers were first allowed to contact sandy loam treated with fipronil at 0-5.0 ppm fipronil/sandy loam (m/m) for one hour and then mixed with unexposed workers.

Materials and methods

Since the detailed materials and methods were previously reported elsewhere (Tsunoda 2006a, 2006b), brief descriptions are given here. The experiment was composed of the exposure of termites to the treated soil (direct exposure) and the subsequent contact of unexposed termites with exposed termites (indirect exposure).

Initial exposure

A constant amount of 10 g fipronil solution was added to 50 g dry sandy loam and mixed well so that the desired concentrations of fipronil (Termidor SC, Bayer CropScience, Tokyo, Japan) [0-5.0 ppm (m/m) (fipronil in the treated sandy loam)] were prepared with water. Twenty sound workers of *C. formosanus* were expose d to the treated soil in a test container (6 cm plastic Petri dish) at 28 ± 2℃ and >80% relative humidity in the dark for one Five replicates were prepared for each test concentration so that the required number of exposed workers (donors) was easily obtained for the subsequent exposure tests.

Indirect exposure test

Indirect exposure tests were conducted by the use of plastic Petri dishes of 6 and 9 cm for mixing ratios of 1:1 [exposed workers (donors) vs. unexposed workers (recipients)] and 1:10, respectively.

Ten exposed workers were used in each test container, regardless of mixing ratios. They were mixed with unexposed termite workers previously marked with Nile Blue A (Aldrich, Milwaukee, WI) by a fast marking technique (Evans 2000). Three replicates of each treatment were tested. Assembled dishes with lids on were placed in a container with moistened cotton pads at the bottom, and maintained at 28±2℃ and >80% relative humidity for 4 wk. During the incubation period, numbers of dead termites were regularly counted.

Results and discussion

Indirect exposure test at a mixing ratio of 1:1 (exposed vs. unexposed)

There was no conspicuous increase in mortality of workers marked with Nile Blue A between the freshly prepared sandy loam and the 18 month-stored sandy loam and termite mortality rates raged from 0 to 10%. However, the mortality rates of the 40 month-stored sandy loam were largely overcame the previous values and ranged from 19 to 83% as shown in Fig. 1. Since the experiments were carried out by the same personnel using termites from the same laboratory colony, no reasonable explanations were given to theses discrepancies.

Mortality rates of exposed workers that were exposed to fipronil-treated sandy loam after storage for 40 months were as high as those with freshly treated sandy loam, whereas the unexposed termites after 40-months'storage died fewer than those without storage. Consequently, the mortality rates after the storage for 40 months were thoroughly different from those after 18 months' storage (Fig.2)

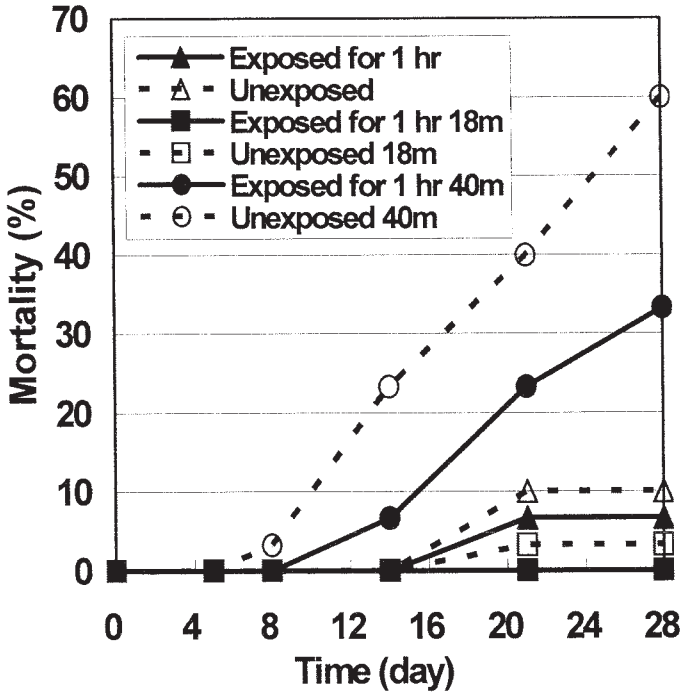

Fig. 1 Mortality of exposed and unexposed termite workers after incubation on untreated sandy (mixing ratio of exposed vs. unexposed=1:1)

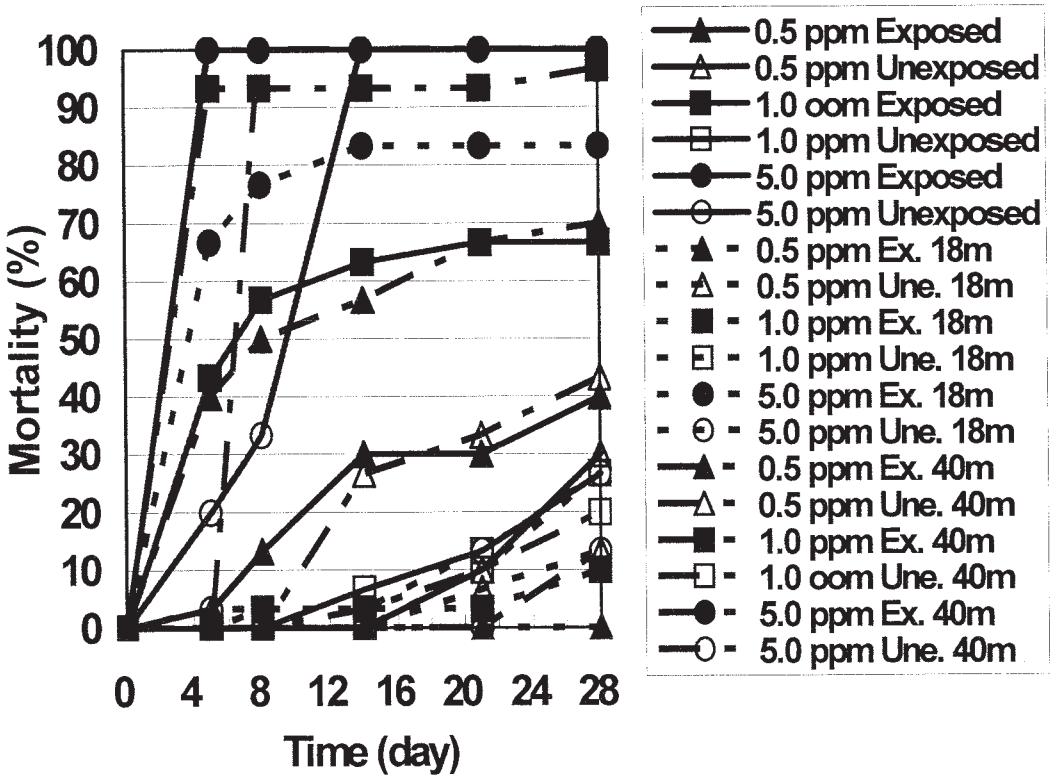

Fig. 2 Effect of storage length after treatment of sandy loam on the mortality of termite workers through transfer of fipronil from exposed to unexposed workers (mixing ratio of exposed vs. unexposed=1:1)

Although comparison of mortality rates indicated the inconsistency of the results at different times, the mortality of unexposed workers showed a trend similar to that of exposed workers. A larger decrease in mortality (87%) was recorded at 5.0 ppm, whereas 13 % increase and 7% reduction were seen at 1.0 and 0.5 ppm, respectively. It should be noted that there was no prominent difference in mortality among the test concentrations and that mortality figures recorded with unexposed workers for treated sandy loam were higher than those for untreated sandy loam with an exception of the 40 month-stored 5.0 ppm sandy loam (Figs. 1 & 2). Therefore, it is generally concluded that fipronil was transferred from exposed to unexposed termite workers at a mixing ratio of 1:1 (exposed vs. unexposed) even after 40-months' storage. However, the exact cause of the decreased mortality of both exposed and unexposed workers after storage for 18-40 months remains undetermined. Because the chemical analysis did not clearly answer the question how mortality rates of worker termites vary from time to time.

Indirect exposure test at a mixing ratio of 1:10 (exposed vs. unexposed)

The termite mortality after 40-months' storage was higher than that with a freshly prepared soil when termites were incubated on untreated sandy loam. Although there was no significant difference in mortality rates of unexposed termites among sandy loam samples of different storage durations (Tsunoda 2006b) as shown in Fig. 3.

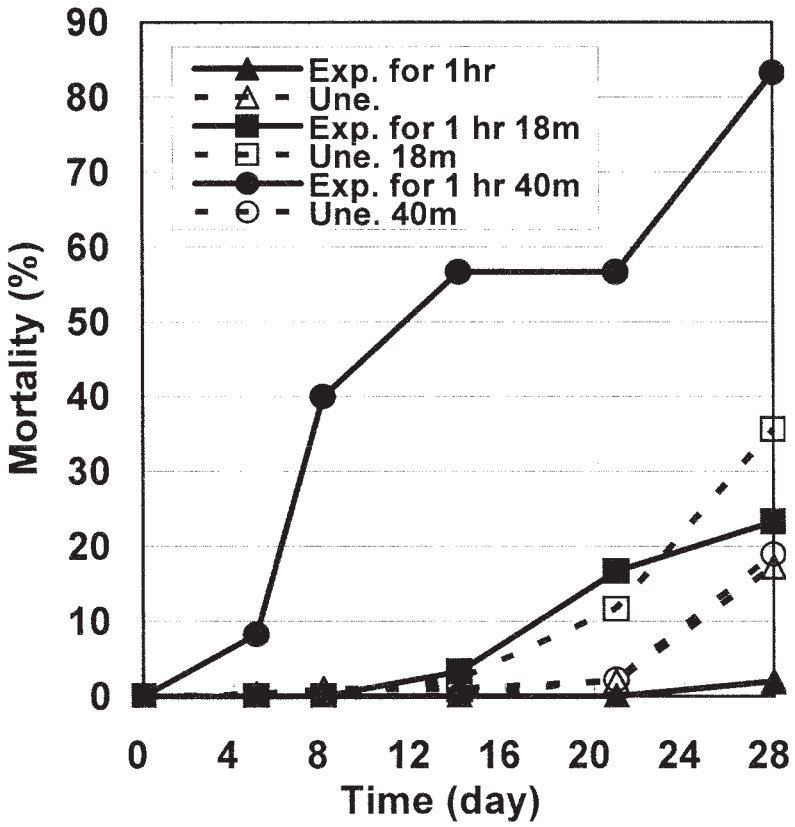

Fig. 3 Mortality of exposed and unexposed termite workers after incubation on untreated sandy loam (mixing ratio of exposed vs. unexposed=1:10)

Figure 4 shows the effect of storage after treatment of sandy loam on the mortality of termite workers by the transfer of fipronil from exposed to unexposed workers (mixing ratio of exposed vs. unexposed=1:10).

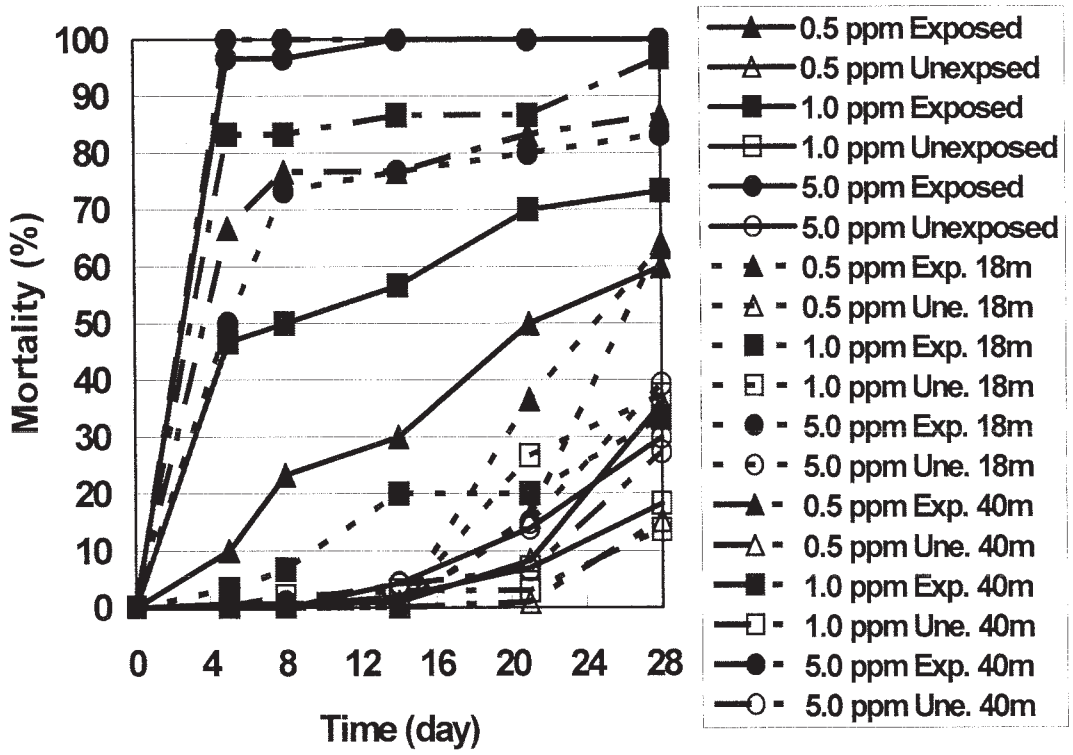

Fig. 4 Effect of storage length after treatment of sandy loam on the mortality of termite workers through transfer of fipronil from exposed to unexposed workers (mixing ratio of exposed vs. unexposed=1:10)

As previously reported, there was little difference in the mortality of exposed workers regardless of the mixing ratio, when testing was performed with freshly treated sandy loam (Tsunoda 2006a). The similarity was noticeable for the cases after 40-months' storage (Figs. 2 & 4), although termite mortality rates recorded with exposed workers on sandy loam stored for 18 months were higher at a mixing ratio of 1:10 (exposed vs. unexposed) than at 1:1 at 0.5 and 1.0 ppm, and appeared to coincide with those of freshly treated sandy loam samples. Different effects of storage length still remained unsolved.

Although a simple comparison of the mortality of unexposed workers on sandy loam stored for 18-40 months did not suggest any prominent effect of the storage length, the longer storage seemed to cause no negative termiticidal efficacy (Fig. 4) at a mixing ratio of 1:10.

Based on the present results, the potential of fipronil consecutive transfer from one to other nestmates of *C. formosanus* to other nestmates would be similar to the transmission of fipronil among workers and soldiers of the same termite species when topically treated with fipronil (Ibrahim *et al.* 2003). These results also suggest that a

relatively high proportion of nestmates should come in contact with fipronil-treated soil to circulate the toxicant among members for inducing the elimination of the whole colony (Shelton & Grace 2003; Remmen & Su 2005a, 2005b)

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Evaluation of Bistrifluron as an Above-Ground Bait against the Asian Subterranean Termite, *Coptotermes gestroi* (Wasmann) in Malaysia

by
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Abstract

A new benzoylphenylurea compound, bistrifluron, was evaluated as a toxicant at concentration of 0.5 and 1.0% in above-ground termite baits against Asian subterranean termite, *Coptotermes gestroi* (Wasmann) infestation in buildings and structures in Malaysia. A total of 9 premises with active infestation were chosen for the study. Four premises were each treated with 0.5% and 1.0% bistrifluron baits, respectively while the remaining one served as control. Results indicated that termites in all 4 sites treated with 1.0% bistrifluron bait were eliminated within 4 – 5 weeks post-baiting, while premises treated with 0.5% bistrifluron bait showed no sign of active termites after 6 – 8 weeks post-baiting. No feeding deterrency was observed on both concentrations as termites were readily feeding on the baits. A total amount of 1.06 – 8.34 g of bistrifluron was required to eliminate termite colonies in the study. The implication of the findings on novel termite management strategies against the Asian subterranean termite in Malaysia is discussed.

Key words: *Coptotermes gestroi*, baiting, bistrifluron, above-ground station, Malaysia.

Introduction

The Asian subterranean termite, *Coptotermes gestroi* is the most destructive termite pest species in Malaysia and South East Asia (Lee 2002a, Kirton and Azmi 2005, Yeap *et al.* 2007). Control of this species accounted for a substantial proportion of the total business turnover of the pest control industry in Malaysia. Most termite control strategies in Malaysia at post-construction level depended heavily on the use of soil termiticides. However, over the last 6 years, termite baits are gaining popularity in many parts of South East Asia (Ngee *et al.* 2004), namely Sentricon™Colony Elimination System (containing hexaflumuron) and Exterra™ Termite Interception and Baiting System (containing chlorfluazuron).

Bistrifluron, a benzoylphenylurea compound, is a novel chemistry that was discovered by Dongbu Hannong Chemical Co., Ltd, Seoul, Korea. Earlier, Kubota *et al.* (2006) reported its excellent activity against *Coptotermes formosanus* and their laboratory results indicated that bistrifluron is an effective bait toxicant at 5000 ppm. This study was intiated to evaluate the performance of bistrifluron termite bait against *Coptotermes gestroi* in infested premises in Malaysia.

Materials and methods

Baits: Two concentrations of bistrifluron termite bait in the form of above-ground station bait were evaluated, namely 0.5% and 1.0%. They were provided by Sumitomo Chemical Co., Ltd., Tokyo, Japan. Blank bait was also evaluated as control.

Sites: Buildings or structures with active infestation of the Asian subterranean termites, *Coptotermes gestroi* were used in this study. All sites were located in Penang Island, Malaysia with exception to one which was located in Taiping. With exception to Feed house and Stadium, all the evaluated sites are residential premises. Stadium USM is an indoor badminton stadium located in the Universiti

Sains Malaysia Minden campus, while Feed house is a building in the USM Minden campus which is used to store fish feed products. All sites had very serious termite infestations and had gone through many soil treatments by pest control professionals over the years.

Above-Ground Stations: A modified method of above-ground monitoring stations (Su *et al.* 1997) were used. Placing of a plastic container (as monitoring station) with toilet rolls as baits over active termite mud tubes were carried out. This consisted of one to two rolls of paper that were pre-weighed before placement in the stations. The bases of the containers were hollow so as to allow the entry of termites, and taped with black-coloured tape to reduce the penetration of lights into the inner portion of the containers.

The stations were inspected weekly and fresh toilet rolls were replaced. Any remains of previous toilet rolls were retrieved, cleaned and oven-dried at 80°C for 24 h then, weighed. The weight difference of the toilet rolls were used to assess the feeding activity of the colonies studied.

Baiting Procedures: After the termite activity in the infested premises were characterized, bistrifluron-based baits were placed into 30 – 50% of monitoring stations set up earlier. Monitoring stations without bistrifluron baits were continuously provided with fresh toilet rolls on weekly basis.

All monitoring stations were monitored weekly. Baits with less than 20% remaining matrix were replaced. The remaining matrix was brought back to the laboratory, dried and weighed to determine the amount of bait matrix consumption.

Baiting activities were terminated when termites were no longer detected in all monitoring stations. However, monitoring stations with baits were still provided with toilet rolls for continuous monitoring process.

Results and discussion

Results indicated that termites in all 4 sites treated with 1.0% bistrifluron-based bait were eliminated or suppressed within 4 - 5 weeks post-baiting (Table 1). This is considered a very outstanding performance since the existing bait product in the market containing 0.5% hexaflumuron required 6 – 9 weeks post-baiting to achieve colony elimination (Lee 2002b, Sajap *et al.* 2000). As for the 0.5% bistrifluron-based bait, it showed comparable results to those reported for 0.5% hexaflumuron-based bait, of which colony elimination was achieved in 6 – 8 weeks (Table 1).

The termite colony in Site 1 (Pulau Tikus) consumed a total amount of 202.5 g bait matrix which equates to an amount of 2.025 g of bistrifluron, while a total amount of 833.9 g bait matrix was consumed at the Stadium USM site (equates to an amount of 8.339 g bistrifluron). Two other sites (Bayan Lepas and Air Itam) which were baited with 1.0% bait also showed relatively similar results with 195.1 and 172.9 g of bait matrix consumed, respectively.

As for 0.5% bistrifluron-based bait, it performed slower than the 1.0% bait. It took 7 weeks before the termite activity in the Taiping site ceased. A total amount of 534.8 g bait matrix was consumed which equates to 2.674 g. As for the Teluk Kumbar site, it also took 7 weeks before the termite activities diminished. A total amount of 463.5 g bait matrix was consumed (equivalent to 2.3175 g of active ingredient). A relatively similar amount of time for colony elimination was recorded for both Tanjung Bungah (8 weeks) and Green Lane sites (6 weeks) which were baited with 0.5% bistrifluron-based baits.

Control bait did not show an impact on the colony of *C. gestroi* in the Feed house site in USM Minden campus. The slight fluctuation observed in the feeding rate was normal, and was attributed to the

regular removal and replenishing of control bait matrix (a total amount of 911.2636 g bait matrix was consumed over 7 weeks since baiting started).

I did not observe any feeding deterrence shown by the termites towards the baits used in this study as termites were observed to readily feed on them. This implied that bistrifluron at 0.5% and 1.0% do not have any repellent effects on the termites.

The Asian subterranean termite, *C. gestroi* is a notorious termite species which causes serious infestations in buildings and structures in the Southeast Asia region (Yeap *et al.* 2007). It is the most important subterranean termite species in Thailand, Malaysia and Singapore and causing multi-million dollars of building damages to property owners each year. The above-ground bait station is a no-hassle and ready-to-use baiting technology which can be deployed by trained pest control professionals. The current bait formulation is also highly palatable to the termites.

In summary, the 1.0% bistrifluron-based above ground termite bait demonstrated superb performance against the Asian subterranean termite, *C. gestroi* in the field, while the 0.5% bait showed comparable performance as the existing termite bait (0.5% hexaflumuron). The 1.0% bistrifluron-based above-ground termite bait certainly has great potential to be used against *Coptotermes* species in the Southeast Asia region.

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Table 1: Performance of above-ground bistrifluron termite baits against *Coptotermes gestroi* in Northern Peninsular Malaysia.

Site[1]	Concentration (%)	Amount consumed (g)		Period required for colony elimination (weeks)	No. bait placement
		bait matrix	active ingredient		
Feed house (USM)	0	911.3	-	-	2
Taiping (Perak)	0.5	534.8	2.674	7	2
Teluk Kumbar (Penang)	0.5	463.5	2.318	7	1
Tanjung Bunga (Penang)	0.5	645.2	3.226	8	2
Greenlane (Penang)	0.5	211.4	1.057	6	1
Bayan Lepas (Penang)	1.0	195.1	1.951	5	1
Pulau Tikus (Penang)	1.0	202.5	2.025	4	1
Stadium (USM)	1.0	833.9	8.339	5	2
Air Itam (Penang)	1.0	172.9	1.729	4	1

[1]With exception to Feed house and Stadium which were located in Universiti Sains Malaysia campus, all other sites were residential premise

Termiticidal Efficacy of Bistrifluron to the Japanese Subterranean Termites

by

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Abstract

Termiticidal efficacy of a benzoylphenylurea compound bistrifluron against economically important *Coptotermes formosanus* and *Reticulitermes speratus* was evaluated in the laboratory. Gradual increase of mortality of the two termite species was observed over the 6-weeks duration in the no-choice feeding test in which termite workers were exposed to 5,000 ppm bistrifluron bait. The results appeared to indicate that bistrifluron is slow-acting on both *C. formosanus* and *R. speratus* and that it is possible to apply bistrifluron to the subterranean termite management in Japan.

Key words: bistrifluron, bait toxicant, *Coptotermes formosanus*, *Reticulitermes speratus*

Introduction

It has been demonstrated that BPUs is a slow-acting bait toxicant effective in the elimination of colonies of the subterranean termites (Su 1994, Su *et al.* 1995). Bistrifluron is one of BPUs (Kim *et al.* 2000) and is suggested to be a promising bait toxicant against *Coptotermes formosanus* Shiraki (Aki 2005, Kubota *et al.* 2006). In Japan, there are two economically important species of subterranean termites: *C. formosanus* and *Reticulitermes speratus* (Kolbe). The current research was planned to carefully evaluate the slow-acting characteristics of bistrifluron against both termite species for discussing the potential of application of this compound to the termite management in Japan.

Materials and Methods

1) Termites

Coptotermes formosanus

Undifferentiated larvae (workers) of *C. formosanus* were obtained from the colony that was originally collected in Kagoshima Prefecture, Japan, in 2002 and has been maintained at the Agricultural Chemicals Research Laboratory of Sumitomo Chemical Co., Ltd (SCC-ACRL), Hyogo prefecture, Japan).

Reticulitermes speratus

A field population of *R. speratus* infesting a dead branch was collected in May 2006 in Osaka Prefecture, Japan. They had been maintained at SCC-ACRL, supplying them with the sufficient amount of water regularly until they were used for the bioassay (within one month). The workers older than third instar were distinguished from their body sizes and yellowish body color (Takematsu 1992) and used for the present study.

2) No-Choice Feeding Test

Coptotermes formosanus

A disk of filter paper (No.1026, ϕ 33 mm, *ca.* 0.2 g) (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) was treated with 1 ml acetone solution of a given concentration of bistrifluron and air-dried to prepare 5,000 ppm (wt/wt) bistirfluron bait. Untreated control disks were also prepared.

Each disk was placed in a small plastic cup (*ca.* 14 ml) with small entry holes for termites. This container was then put in a larger plastic container (*ca.* 200 ml) together with 100 worker termites. The bottom of the larger container had several small holes and was covered with 2-3 mm of plaster. These assembled units were placed on a damp cotton pad in an incubation chamber so that termites could uptake water through the plaster. Fifteen units were prepared for both bistrifluron treatment and control. The units were kept at *ca.* 25℃ for 9 weeks. Five units were disassembled at 3rd, 6th and 9th weeks to determine the change in mortality over time.

Reticulitermes speratus

A paper towel (Tohyo Co., Ltd., Ehime, Japan) was cut into disks with a diameter of 90 mm (*ca.* 0.5 g). Each disk was treated with 1 ml acetone solution of a given concentration of bistrifluron and air-dried to prepare 5,000 ppm (wt/wt) bistirfluron bait. Untreated control disks were also prepared. Four disks were laid one on the top of another and then put into a plastic Petri dish (90 mm in diameter). Six milliliter of distilled water was added to the layer of disks: The amount of water was three times as much (wt/wt) as total weight of disks (*ca.* 2 g) that were deemed to be both their foods and living spaces (Watanabe & Noda 1991).

Twenty worker termites were released into the Petri dish and the dish was covered with a lid and sealed with parafilm. Twelve units were prepared for both bistirfluron treatment and control. The units were kept at *ca.* 25℃ for 6 weeks. Four units of each treatment were disassembled to determine the change in mortality at 2nd, 4th and 6th weeks.

3) Statistical analysis

Termite mortality at given observing times were compared between bistrifluron treatment and control by *t* test ($P < 0.05$). Percentage mortality was transformed into the arc sine of the square root for statistical analysis (Yamamura 2002).

Results and Discussions

Mortality of *C. formosanus* exposed to 5,000 ppm bistrifluron bait significantly increased between the 3rd and the 6th week in comparison to control (Table 1). Mortality of *R. speratus* exposed to 5,000 ppm bistrifluron was significantly higher than control even at the 2nd week and increasing gradually for the following period (Table 2). Trace of feeding was shown on the baits in both tests, indicating bistrifluron was taken more or less by feeding. Thus, bistrifluron seems to have a slow-acting termiticidal effect on both Japanese subterranean termite species. Subsequent biological tests should be conducted to evaluate its performance in the field.

Table 1. Mortality of *C. formosanus* exposed to filter paper bait in the no-choice feeding test

AI	Conc. (% wt/wt)	Mortality (%±SE) over time (week)		
		3	6	9
Bistrifluron	5,000	28.0±18.09	82.8±10.70*	100.0±0.00*
Control	-	6.4±1.50	7.8±1.59	15.0±1.26

* Significantly different by *t* test ($P < 0.05$)

Table 2. Mortality of *R. speratus* exposed to paper towel bait in the no-choice feeding test

AI	Conc. (% wt/wt)	Mortality (%±SE) over time (week)		
		2	4	6
Bistrifluron	5,000	27.5±7.77*	52.5±4.79*	86.3±8.00
Control	-	7.5±4.33	20.0±3.54	25.0±3.54

* Significantly different by *t* test ($P < 0.05$)

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Defensive Secretion of a Longicorn Beetle, *Chloridolum loochooanum* (Coleoptera; Cerambycidae) and its Repellent Effect on Termites

by

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Abstract

Adults of the longicorn beetle, *Chloridolum loochooanum*, emit a white frothy secretion from their metasternal glands. The chemical structure of these defensive substances of *C. loochooanum* were elucidated using GC-MS and NMR analyses by comparing these substances with synthesized chemicals. The results suggested that they were cyclopentanoid monoterpenes, an iridodial, and they were found to have repellent effect on the termite, *Coptotermes formosanus*.

Keywords: chemical defense, iridodials, longicorn beetle, termite, repellency

Introduction

Insects have established special strategies to protect themselves from predators by chemicals, surface hardness and/or mimicry. Defensive chemicals especially have been well investigated because of their diversity and species-specificity. Most of the defensive chemicals of Coleoptera beetles have odoriferous but stinky smell such as formic acid, benzoquinones, cresols and others (Kanehisa and Kawazu, 1985; Eisner et al., 1977; Markarian et al., 1978; Balestrazzi et al., 1985). Exceptionally, Cerambycid beetles in the tribe Callichromini are famous for emitting an odor recognizable to human as fragrant. They secrete fragrant substances from their metasternal glands when attacked by their predators. Vidari et al. (1973) examined the secretion of the European musk beetle, *Aromia moschata* (Linnaeus), and reported that the secreted chemicals were rose oxide and iridodials.

The longicorn beetle, *Chloridolum loochooanum* Gressitt is distributed only on Amami and Okinawa Islands, Japan. During flight, *C. loochooanum* emits a scent (Makihara and Ohmura, 2005), which is famous for its delicacy. We were interested in the defensive chemical of *C. loochooanum*, and analyzed its structure using GC-MS and NMR.

An additional interest is related to the attention being given to natural compounds that can repel termites as a result of the health and environmental impact by synthetic pesticides. In our study, we also examined the repellent ability of the chemicals from this beetle against a termite, *Coptotermes formosanus*, in order to understand their potential as termite repellents.

Materials and methods

Chloridolum loochooanum

Freshly hatched adults of *Chloridolum loochooanum* Gressitt (Fig.1) were obtained from the fallen *Viburnum awabuki* from Okinawa Island on May, 2002. They were maintained at room temperature until the defensive secretion was collected.

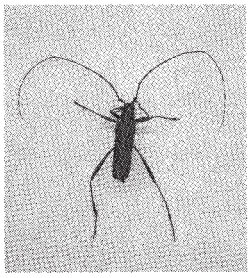

Fig.1 *Chloridolum loochooanum*

Termite

The test termite, *Coptotermes formosanus* Shiraki, was collected from a laboratory colony maintained at the Forestry and Forest Products Research Institute. The colony was collected in Kurashiki, Okayama, and has been reared on wood pieces of *Pinus densiflora* in the dark at 26 ± 2 °C, 65% R.H., for 4 years. Only pseudergates (workers) above the third instar were used in the termite repellent test discussed below.

Collecting the defensive secretion

When disturbed, *C. loochooanum* promptly emits white liquid secretions from their metasternal glands. The emitted secretion was absorbed in glasswool and was then extracted with $CHCl_3$ or was directly absorbed by SPME (100 μm PDMS, SPELCO Co.Ltd.)

Synthesis of iridodials

Iridodials were synthesized according to Clark et al (1959). A mixture of citronellal (25 ml), ethyleneglycol (15 ml), *p*-toluene sulphorate (30 mg) and benzene (240 ml) was refluxed in a Dean-Stark water separatory for 24 hr to give the cyclic acetal. Selenium oxide, dissolved in 50 mL of EtOH was added to the acetal in 30 mL of EtOH under reflux at 100°C for 20 hrs. The obtained aldehydic cyclic acetal was hydrolyzed by refluxing with 50% acetic acid in N_2 for 4hr. The reaction mixture was fractionated using silica-gel column chromatography (Acetone/CH_2Cl_2=50/1) to obtain each iridodial isomer.

Chemical analysis

Capillary gas chromatography mass spectrometry (GC-MS) analysis was carried out with a Hewlett Packard HP-6890 GC system connected with a HP-5973 MS spectrometer under the following conditions: column, HP-5 (30m×0.25mmφ); injection port temperature, 240℃; detector temperature, 260℃; carrier gas, helium. The column temperature was kept at 40℃(1 min, hold), 40 ℃ to 250℃(5℃/min).

The NMR spectra were measured using JEOL LAMBDA-400 and ALPHA-500 spectrometers. $CDCl_3$ was used as the solvent.

Repellency test against termites

Paper discs (diameter, 13 mm, Whatman International Ltd.) were permeated with 60 μl of the

$CHCl_3$ solutions containing iridodials. The treatment retentions were 1.0% (w/w) per disc. The two discs were put onto the bottom of a Petri dish (diameter, 50 mm; height, 10 mm) positioned 10 mm diagonally from the center of the dish. The dish was supplied with 100 pseudergates of *C. formosanus* and was covered with a lid during the test. The efficacy of the chemicals was confirmed.

Results and discussion

Identification of defensive chemicals

By GC-MS analyses, the 4 peaks of each molecular ion of *m/z*=168 were found to be isomeric at the time of retention (tR) from 13.5 min. to 15.0 min. The diagnostic fragment ions of each peak were *m/z*: 67, 81, 93, 109 or 111, 135, 150.

The ^{1}H-NMR spectrum showed signals for 16 protons, including 2 aldehydes (δ = 9.59, 9.63; d; *J*=3.7 Hz, 2.4 Hz) and 2 methyl groups (δ =1.079, 1.085; d; *J*=6.7 Hz, 7.3 Hz). These chemical analyses indicated that the major components were cyclopentanoid monoterpenes, an iridodial (Fig. 2).

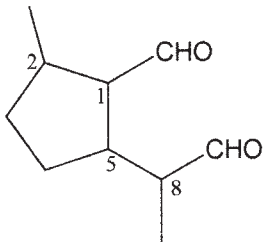

Fig. 2 Iridodial

The coupling constants of $J_{1,2}$ and $J_{1,5}$ were both 8.5 Hz, suggesting a *trans, trans* conformation, although the difficulties were known in determining the relative stereochemistry of a cyclopentanoid skeleton (Constantino and Silva, 1998).

In order to determine the relative conformation of the defensive chemicals of *C. loochooanum*, iridodials were synthesized according to Clark et al. (1959). After fractionated by silica-gel column chromatography, a compound in which the chemical shifts coincide with those of the defensive secretion was subjected to oxidation using PDC, followed by methylation. By comparing the ^{1}H-NMR spectral data of the obtained methyl ether with those of Sakai et al. (1981), we determined the relative conformation of *C. loochooanum* iridodial as *trans,trans*-iridodial. According to Oldham (1994), the configuration of C2 in natural iridoids is exclusively (*S*)-, indicating that the major component in the defensive secretion was 1*R*, 2*S*, 5*S*-iridodial.

Iridodials have been known as one of the defensive chemicals in many insects, such as the longicorn beetle, *Aromia moschata* (Vidari et al., 1973), ants (Cavill et al., 1976; Attygalle and Morgan, 1984; Nascimento et al., 1998), rove beetles (Huth and Dettner, 1990), and a stick insect (Smith et al., 1979). Among the latter insects, the coconut stick insects, *Graeffea crouani*, contain 1*R*, 2*S*, 5*S*-iridodial (Smith et al., 1979).

Most of the longicorn beetles emit human-sensitive semiochemicals, belong to Cerambycinae, and secrete defensive chemicals from their metasternal glands. Common predators of longicorn beetles are thought to be birds and small animals. In future studies, we will consider the efficacy of

semiochemicals of the longicorn beetles against some kinds of small animals.

Termite repellency

Both the defensive secretion of *C. loochooanum* and iridodials synthesized according to the method of Clark et al. (1959), a method known to give the *trans,trans*-isomer as the major component (Smith et al., 1979), were subjected to a termite repellent test using *C. formosanus* as a test termite. The test compounds kept their efficacy for 3 days, indicating that iridodials are a potential termite repellent.

Colnelius et al. (1995) reported the termite repellency of dolichodial, the defensive substances of the Dolichoderin ant, *Iridomymerix glaber*. Dolichodial has a pungent odor similar to α-sanshool in *Zanthoxylum piperitum*; on the other hand, 1*R*, 2*S*, 5*S*-iridodial has an aquatic smell. When we utilize natural compounds against household pests like termites, we should consider both their efficacy against termites and also their effect on human health. From the human feeling point of view, 1*R*, 2*S*, 5*S*-iridodial is thought to be a candidate for a natural repellent for practical use.

Conclusion

The defensive substances of a longicorn beetle, *Chloridolum loochooanum*, were iridodials, which were known to be a part of insect defensive substances. A main substance was 1*R*, 2*S*, 5*S*-iridodial, which has been found in the defensive secretion of a coconut stick insect, *Graffae crounai*. Iridodials showed a repellent effect on the termite, *Coptotermes formosanus*.

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Utilization of *Humicola* sp. as Biocontrol for Subterrranean Termite *Coptotermes* sp.

by

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Abstract

Indonesia is a tropical country, which is appropriate for various fungi and termite environment. Termite is one of the important pests in wood preservation. Recently, chemical substances are used as termite control. However, it is unsafe to human and other organisms. In order to prevent further damage that is caused by chemical treatment, biological termite control research using entomopathogenic fungi was done instead of using chemical substances. Utilization of the entomopathogenic fungi has been developed in many advanced countries such as in USA and Japan.

The methods of this research were: (i) isolation and identification of fungi; (ii) fungal screening in order to get isolate that have pathogen effect against termite; (iii) optimation of fungal growth; and (iv) bioassay by using contact (spraying and dusting) and feeding (baiting) mechanism to determine the pathogenic ability of the fungi. The result of the isolation method was three class of fungi: Hyphomycetes, Zigomycetes and Yeast. The fungal screening showed that several fungi, such as *Humicola* sp. could be developed as biological control agents. According to bioassay of *Humicola* sp., the feeding mechanism affect termite mortality higher than contact mechanism, but the difference is not significant.

Key words: isolation, entomopathogen, bioassay, biological control

Introduction

There are many kinds of wood utilization *e.g.* building materials, furnitures and handycrafts. The utilization of wood should be followed by the maintenance of the materials from destruction from fungi and termite attack. Utilization of anti termites chemicals (*chlordane, dieldrin, sodium arsenite, copper naphtenate, pentachlorophenol, sodium pentachlorophenol, and benzene hexachloride*) were effective to control termites population (Prasetiyo and Yusuf, 2004), but it has led to a new problem. The chemical ingredients are harmful for human and it can pollute the environment. For this reason, alternative methods should be found to substitute anti-termites chemical. The methods should be effective, safe, economic and easy to find. Over the past two decades, interest in the use of alternative anti-termites chemical (termiticides) due to environmental and human health concerns has stimulated increasing efforts in the development of microbial control agents for biological control of insects as components of integrated pest management systems.

In Burges (1998) and Butt *et al*, (1999) fungi (*Verticillium lecanii, Metarhizium anisopliae, M. flavoviride, Beauveria bassiana, B. brongniartii, Paecilomyces fumosoroseus* and *Lagenidium giganteum*) can be developed as bio-control such as pesticide. In japan and USA, *M. anisopliae* has been developed as bio-control. In Indonesia, research done to develop entomopathogenic fungi as bio-control has not been improved yet. Therefore, the purpose of this research is to find a new species that has pathogenic effect against termite and suitable for Indonesian circumstances so that it can be used as bio-control against termite. Then the choosen isolate is optimized to increase the fungal growth both culture medium and condition of incubation room including pH and illumination. Then, the

feasibility of choosen isolate to be developed as biological control agent was determined by pathogenic activity test (bioassay) against *Coptotermes* sp. by using contact (dusting and spraying) and feeding (baiting) mechanism.

Materials and methods

Fungi were isolated from soil, pest and termite death body. To grow the fungi, Potato Dextrose Agar (PDA) was used.

Isolation

For isolation the entomopathogenic fungi, two kinds of isolation technique were used:

1. **Direct plating.** In direct plating, samples (examples: termites and soils) are placed directly on solidified agar media (PDA). Then, incubate the plates upright in room temperature (± 25°C) for 5 days. After incubation, examine plates visually (macroscopic and microscopic).
2. **Dilution Plating.** Dilution plating of samples were carried out by immersed samples into sterilized aquadest, at that time samples was blending by vortex to make homogeny suspension and to scrape the spores from samples. Next, a few ml (0,1 ml) of suspension was spread evenly over the agar surface (PDA) with a sterile bent glass rod. The glass rod was sterilized by flaming it with ethanol before use. And then, incubation was done under condition of 25°C for 5 days.

Purification and Identification

After incubation, purification of fungi was done by inoculation fungi onto new media. It intended to obtain pure isolates. Then identification was done by examine fungi visually using microscope (slide culture method).

Fungal Screening

To screen the fungi that have pathogenic effect against termite, bioassay was done to get certain isolate.

Optimation of fungal growth

Culture Media

To choose the appropriate media for fungal growth, some factors are needed to be considered: nutrition compound of media, abundance of raw materials (easy to find) , and cheap. In this research, cereal grains was used to grow the fungi namely white rice, red rice, corn and sorghum.

Rice Medium. Two kinds of rice were used: white and red rice. Rice culture was prepared by adding aquadest into rice in Erlenmeyer. Then it was sterilized with autoclaved for 15 minutes at 121°C.

Corn and Sorghum Medium. Corn and sorghum were heated up for 30 minute. After it become cooler, the cereals were shinned and put into petri dish. Then it was sterilized with autoclave for 15 minutes at 121°C.

Incubation

pH. There are four pH that are used to grow the fungi: 5, 6, 7 and 8.

Illumination. daylight, nonstop lighting and dark were used to grow the fungi.

This step purpose to optimize the condition of incubation periods in order to get optimum growth.

Bioassay

Two concepts of bioassay were used: contact (spraying and dusting) and feeding (baiting) mechanism.

Contact

The specimen test consist of fifty workers of *Coptotermes* sp. that were sprayed with fungal suspension (spraying method) or were dusted with fungal powder (dusting method).

Feeding

Fifty workers of *Coptotermes* sp was fed up or baited with treated paper disc by immersing it with fungal suspension.

Then, the specimen test was placed in petri dish and kept at 28°C under humid condition in the dark place for 14 days. Each treatment carried out in three-replicates. The untreated termites were used as a control. To examine the pathogenic effect of fungi against termite, mortality rate of termite was observed every two days during 14 observation days.

Results and discussion

Isolation and identification of fungi isolated from soil, termites and plant pests resulted some genera of fungi that are classified into three groups of fungi ; Hyphomycetes, Zigomycetes and Yeast. The fungi are then tested on termite in order to determine the pathogenic ability of fungi. The test is used to screen fungi is assumed pathogen to termite. The following figure show the mortality of termite after fungal infection during 14 days observation.

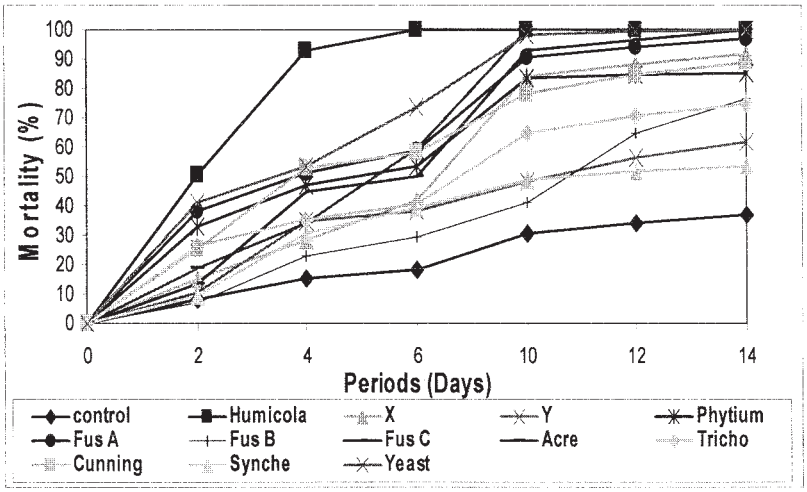

Fig 1. Mortality of termite after fungal infection during 14 day observation

This figure shows the effect of fungi to termite mortality. The screening process of fungi resulted three fungi, *Humicola, Yeast, and Acremonium*, that have potential to developed as bio-control to termite because they can cause termite mortality until 100% in just ten days. However, *Humicola* sp. is the better if compare to other fungi because it caused 100% of termite mortality after six days.

Humicola frequently found in soils and litters, is classified into Hypomycetes. There are several kinds of *Humicola* sp. such as *Humicola fruscoata* and *Humicola grisea*. To grow well, *Humicola* need high carbon (C), e.g. *glucose, sucrose, fructose, inulin, starch,* and *xylan*. In additioon, to increase the mass of mycelium, it is supported by mangan, zinc, and calcium content (Domsch, K. H. *et al*, 1980). Therefore, the *Humicola* growth needs to be optimized so that it can be developed as termite bio-control. The fungal growth increases rapidly in size or cell number. Measurement of filamented organism like fungi is determined based on fungal population counting for each mL or gram media. The growth of fungi will be influenced by many factors such as nutrition availability, pH, and light condition of incubation room. The following figure is the growth of *Humicola* represented by number of conidias in various illuminations and pH of media.

Based on Domsch *et al,* the optimum pH for *Humicola* is about 7 – 8. However, it is not suitable for any *Humicola* because the difference in species and its natural environment. And for illumination/lighting, the best condition is continuous lighting, followed by daylight and dark. The fungi may be divided into five groups on the basis of their response to light: (1) those that are apparently indifferent to light; (2) those in which sporulation decrease or prevented on exposure to light; (3) those that require alternating light and darkness to sporulate; (4) those that are

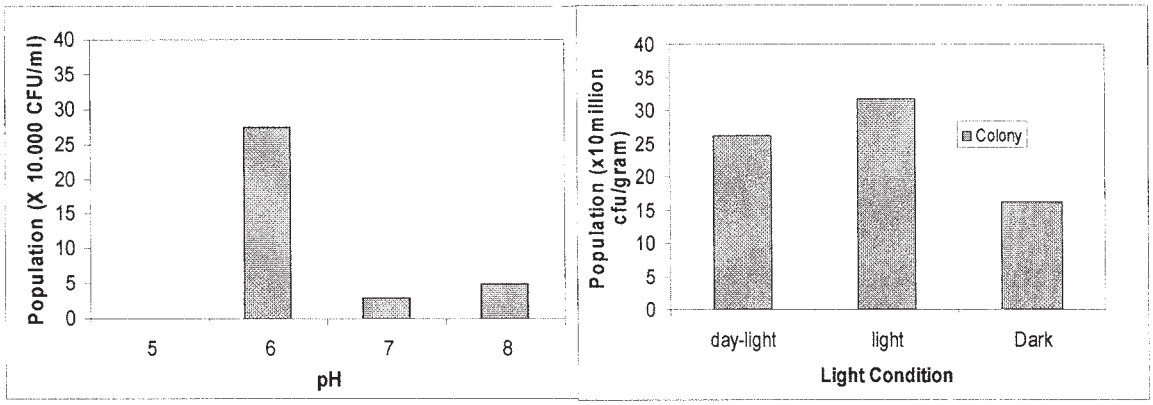

Fig 2. Colony population of *Humicola* sp. in different pH and illumination

able to produce viable spores in complete darkness but sporulate more abundantly with exposure to light; and (5) those that require light in order to produce spores (Moore-Landecker E. 1996).

Besides pH and illumination, the growth of fungi is influenced by nutrition availability in substrate. Quantity and quality of nutrition influence the growth of fungi. Quantity (concentration) relate to availability of nutrition or essential elements to support fungal growth, such as carbon (C), nitrogen (N) and sulfur (S). There are several fungi that require other elements to optimize their growth, like vitamins and minerals, e.g. mangan, zinc and calcium. For this reason that is the significance of nutrition to support fungal growth, needed to have research in media fungal preference. In this research, cereals like rice, corn and sorghum were chosen as fungal substrate because it might have appropriate nutrition for fungal growth. Most entomopathogenic fungi grow fast in substrates containing sufficient carbon and nitrogen.

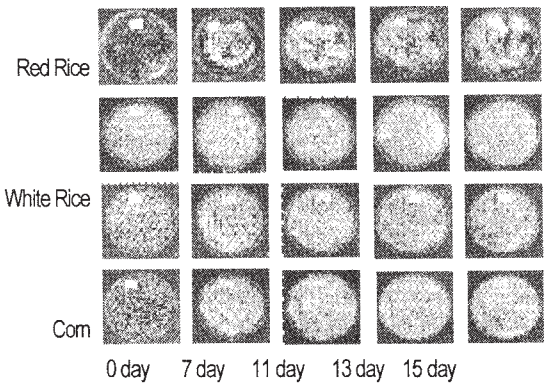

Figure 3. The preference of *Humicola* to grow

In this study, fungal growth is observed visually by morphological character both colonies mass and number of conidia. The following figure (Fig. 1) shows the colonies mass of *Humicola* in four kinds of cereals substrate. Visually, the preference of *Humicola* to grow in particular substrate is different according to substrate. In this figure, sorghum gives the best growth for *Humicola.* On the seventh day of incubation time, the colonies of *Humicola* almost covers whole surface of substrate, and it completely covers whole surface of substrate on the 11^{th} to 15^{th} day of incubation.

It can be compared with other cereals (corn, white rice and red rice). On the 15^{th} day of incubation, fungal mass has not completely covered the whole surface of substrate. First, fungi started to grow by spore germination, then it formed hyphae that comprised branches to any directions and formed group of hyphae called mycellium. To live, fungi continue to absorb nutrition in substrate until substrate become poor or even loss nutrition. Finally, this condition result in spore or conidia formation (Moore-Landecker, 1996). To infect its host, spore or conidia propagule to initiate infection process. In this research, the number of conidia on each substrate is different. In Table 2 the number of fungal conidia and colony grown on four kinds of substrates is illustrated.

Table 2. The number of conidia and colony of *Humicola* grown on various substrate.

Media	White Rice	Corn	Sorghum	Red Rice
Conidia (conidia/g)	$2{,}14 \times 10^7$	$3{,}6 \times 10^7$	7×10^7	$1{,}25 \times 10^7$
Coloni (cfu/g)	$8{,}96 \times 10^6$	$19{,}20 \times 10^6$	$28{,}76 \times 10^6$	$10{,}67 \times 10^6$

Table 2 illustrate the number of conidia of *Humicola* on sorghum media is 7×10^7 conidia/g. It is the highest number compared to other media. The plenty of fungal conidia may be assumed that it has enhanced its pathogenic level because conidia is propagule to initiate host infection. Besides the number of conidia, fungal growth can be determined by the number of fungal colony. This study examine that the number of fungal colony on sorghum media is more than the others, i.e. $28{,}76 \times 10^6$ CFU/g. It assume that sorghum is a proper media to support *Humicola* growth both in number of conidia and the colony.

After the optimum condition to support *Humicola* growth was known, production of anti-termite bio-control (biotermiticide) using *Humicola* was done by combining all factors. The formulation of biotermiticide is blended materials, i.e. fine powder. Then, bioassay on termite was done to apply and determine the biotermiticide pathogenic level. In Figure 4, termite mortality caused by the biotermiticide is illustrated.

Bioassay was done by applying three methods, that are spraying, dusting, and baiting methods. The result shows that *Humicola* may cause higher termite mortality for each method compared with control. After 14 days observation, baiting method cause termite mortality up to 60% followed by dusting and spraying method, 45% and 40% repectively. The variation may be caused by fungal infection mechanism. Mostly, fungi invade directly through cuticle. Unlike other biocontrol agents, fungi do not have to be ingested to infect their host. In this research, application of biotermiticide to infect termite was done both by contact mechanism (spraying and dusting) to invade directly through cuticle and by feeding mechanism (baiting) to be ingested to infect their host.

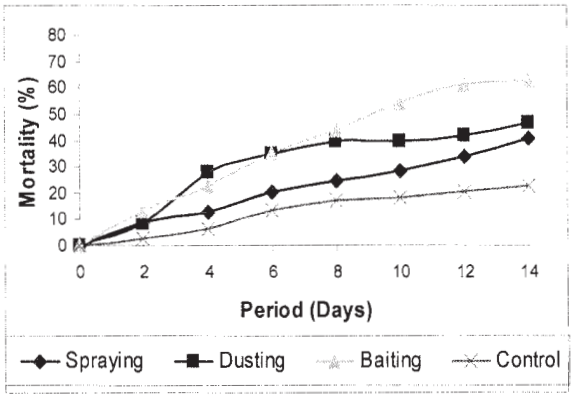

Figure 4. Mortality of termite after fungal infection during 14 observation days

The feeding mechanism affect termite mortality higher than contact mechanism, but the difference is not significant. In this case, *Humicola* is assumed to have particular substances that affect termite mortality. In ARSef (USDA-ARS *Collection of Entomopathogenic Fungal Cultures*), *Humicola* is registered as entomopathogenic fungi to host Lepidoptera: Lymantriidae (Humber & Hansen 2005). Hyphae or mycelium of *Humicola grisea* also decompose chitin and ceratin, besides mycelial extract showed toxic effects against brine shrimps (Domsch *et al.* 1980).

For termites infection caused by *Humicola*, there is no much information about the mechanism. The result shows that *Humicola* prefers to infect the termite by ingesting rather than invading cuticle directly. It can be identified from the value of termite mortality by feeding that is higher than by contact mechanism. Therefore, the characteristic of *Humicola* should be evaluated for further information about infection and pathogenic ability against termite.

Conclusion

1. Humicola can be developed as bio-termiticide.
2. The optimum condition for growing *Humicola* sp is continuous lighting condition, pH 6 and Sorghum as media to support *Humicola* sp. Growth..
3. In bio-assay, baiting is the best method compared to other contact (spraying and dusting).

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Control of the Dry-Wood Termite *Incisitermes minor* (Hagen) Infestation by Bait System

by
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Abstract

Three types of experiments were designed to evaluate the performance of a bait system intended to control *I. minor*. In Type I, the effectiveness of the bait in a small wood specimen was evaluated. In Type II, the bait effectiveness was evaluated in a bigger wood specimen. Feeding arena lumber with artificial galleries was prepared for the Type III experiment so that the response of the insects to the gel could be observed. In general, the average percentage termites that died after being exposed to the gel formulation in Types I and III of experiment was more than 60%, and more than 85% for Type II. In the gel control the average percentage of live termites was more than 95% in Types I and III, and more than 75% in Type II. These results suggest that the gel bait system used in this study has the potential to eliminate *I. minor* colonies. Further investigation will be indispensable to increase the reliability of the bait system as a control strategy for dry-wood termites.

Key words: Control strategy, Bait system, Dry-wood termite, *Incisitermes minor* (Hagen)

Introduction

Chemical treatments with a liquid formulation have been widely used to prevent the infestation of dry-wood termites in buildings. However, such chemical treatments are problematic due to health and the environmental considerations. Therefore, it is important to develop remedial treatments that do not pose environmental hazards. Whole-structure treatments and local remedial treatments have been developed as dry-wood termite control measures that use fewer or no chemicals (Lewis, 1996). In recent years, the introduction of bait systems that use fewer chemicals to the methods of subterranean termite control (Su, 1994; Su and Scheffrahn, 1996; Su *et al*, 1982; Su *et al*, 1997; Su *et al*, 1998; Tsunoda *et al*, 1997) may help us to develop new strategies for eliminating colonies of dry-wood termites.

The dry-wood termite, *Incisitermes minor* (Hagen), is a native to the western region of the United States, and is one of the five economically important and destructive termites in that country (Su and Scheffrahn, 1990). The colony is found in both natural and artificial environments (Su and Scheffrahn, 1990). This study was conducted to develop a control strategy for dry-wood termite *I. minor* infestation by a bait system.

Materials and methods

Termites

As test organisms, *I. minor* was collected from infested timbers in Yokohama City, Kanagawa Prefecture, Japan. The termites were kept in the Deterioration Organism Laboratory (DOL) of the Research Institute for Sustainable Humanosphere, Kyoto University, at 28±2°C, >85% RH, for at least one week before testing to ensure that only healthy termites would be used in the experiment.

Test timber

Three types of experiments were carried out in the current investigation. In Type I, a pair of air-dried sapwood specimens of spruce (*Picea abies* Karst.), measuring 30 (R) x 30 (T) x 50 mm (L), were used. A hole, measuring 50 mm in depth and 10 mm in diameter, was drilled in the center of a specimen to accommodate the termites. Another hole, measuring 40 mm in depth and 10 mm in diameter, was also drilled in the center of the other specimen. These two specimens were combined, and a hole, measuring 15 mm in depth and 10 mm in diameter, was then drilled in the center of the top surface of the combined specimen for the termites to be placed inside (Fig. 1).

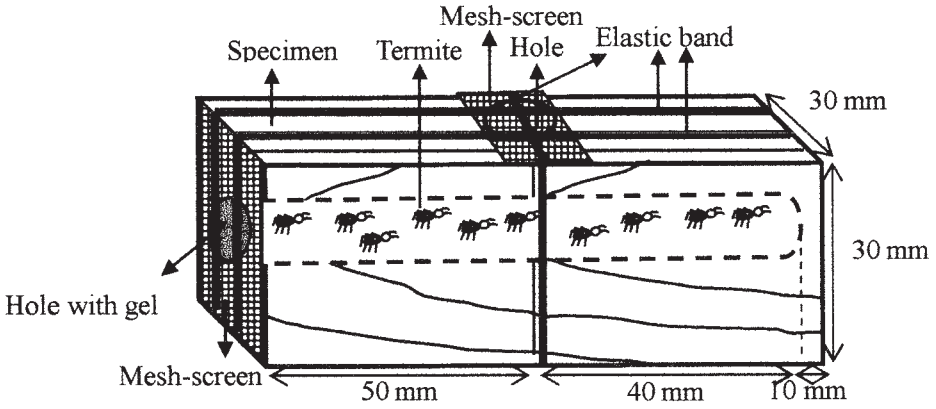

Fig. 1. Test apparatus for the testing of a bait system against *I. minor* (Type I)

In Type II, the sample preparation was similar to that described for Type I except for the number of specimens for each set (six specimens) (Fig. 2).

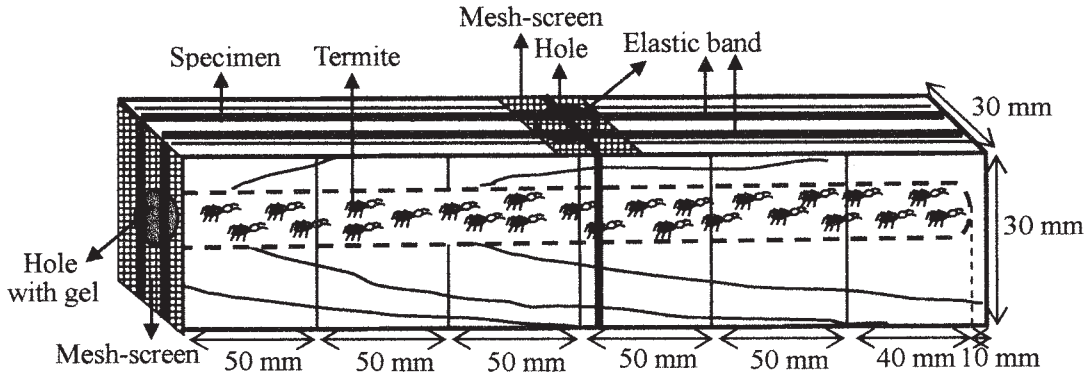

Fig. 2. Test apparatus for the testing of a bait system against *I. minor* (Type II)

In the case of Type III, a feeding arena was prepared by a spruce sapwood lumber (200 (R) x 20 (T) x 300 mm (L)). Artificial galleries were then grooved as shown in Fig. 3. The width and depth of the galleries were 10 mm and 5 mm, respectively.

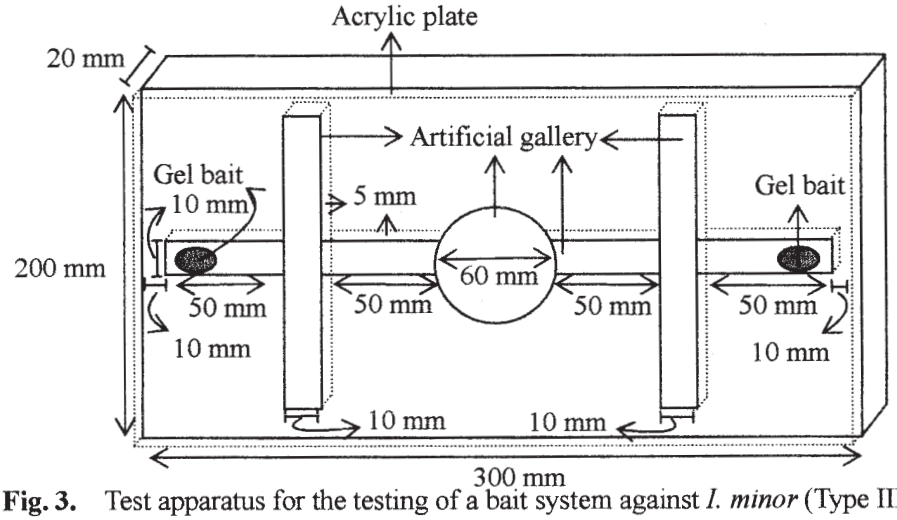

Fig. 3. Test apparatus for the testing of a bait system against *I. minor* (Type III)

Gel formulation

A commercial gel formulation with an active ingredient (2.15% hydramethylnon) and food attractants was used for the testing (Types I, II, and III). For the control in Type I, we used a commercial gel product without any insecticidal ingredient for the horticultural plantation. The gel control was dipped in the distilled water for approximately 4 hours before the experiment. The formulations without an active ingredients were employed as the controls in Types II and III.

Test apparatus

A 0.4 gram gel formulation was put into the hole drilled in the side of the specimen for the Type I and Type II experiments. The hole was covered with a fine mesh screen that was tightly attached using two elastic bands to prevent the termites from coming out of the hole.

For the Type I and Type II experiments, ten and thirty termites, respectively, were put into the center holes of the top surfaces of the wood specimens and the holes were then covered with a fine mesh screen that was attached tightly with an elastic band (Fig. 1 and Fig. 2).

A similar control experimental set up was employed, except that a much greater amount of gel (two grams) was used in the Type I control. For the control in Type II, the experimental procedure and the amount of gel were the same as described above except that the gel used had no active ingredient.

On the other hand, one gram of the gel formulation was placed at the center of one of the 300 mm sides of the specimen for the Type III experiment (Fig. 3). For the control in Type III, the same experimental procedure as that described above for Type II was used.

Forty *I. minor* for Type III were put in the center of the artificial gallery. The assembled arena was then covered with an acrylic plate (2 mm in thickness), which was fastened by four paperclips.

All the experiment units (Types I, II, and III) were kept in the DOL for two weeks. Three replicates were served for each type. The mortalities of the termites were evaluated for all types after two weeks. For Type II, the test set-up was disassembled and the location of the test insects was observed at the end of the experiment. On the other hand, for Type III, the location of the termites inside the test arena was observed daily.

Results and discussion

Mortality of termites

The percentages of live, moribund, and dead *I. minor* after being exposed to the gel formulation in Types I, II, and III for two weeks are shown in Tables 1, 2, and 3, respectively.

Table 1. The response of pseudergates of *I. minor* exposed to the gel formulation after two weeks (Type I)

Treatment	Run	Condition of termites		
		Live (%)	Moribund (%)	Dead (%)
Gel formulation	1	0	0	100
	2	0	40	60
	3	60	20	20
	Average	**20**	**20**	**60**
Gel control	1	100	0	0
	2	90	0	10
	3	100	0	0
	Average	**96.7**	**0**	**3.3**

Table 2. The response of pseudergates of *I. minor* exposed to the gel formulation after two weeks (Type II)

Treatment	Run	Condition of termites		
		Live (%)	Moribund (%)	Dead (%)
Gel formulation	1	0	0	100
	2	0	0	100
	3	33.3	6.7	60
	Average	**11.3**	**2.2**	**86.7**
Gel control	1	73.3	0	26.7
	2	96.7	0	3.3
	3	60	0	40
	Average	**76.7**	**0**	**23.3**

The average percentages of dead termites after two weeks of exposure to the gel formulation were more than 60% for Types I and III, and more than 85% for Type II (Tables 1, 2, and 3). On the other hand, the average percentages of dead termites in the gel control were less than 4% for Types I and III, and less than 25% for Type II in the same period (Tables 1, 2, and 3). These results suggest that the present test methods are suitable for evaluating the performance of the bait system against *I. minor*, and that the gel formulation used in this study has considerable potential as

a control strategy for dry-wood termites.

In Type III the percentage of live termites in Run 2 was 97.5% at the end of the experiment (Table 3), while the rest of the runs (Runs 1 and 3) did not show any sound termites after two weeks (Table 3). The fact that the termites in Run 2 of the Type III experiment built a barrier using their fecal pellets and wet feces near the gel might explain this phenomenon. The insects could not come into contact with the gel through the barrier.

Table 3. The response of pseudergates of *I. minor* exposed to the gel formulation after two weeks (Type III)

Treatment	Run	Condition of termites		
		Live (%)	Moribund (%)	Dead (%)
Gel formulation	1	0	2.5	97.5
	2	97.5	0	2.5
	3	0	0	100
	Average	**32.5**	**0.8**	**66.7**
Gel control	1	100	0	0
	2	92.5	0	7.5
	3	100	0	0
	Average	**97.5**	**0**	**2.5**

Location of termites

When exposed to the gel formulation, the insects were spread evenly in all parts of the sample at the end of the experiment in Type II (Table 4). Although the termites were not accumulated in the bait, the mortality of these test insects was 86.7% on average (Table 2). This clearly indicates that the gel formulation does not have any special attraction or repellent effect on *I. minor*, and that trophallaxis activity, one of the characteristic behaviors of termites and other social insects, may contribute to the higher mortality.

Table 4. Location of termites after two weeks in test apparatus in Type II

Run	Location of termite					
	Part of sample					
	1	2	3	4	5	6[1]
Gel formulation						
1	3 D	5 D	7 D	11 D	1 D	3 D
2	7 D	2 D	6 D	2 D	5 D	8 D
3	2 L, 2 M, 2 D	6 L, 1 D	2 D	6 D	2 L, 5 D	2 D
Gel control						
1	21 L, 3 D	2 D	1 L, 1 D	1 D	-	1 D
2	4 L	2 L	11 L	7 L, 1 D	5 L	-
3	2 D	4 D	2 D	6 L	2 L, 3 D	10 L, 1 D

[1] Part in which the gel was applied; D: dead termite; L: live termite; M: moribund termite;

In the test arena, Type III, in Run 2, the gel formulation resulted in a very low mortality among the termites (2.5%) after two weeks (Table 3). The termites avoided the bait and built a barrier using their fecal pellets and wet feces in this case. But in other cases, Runs 1 and 3, in which

the mortalities were 97.5% and 100%, respectively (Table 3), the termites were spread evenly in all parts of the test arena (Fig. 4). These varied results support the above-mentioned assumption well.

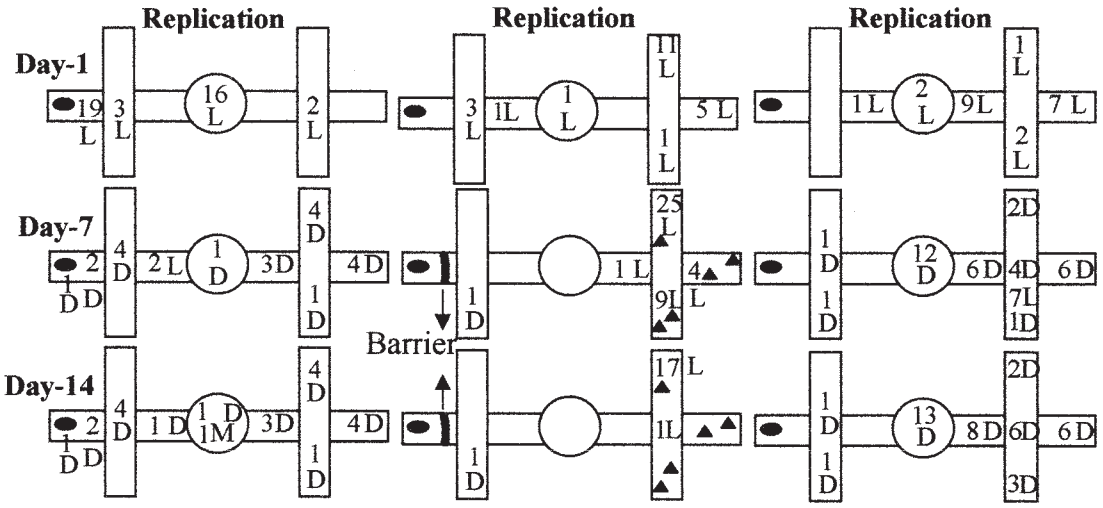

Fig. 4. Location of termites in the test arena exposed to the gel formulation in Type III after 1, 7, and 14 days. Numbers in the galleries represent numbers of termite. Filled oval: gel; Filled triangle: perforations by termite; D: dead; L: live; M: moribund.

From the present investigation, it can be conclude that the gel bait has the potential to be used as a remedial control strategy against *I. minor*, and that consideration of its varied performance will be a key factor in constructing a reliable bait system. The search for special attractants substances that are spread into the entire attacked area should be the next step in this research.

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Tunneling Patterns of Subterranean Termites *Coptotermes gestroi* (Wasmann), *Coptotermes curvignathus* (Holmgren) and *Coptotermes kalshoveni* Kemner (Isoptera: Rhinotermitidae)

by
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Abstract

Coptotermes is an important genus of subterranean termites that infests buildings and wooden structures worldwide. *Coptotermes gestroi* (Wasmann), *Coptotermes curvignathus* (Holmgren) and *Coptotermes kalshoveni* Kemner are among the economically important *Coptotermes* species in Peninsular Malaysia. Due to the varying prevalence of infestation of the three species, this study was initiated to study their tunneling behaviour with the hope to shed light into their food-seeking behaviour. A laboratory study was conducted using two different tunneling mediums: agar and moisten sand. The tunneling activities were semi-qualitatively ranked. All three species showed different tunneling pattern. *Coptotermes curvignathus* was found to be the most aggressive species with extensive tunneling activity, followed by *Coptotermes gestroi* and *Coptotermes kalshoveni*. *Coptotermes kalshoveni* showed the highest wood consumption rate within the 28-day evaluation period.

Key words: subterranean termite, *Coptotermes gestroi*, *Coptotermes curvignathus*, *Coptotermes kalshoveni*, tunneling behavior, wood consumption.

Introduction

Coptotermes is an important genus of subt erranean termites found infesting buildings and wooden structures. The three economically important species of *Coptotermes* in Peninsular Malaysia are *Coptotermes gestroi* (Wasmann), *C. curvignathus* (Holmgren) and *C. kalshoveni* Kemner. *C. gestroi* is the most economically important species in South East Asia (Kirton & Brown 2003). Studies on subterranean termite foraging populations, damage and control strategies has been reported by numerous authors (Sornuwat *et al.* 1996, Lee 2002, Ngee *et al.* 2004). Earlier, Kirton & Azmi (2005) noticed different prevalence of infestation of the three *Coptotermes* species in buildings and structures in Malaysia. We hypothesised that this could be due to differences in tunneling and feeding behaviour of the various species of *Coptotermes*. This study was initiated to address the above hypothesis.

Materials and methods

C. gestroi, *C. curvignathus* and *C. kalshoveni* were each collected from in-ground monitoring stations that were established earlier in Minden campus, Universiti Sains Malaysia, Penang Island, Malaysia. Two hundred worker termites together with 10 soldiers were released into a petri dish (15 cm diam.) that was pre-filled with 5% agar (1 - 1.3 cm height). Four pieces of rubber wood were placed at the position of 0°, 90°, 180° and 270° of the petri dish prior to the formation of the agar (tunneling medium). Termites were allowed to forage freely. Tunneling activities were observed 28 days post-introduction and semi-qualitatively ranked. Experiment was replicated 10 times for each species. Termite mortality was also being estimated.

Another method used moisten sand and was similar to that described by Grace *et al.* (2004). A piece of rubber wood (*Hevea brasilliensis*) and pine wood (*Pinus* sp.) was provided in each glass jar to serve as food source. Two hundred workers with 10 soldiers were introduced into the jar containing moisten sand and food. They were allowed to forage freely for 28 days. Upon that period, termite mortality and total amount of wood consumed were determined at the end of experiment. Each experiment was replicated 10 times.

Results and discussion

There were noticeable differences in the tunneling geometry between the three *Coptotermes* species. It was found that *C. curvignathus* made wider and highly branched tunnels, followed by *C. gestroi*. *C. kalshoveni* however, showed the least tunneling activity; most of its tunnels were straight and hardly branched. Su (2005) had reported that termites would tunnel along the edge of testing arena when no food attractant was present. We found similar results in this study, where all three termite species foraged along the perimeter of the petri dish in their effort of searching new food source. Termite survivorships were high (>75%) in all replicates in both evaluation methods.

Despite its aggressive tunneling behaviour, *C. curvignathus* did not show the greatest wood consumption among the three *Coptotermes* species. In contrast, *C. kalshoveni* that tunneled the least had the highest wood consumption rate, indicating their high resource fidelity. Based on amount of wood consumed, *C. curvignathus* showed no wood preference; however, both *C. gestroi* and *C. kalshoveni* clearly preferred rubber wood over pine wood.

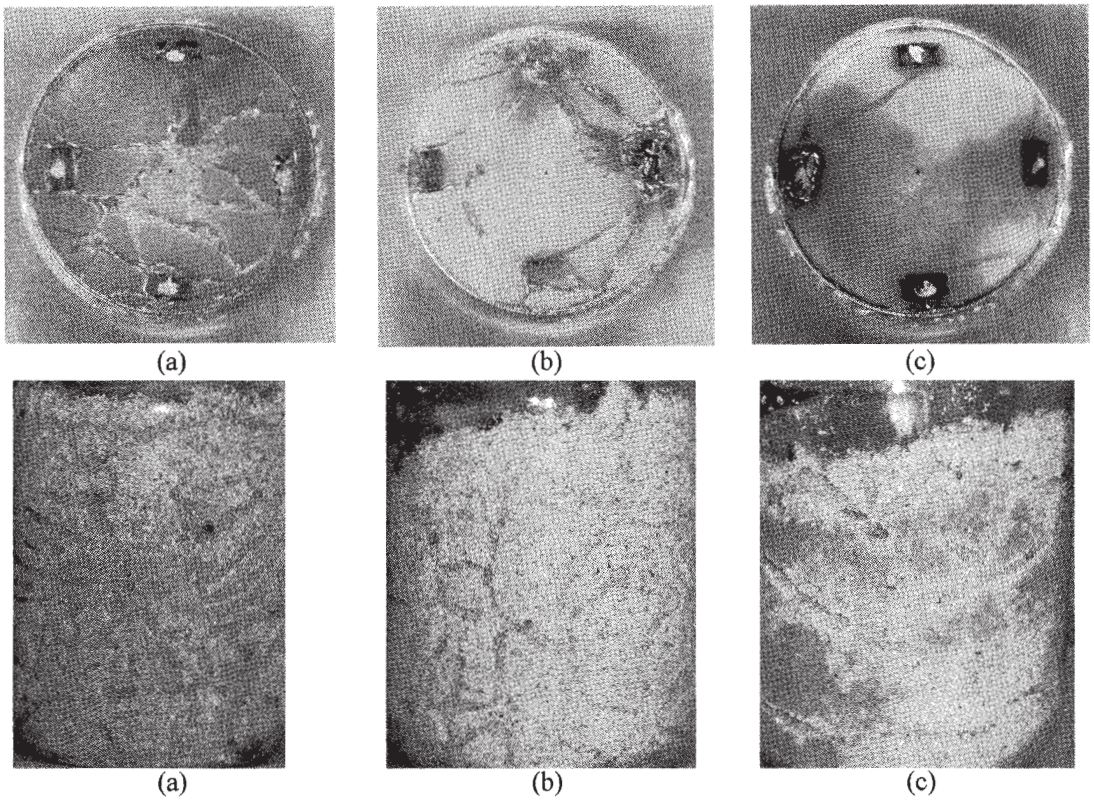

Figure 1: Termites tunneling activities of (a) *C. curvignathus*, (b) *C. gestroi*, and (c) *C. kalshoveni* in two different tunneling medium.

Table 1: Tunneling activity, termite mortality and wood consumption by *Coptotermes curvignathus*, *Coptotermes gestroi* and *Coptotermes kalshoveni*, after 28 days evaluation period (n=10)

Termite species	Tunneling activity[1]	Termite mortality[2]	Wood consumption[3] (g)	
			rubber	pine
Coptotermes curvignathus	3	Low	moderate	moderate
Coptotermes gestroi	2	Low	low	low
Coptotermes kalshoveni	1	Low	high	high

[1]Tunneling activity: 0-30% area = 1, 31-60% area = 2, 61-100% area = 3.

[2]Termite mortality: 0 – 25% = Low; 25 – 70% = moderate; >75% = High.

[3]Relative comparison

Summary and conclusion

C. curvignathus was found to be the most aggressive species with extensive tunneling activity, followed by *C. gestroi*. This may indirectly provide an explanation to support Kirton and Azmi (2005) findings that the two species are the top two most destructive species of subterranean termites in Malaysia. On the other hand, *Coptotermes kalshoveni* showed the least tunneling activity, but demonstrated the highest wood consumption rate within the 28 days evaluation period.

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Inter-colony Transmission and Environmental Acquisition of Symbiotic Methanogenic Microbes by the Termites, *Neotermes koshunensis*

by
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Abstract

Despite the obligate mutual relationships between termites and microbes in the guts, it still remains unclear where the symbiotic microbes come. We investigated this by using the termite, *Neotermes koshunensis*, and their symbiotic methanogenic microbes. Firstly, *N. koshunensis* colonies were examined for the presence or absence of symbiotic methanogens by microscopic observation and PCR amplification of the 16S rDNA. As a result, methanogens were not detected from some colonies. By keeping termites of these colonies with termites of other *N. koshunensis* colonies or *Coptotermes formosanus* colonies (from which methanogens were detected), methanogens were detected from the termites that initially lacked methanogens. These strongly suggest the horizontal transmission of methanogens among termites.

Key words: methanogen, horizontal transmission, termite colony

Introduction

The ability of termites to utilize woody material depends on symbiotic relationships with the gut microbes—Eucarya (protozoans and fungi), Eubacteria and Archaea. One of the most common symbiotic microbes is methanogenic Archaea (methanogens) of the genus *Methanobrevibacter* (Ohkuma *et al.* 1999; Shinzato *et al.* 1999; Tokura *et al.* 2000; Donovan *et al.* 2004). H_2-dependent CO_2 reduction by these methanogens (Leadbetter & Breznak 1997; Leadbetter *et al* 1998) represents an important sink of the H_2 emitted during cellulose degradation in termite guts and probably contributes to energetic economy of the termites. Despite the apparently obligate symbioses between termites and methanogens, it still remains unclear where and how termites acquire their symbiotic methanogens. In a broad sense, termite symbiotic methanogens are phylogenetically distant from the other methanogens (Ohkuma *et al.* 1999; Shinzato *et al.* 1999; Tokura *et al.* 2000; Donovan *et al.* 2004), meaning long-time isolation of termite symbiotic methanogens from the others. According to Shinzato *et al.* (2001), several termite species especially of the families Rhinotermitidae and Kalotermitidae harbor the methanogens that are phylogenetically placed in the almost same position to each other. This could be explained by horizontal (from-colony-to-colony and/or from-species-to-species) transmission of methanogens among the termites. In addition, the detection of the methanogens closely related to termite-symbiotic ones from the environments such as nest material (Shinzato *et al.* 2001; Donovan *et al.* 2004) may interpret horizontal transmission of methanogens via the environments.

The termites, *Neotermes koshunensis*, are widely distributed from Okinawa Island, through Taiwan Island, to China (Huang *et al.* 1989). This species belong to the family Kalotermitidae and so-called one-piece nesters (Abe 1987), and make single-nest colonies, of which the mean population is approximately 500 individuals except for extremely large colonies (Maki and Abe 1986). Shinzato *et al.* (1993) reported that methane emission was not detected from some colonies of *N. koshunensis* and that methanogens appear not to be present in the guts of these termites on the basis of microscopic observation. Therefore, *N. koshunensis* could be used as "methanogen knock-out" termites. Using *N. koshunensis* colonies, we tested horizontal pathways of methanogen transmission by laboratory experiments and field observations.

Materials and methods

Differentiation of* N. koshunensis *colonies between MC and MFC

We collected *N. koshunensis* colonies from Okinawa Island and Iriomote Island, and examined them for the presence or absence of gut-symbiotic methanogens by microscopic observation and PCR (polymerase chain reaction) amplification. Methanogens are generally known for their F420 autofluorescence (Doddema & Vogels 1978), which can be visually detected by the epifluorescence microscope with a WBV filter. In the present study, the whole gut of an individual was pulled out with forceps and squeezed into a 50-μl solution U buffer (Trager 1934), of which 15 μl was spread on a slide grass and thoroughly observed under the OLYMPUS BX41 microscope with the U-MWBV2 filter. Three individuals were used for each colony.

Termite individuals of the same colonies were used for the PCR detection of 16S rDNA of methanogens. Genomic DNA was extracted from the whole guts of three individuals of each colony by using ISOPLANT II (Wako, Japan) and DNeasy tissue kit (QIAGEN, USA). Nested (two-step) PCR methods were employed in the present study due to the high sensitivity. The first PCR was conducted under the condition: 30 cycles at 94°C for 30 sec, 53 °C for 30 sec and 72 °C for 2 min, with the methanogen-specific primers 23F (5'-TTGATCCYGSCRGAGG-3') and 1392R (5'-ACGGGCGGTGTGTC-3'). Using the PCR products, the second PCR was conducted for 30 cycles at 94 °C for 30 sec, 55.5 °C for 30 sec and 72 °C for 2 min, with the internal primers MB520F (5'-CAGCMGCCGCGGTAAC-3) and M1382R (5'-GTGTCAAGGAGCAG-3'). In addition, PCR amplification of the bacterial 16S rDNA was done to check the quality of the extracted DNA.

Methanogen transmission within colony members

A total of 500 individuals of a MFC (methanogen free colony, see below) and one marked individual of a MC (methanogen colony, see below) were put in a plastic container with moist filter paper and monitored for the occurrence of methanogen transmission. For two weeks at an interval of one day, ten individuals of the MFC in the container were randomly picked up and observed for gut-symbiotic methanogens by the microscope as described above. Additionally, three months later, we examined the remaining termites for the retention of methanogens. Two MFCs were used for this experiment.

Inter-colony transmission and environmental acquisition of methanogens

We tested the possibility of methanogen transmission from potential sources. In a petri dish with moist filter paper, 10 individuals of a MFC were kept with 1) 10 individuals of a MC, 2) 20 individuals of *Coptotermes formosanus* (containing methanogens), 3) filter paper soaked with gut contents of a MC, 4) no potential source (as a negative control). Ten days after these experiments were started, the individuals of the MFC were examined for the presence or absence of methanogens by fluorescence microscope as described above.

Population size and frequency of MFCs under natural environments

We compared population size of *N. koshunensis* colonies between MCs and MFCs. A total of 48 colonies were collected from Iriomote Island in the Ryukyu Archipelago and brought back to the laboratory. The entire population (total number of individuals) of each colony was determined by breaking the nesting wood with a hatchet. Three individuals of each colony were examined for the presence or absence of methanogens by fluorescence microscope as described above. Of the 48 colonies, there were 4 colonies that were not able to be observed by microscope due to their very small populations.

Results and discussion

Termites are well-known to harbor symbiotic methanogens in their guts and emit methane at significant levels (Ohkuma *et al.* 1999; Shinzato *et al.* 1999; Sugimoto *et al.* 2000). However, Shinzato *et al.* (1993) reported that some colonies of *N. koshunensis* in the Ryukyu Archipelago do not emit methane at a detectable level and that members of the colonies seem to lack symbiotic methanogens based on epifluorescence microscopic observation. In the present study, we re-examined *N. koshunensis* colonies for the

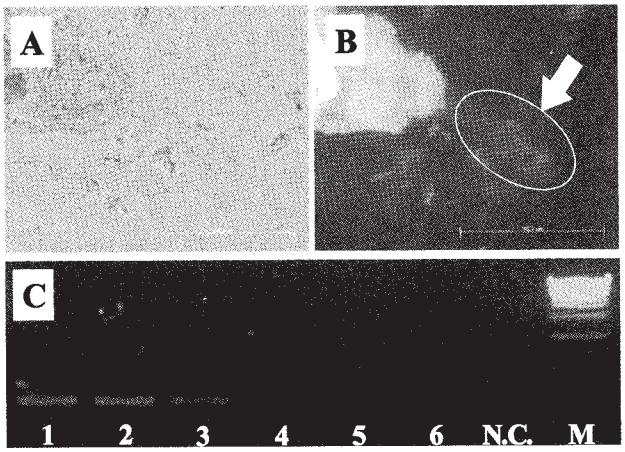

Fig.1. Epifluorescence microscopy and detection of 16S rDNA of methanogens by PCR amplification. A) Light micrograph of the flagellate *Oximonas* sp. in the gut of *N. koshunensis* (×400). B) Epifluorescence micrograph of the same field as in A. Arrow points F420 autofluorescence of methanogens. C) Electrophoretic analysis of second PCR amplification products. 16S rDNA of methanogens were detected from three individuals of each colony by Nested PCR methods. Lane 1-3: 16S rDNA of methanogens were detected. Lane 4-6: 16S rDNA of methanogens were not detected. Lane N.C.: Negative control. Lane M: DNA marker.

Table.1. Detection of methanogens by two methods.

colony	NKY-A1	NKO-20	NKO-22	NKY-A3	NKO-19	NKO-21
Epifluorescence microscopy	+	+	+	−	−	−
PCR detection of 16S rDNA	+	+	+	−	−	−

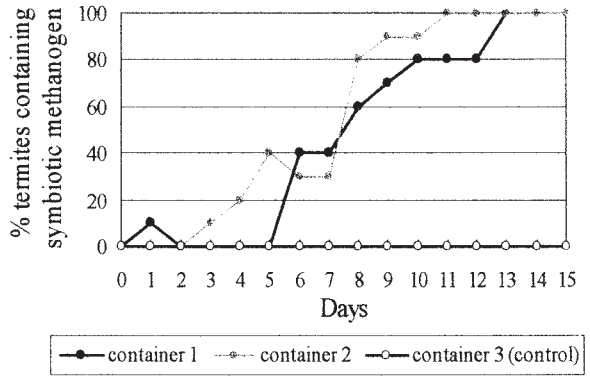

Fig.2. Methanogen transmission within colony members. A total of 500 individuals of a MFC and one marked individual of a MC were put in a plastic container and monitored for the occurrence of methanogen transmission. Two MFCs were used for this experiment (container 1,2). In addition, only 500 individuals of a MFC were put in a container and monitored as negative control (container 3).

presence or absence of symbiotic methanogens by microscopic observation as well as PCR amplification of the 16S rDNA (Fig. 1). Our results agree with the previous study (Shinzato et al. 1993) and clearly indicate that methanogens are not detected from termite individuals of some *N. koshunensis* colonies by both of the methods (Table 1). There was not such a colony that methanogens were not detected from the members by one method but detected by the other. Hereafter, on the basis of the microscope observation, we divide *N. koshunensis* colonies into the two types, MC (methanogen colony) and MFC (methanogen free colony).

There is a doubt whether methanogens are entirely absent from or present at a very low density in the termite guts of MFCs. In the latter case, the gut environments might inhibit the growth of methanogens by certain factors such as concentrations of some materials; in other words, an infection of methanogens to these termites would not occur even if a potential source is provided for them. We clarified this issue by putting one termite of a MC into 500 termites of a MFC. Our results show that methanogens have been fully detected from the 500 termites within approximately two weeks (Fig. 2). This strongly suggests the entire absence of methanogens in MFCs and that methanogens are inter-colonially transmitted in *N. koshunensis*. The results also indicate that all the termites of MFCs lack methanogens and *vice versa*. Furthermore, during colonies kept intact, the disappearance of methanogens seems not to occur.

Phylogenetic relationship of symbiotic methanogens of termite gives an insight into horizontal transmission and environmental acquisition of methanogens by termites (Shinzato *et al.* 2001; Donovan *et al.* 2004). It was shown that

methanogens are horizontally (intra-specifically) transmitted at least in experimental conditions. In addition, the possibility of horizontal (inter-species) transmission was tested by keeping termites of MFCs with those of *Coptotermes formosanus* (from which methanogens were detected). As in the case of *N. koshunensis* colonies, methanogens were detected from the termites of a MFC (Fig. 3). This means that inter-colony (intra-species) transmission occurred between the termites of *N. koshunensis* and *C. formosanus*, while all the *C. formosanus* termites were dead 10 days after the experiment began. Thus, this transmission could have been mediated not by trophallaxis, but by the dead bodies and/or faeces of *C. formosanus*. Supporting this, termites of a MFC had methanogens at the end of the experiment where the termites of a MFC were fed with gut contents of a MC. These combined, regardless of termite species, methanogens could be transmitted to termites of MFCs.

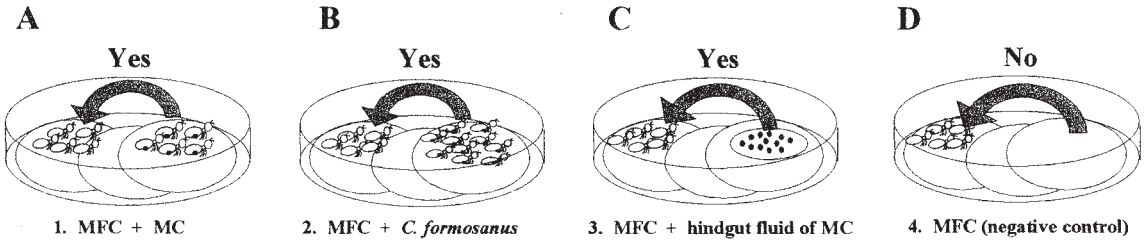

Fig.3. Horizontal transmission of methanogens in Petri dishes. Termites were bred with various methanogen donors in Petri dishes. A,B,C: Methanogens were transferred from potential sources; D: Methanogens were not transferred.

Colonies of *N. koshunensis* are usually found to be the same woods that are inhabited by other colonies and/or other termite species. In Iriomote Island, we collected a total of 48 *N. koshunensis* colonies, of which 5 colonies were found to coexist with other colonies of termites comprising *C. formosanus* and *Glyptotermes fuscus* or with foraging termites of *Nasutitermes takasagoensis*. As shown above, these termites could be potential sources of symbiotic methanogens. Simply, population size of *N. koshunensis* colonies will represent the elapsed time from the colony funding and a longer elapsed time will increase the chance of contact with methanogen sources. Here, we compared population size of the 48 *N. koshunensis* colonies between MCs and MFCs (Fig. 4). The mean population size was apparently different, though there was no significant difference possibly due to the small number of large colonies.

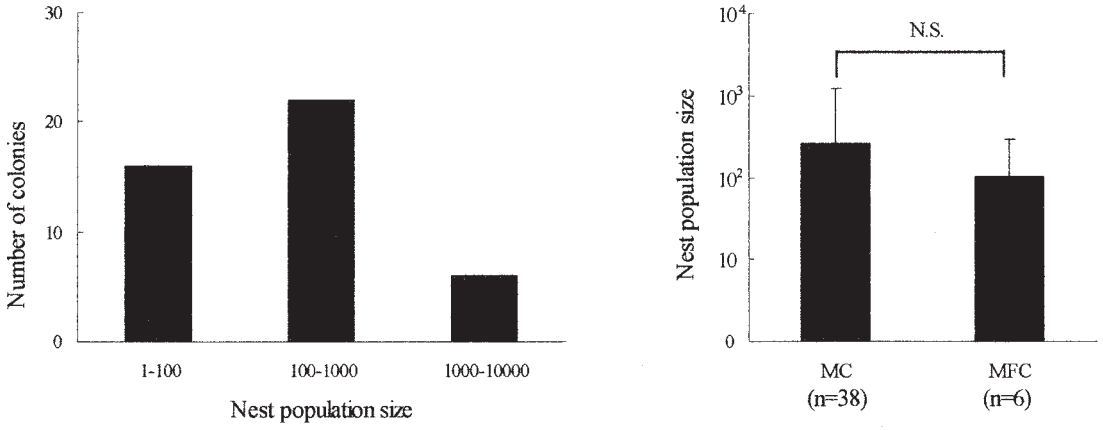

Fig.4. Comparison of the nest population between MC and MFC. A, Frequency distribution of the nest population of *N.koshunensi.s* B, Comparison of the nest population between MC and MFC—Values are mean ± S.D. (They are transformed into the natural logarithm)

Based on the results, we strongly suggest the absence of methanogens in some *N. koshunensis* colonies and the occurrence of methanogen transmission from the other termites. Here, a question is given: why do MFCs exist? Without any process losing methanogens, all colonies of *N. koshunensis* would be MCs soon. A possible explanation could be provided if some alates from MCs will lack methanogens. Such alates can be expected to found a MFC colony. In order to elucidate the relationships between *N. koshunensis* and methanogens this aspect should be examined by further experiments.

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A Carbon Source Based Perspective on the Global and Geographical Patterns of Feeding Group Composition in Termites

by

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Abstract

A recent report (Yamada *et al.* 2005) has suggested that the carbon (C) source competition between termites and litter-layer microbes may be employed for understanding the C mineralization processes in tropical forests, but the situation in dry tropical ecosystems (e.g., savannas) and the possible intergroup competition in termites remain unclear. Here, we observed termites in a dry tropical ecosystem (dry deciduous forest (DDF)) and a high-altitude tropical forest (hill evergreen forest (HEF)) in Thailand and estimated the C fractions in the annual aboveground litterfall (AAL) mineralized by the termites. In the DDF, 5.1% of the AAL was mineralized by termites, with dominant contribution from the fungus-growing group. In such dry tropical ecosystems, fire rather than litter-layer microbes is likely to be the most important limiting factor for the C source that can be used and mineralized by termites. On the other hand, termites contributed to the C mineralization of 4.2% of the AAL in the HEF, while the soil-feeding group played a substantial role. Comparisons of the importance of each termite group in Asian and African tropical forests indicate that the coincidence of the smaller contribution of the fungus-growers and the larger contribution of the soil-feeders in the HEF, suggesting the presence of an asymmetric C source competition between the fungus-growers and soil-feeders through the C flow from the litter layer into the soil.

Key words: fungus-growers, soil-feeders, termites, litter-layer microbes, fire, savannas, tropical forests, carbon source competitions

Introduction

Termites dominate the invertebrate communities in tropical ecosystems (Wood & Sands 1978, Eggleton *et al.* 1999, Inoue *et al.* 2001, Yamada *et al.* 2003). The role of ecosystem engineers played by the termites is reflected by their extensive consumption of organic matter and its subsequent physical and chemical modifications that facilitate its environmental microbial mineralization by producing carbon dioxide (CO_2).Termites comprise two major feeding groups—wood/litter-feeders (including fungus-growers) and soil-feeders. Wood/litter-feeders are involved in the decomposition of aboveground organic matter (i.e., fine litter as well as woody litter), while soil-feeders contribute to the decomposition of belowground organic matter (i.e., soil organic matter). In addition, since a substantial portion of the soil organic matter is derived from the aboveground organic matter, wood/litter-feeders and soil-feeders participate in the early and late processes of decomposition, respectively. This further suggests the presence of asymmetric competition for the carbon (C) source between the two feeding groups.

Respiration measurements in termites show that they are important contributors to the C mineralization of organic matter (Holt 1987, Martius 1994, Bignell *et al.* 1997, Eggleton *et al.* 1999,

Konaté *et al.* 2003, Yamada *et al.* 2005). Yamada *et al.* (2005) estimated the amount of C emitted as CO_2 by termites as well as litter-layer microbes in a tropical dry evergreen forest (DEF) in northeast Thailand and showed that almost all of the C contents in the annual aboveground litterfall (AAL) was mineralized by these termites (particularly the fungus-growers that have the high biomass of fungus combs) and litter-layer microbes. Based on these results, the authors speculated that the termites and litter-layer microbes compete with each other for the C source. At the same time, they found a negative correlation between the annual rainfall in a tropical forest and the AAL fraction that the termites mineralize (Yamada *et al.* 2005). Given the fact that the respiration rate of the litter-layer microbes largely decreases during dry season when compared with that during rainy season (Yoda & Nishioka 1982), higher rainfall under a certain level elevates the litter-layer microbial activity and will result in the C mineralization of a larger AAL fraction by the litter-layer microbes. The observed negative correlation, therefore, strongly supports the termite-microbe competition. Accordingly, a study that incorporates both termites and litter-layer microbes may provide a better understanding of the decomposition processes in tropical ecosystems.

As mentioned above, Yoda & Nishioka (1982) indicate that dry conditions reduce the litter-layer microbial activity. According to the termite-microbe competition, it is expected that a potential increase in the C source available for termites occurs in dry tropical ecosystems such as savannas. However, the AAL fractions mineralized by the termites in the African savannas (annual rainfall: 435–1297 mm, Wood & Sands 1978) appear to remain at approximately the average level of those in the Asian tropical forests that receive low rainfall (up to approximately 2000 mm; e.g., the DEFs) (Yamada *et al.* 2005). This may imply the presence of a different factor that limits the C source that can be practically used and mineralized by the termites. Although the savannas are virtually nonexistent in the Asian tropics, the dry deciduous forests (DDFs) in Southeast Asia can be considered as savanna-like ecosystems due to the frequent occurrence of forest fires

In contrast to the dominant contribution of fungus-growers to the C mineralization by termites in the savannas and Asian tropical forests, extremely abundant soil-feeders were found to dominate the C mineralization by termites in a Cameroonian forest in Africa (Yamada *et al.* 2005). This supports the validity of the above-mentioned C source competition between wood/litter-feeders (fungus-growers) and soil-feeders because the presence of such competition facilitates the prediction that a smaller contribution by the fungus-growers to C mineralization coincides with a larger contribution by the soil-feeders. However, the Cameroonian forest is the only case where the relative importance of fungus-growers is known to be comparatively low (Yamada *et al.* 2005). In addition, there may be doubts regarding the soil-feeder domination being confined to Africa.

Inoue *et al.* (2006) showed a significant decrease in the abundance of fungus-growers with increase in altitude. This indicates that in a relatively high-altitude forest, the fungus-growers may have a comparatively low biomass, thereby decreasing their contribution to C mineralization. Here, we observed the termites inhabiting a DDF and a hill evergreen forest (HEF) in Thailand and estimated the AAL fractions mineralized by them. In the HEF, the asymmetric competition between the two feeding groups further predicts the smaller contribution of the fungus-growers would coincide with the larger contribution of the soil-feeders to C mineralization. Based on a comparison of the AAL fractions mineralized by termites between the DDF and savannas and between the HEF and other tropical forests, we propose a C source competition-based perspective for explaining the global patterns of both the feeding group composition of termites and the C mineralization processes.

Study sites and methods

Observations were carried out in a DDF and an HEF in Thailand in September 2000 and August 2002, respectively. The DDF site was chosen at the Sakaerat Environmental Research Station (latitude: 14°30′N, longitude: 101°56′E, altitude: 450 m) where we had previously set the DEF site (Yamada *et al.* 2005). The DDF is an open area covered by trees of the *Hopea ferrea* species and the dominant grassy pygmy bamboo *Arundinaria pusilla*. The mean annual temperature and rainfall are 27.5°C and 1144 mm, respectively; the typical monthly rainfall is less than 40 mm during the dry season from November to March. The HEF site was located at the Kog-Ma Experimental Watershed (latitude: 18°47′N, longitude: 98°37′E, altitude: 1160 m). The forest canopy is closed by the dominant tree of the family Fagaceae. The mean annual temperature and rainfall are 20.0°C and 2084 mm, respectively, and the dry season extends from December to March.

The termites distributed in the soil, deadwood, and epigeal nests were observed for estimating the biomass and abundance, as described previously (Inoue *et al.* 2001b, Yamada *et al.* 2003). The respiration rates reported by Yamada *et al.* (2005) were used for calculating the amount of C mineralized by termites (including fungus combs). The AAL in the DDF and HEF was estimated as described previously (Yamada *et al.* 2005) based on the data from Boonyawat & Ngampongsai (1974) and T. Toda *et al.* (unpublished). For comparing the aridity between the DDF and DEF, we determined the moisture content of surface soil (0–6 cm) cores and pieces of deadwood (Yamada *et al.* 2006).

Results and discussion

Savannas and savanna-like ecosystems

The African tropics include broad expanses of savannas—open areas where grasses dominate and where seasonal droughts and frequent fires are normal ecological factors. In these areas, fungus-growers are the dominant termite group and are responsible for an important part of the C mineralization of the AAL. Previous studies have estimated that fungus-growers (including fungus combs) mineralize approximately 5%–10% of the AAL (Konaté *et al.* 2003, Yamada *et al.* 2005).

Since at least the present DDF where the termites were observed was dominated by grasses and burnt down by fires at the end of the dry season, our study is, as far as we know, novel in that it is the first to study the role of termites in C mineralization in an Asian savanna-like ecosystem. In the present DDF, the termites contributed to the C mineralization of 5.1% of the AAL, while the fungus-growers mineralized 4.5% (Fig. 1A). The total AAL fraction corresponds to the lower end of the values reported from the African savannas (Yamada *et al.* 2005). In addition, the soil-feeders made a negligible contribution to C mineralization (0.04% of the AAL, Fig. 1A), as in the case of the African savannas (up to 0.3% of the AAL, Yamada *et al.* 2005). Although the present DDF is the only Asian savanna-like ecosystem where the ecological role of termites was revealed, our findings may indicate the common characteristics of the role of termites in the C mineralization in the African savannas and Asian savanna-like ecosystems (Fig. 2A).

As mentioned above, the termite-microbe competition for the C source does not appear to be applicable to the savannas and savanna-like ecosystems. Instead of the litter-layer microbes mineralizing a major fraction of the AAL in tropical forests, fires intensively mineralize the accumulated organic matter on the ground in these ecosystems. For example, more than half of the AAL has been burned by fires in the Serengeti savanna in Africa, while a much larger AAL fraction was consumed by

"decomposers (including termites)" in a fire-protected savanna in Nigeria than that in the Serengeti savanna (Wood & Sands 1978). Therefore, fire appears to be the most important factor that limits the C source available for termites, and the intensive C mineralization by fire may reduce the C flow into the soil, resulting in a negligible C source available for soil-feeders.

The AAL fraction mineralized by soil-feeders in the DDF was much smaller than that in the DEF, while fungus-growers dominantly contributed to the C mineralization of the AAL in both the DDF and DEF (Fig. 1A). These results at least partly support the suggestion that fire and fungus-growers leave only a negligible C source for soil-feeders. Tokuchi *et al.* (2001) provided considerable insights into the possible difference in the C flow into the soil between the DDF and DEF. They examined the soil in Thai forests and showed that the soil C biomass in a DDF was approximately half of that in a DEF. On the other hand, although there is a possibility that soil-feeders are affected by soil drying (cf. Dibog *et al.* 1999), our results showed that the surface-soil moisture content was almost the same between the present DDF and DEF.

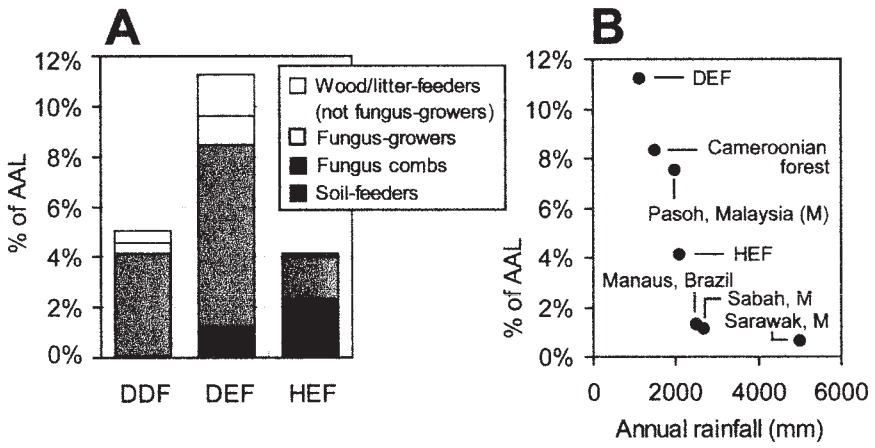

Fig.1. **(A)** Contributions of termites to the carbon mineralization of the annual aboveground litterfall (AAL) in the tropical forests of Thailand. DDF: dry deciduous forest, DEF: dry evergreen forest (data from Yamada *et al.* 2005), and HEF: hill evergreen forest. **(B)** Plot of the AAL fraction mineralized by termites against the annual rainfall in tropical forests (after Yamada *et al.* 2005). The DDF is not included in the plot, because we distinguish the DDF as a savanna-like ecosystem from tropical forests in the present study. Spearman's rank test: $n = 7$, $Rs = -1.00$, $P < 0.05$.

Forest ecosystems

Fungus-growers have been emphasized as contributors to the degradation and C mineralization of the AAL in the African savannas (Wood & Sands 1978, Bignell & Eggleton 2000, Konaté *et al.* 2003). In the Asian tropical forests, their important contributions to C mineralization have been revealed by Yamada *et al.* (2005). For example, the fungus-growers have mineralized 8.4% of the AAL in the DEF, while the total AAL fraction mineralized by termites has been 11.2%. In contrast, the results obtained from the HEF indicated that the fungus-growers were considerably less important for C mineralization and processed only a small fraction (1.8%) of the AAL (Fig. 1A). Instead, soil-feeders mineralized a relatively large fraction (2.3%) of the AAL in the HEF when compared with that in the DEF (1.2%) (Fig. 1A; Yamada *et al.* 2005). The larger fraction of the AAL mineralized by the soil-feeders in the HEF should be related to more C source available for the soil-feeders. In fact, the soil C biomass in an HEF is two times higher than that in a DEF in Thailand (Tokuchi *et al.* 2001), while AAL, which is one of the possible factors

affecting soil C biomass, is slightly lower in the HEF (452 g C m^{-2} y^{-1}) than in the DEF (520 g C m^{-2} y^{-1}, see Yamada *et al.* 2005). As discussed above, soil moisture content may affect the soil-feeder domination in the HEF, but unfortunately we do not have such data.

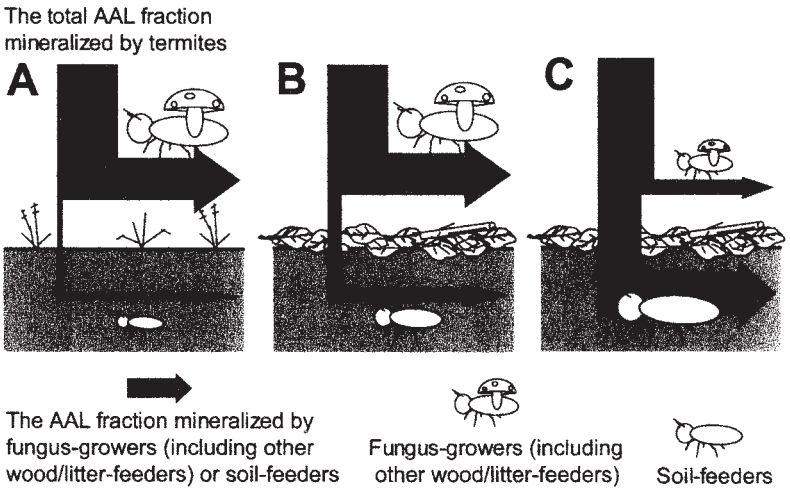

Fig. 2. Schematics of the patterns of feeding group composition of termites involved in carbon mineralization in the Asian and African tropical ecosystems under a specific temperature and rainfall. (**A**): Savannas and savanna-like ecosystems, (**B**): tropical forests with a large contribution from fungus-growers, and (**C**): tropical forests with a small contribution from fungus-growers.

Although the total AAL fractions mineralized by termites are apparently different between the HEF (4.2%) and DEF (11.2%) (Fig. 1A; Yamada *et al.* 2005), the present results answer the question posed earlier and show that soil-feeder domination is not confined to Africa. In the Cameroonian forest, a larger AAL fraction (5.2%) has been mineralized by soil-feeders than that by fungus-growers (1.7% of AAL), while the total AAL fraction mineralized by the termites has been 8.3% of the AAL (Fig. 1B; Yamada *et al.* 2005). These cases in Asia and Africa partly support the above-mentioned asymmetric competition between the fungus-growers and soil-feeders because the smaller contributions of fungus-growers to C mineralization at least coincided with the larger contributions of soil-feeders (Fig. 2B and 2C). For the difference in the AAL fraction mineralized by soil-feeders between the Cameroonian forest and HEF, there may be two possible explanations. One is simply due to less C source for the soil-feeders in the HEF by possibly higher litter-layer microbial activity. The other is due to the higher altitude in the HEF (1160 m) than that in the Cameroonian forest (650 m, Eggleton *et al.* 1996). Collins (1980) showed that the abundance of termites dramatically decreased at the altitude of more than 1000 m.

Conclusions

Combining the present results with those from our previous study (Yamada *et al.* 2005), we conclude that fungus-growers make a dominant contribution to the C mineralization of the AAL in the savannas and savanna-like ecosystems in Asia and Africa, while soil-feeders make negligible contributions. Instead of the litter-layer microbes, fire appears to be the most important factor that limits the C source available for termites, and the intensive C mineralization by fire may reduce the C flow into the soil, resulting in a negligible C source available for soil-feeders. It is suggested that in the Asian and African tropical forests, the contribution of the soil-feeders to C mineralization increases in proportion to the decrease in the contribution of the fungus-growers. Litter-layer microbes strongly affect the total C

source available for the termites, which is exposed to asymmetric competition between the two feeding groups. The present study provides novel insights into understanding the global patterns of not only the feeding group composition of termites but also the C mineralization processes.

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Identification of Microsatellite Loci in the Drywood Termite *Incisitermes minor* (Hagen)

by
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Abstract

We successfully isolated ten polymorphic microsatellite loci in the drywood termite *Incisitermes minor* (Hagen). These loci had 2-7 alleles per locus and their observed heterozygosities ranged from 0.16 to 0.83. These loci were shown to be useful for analyzing genetic structure of colony and population as well as relationships among introduced and native colonies.

Key words: microsatellite loci, drywood termite, *Incisitermes minor*

Introduction

Incisitermes minor (Hagen), native to the southwestern United States, has invaded Hawaii (Su and Scheffrahn, 2000) and Japan (Mori, 1976). In Japan, the habitats of *I. minor* have been spread out and individuals of *I. minor* were found in about twenty sites in 2004 (Indrayani et al., 2004, Ishii, 2005) (Fig. 1). Although the spread of colony is not yet found in Miyagi (Fig. 1-A) (Doi, 2006) and Okinawa (Fig. 1-D) (Yasuda et al., 2003), many colonies have been established in some areas like Kanagawa (Fig. 1-B) (Harunari and Tomioka, 2004) and Wakayama (Fig. 1-C) (Maeda, 1982). Drywood termites rely on water held inside wood and on water obtained from the metabolic breakdown of sugars in wood (Pearce, 1997). *I. minor* lives in sound dry wood without access to water, and is thus transported in wood products such as furniture and packing cases by human activity.

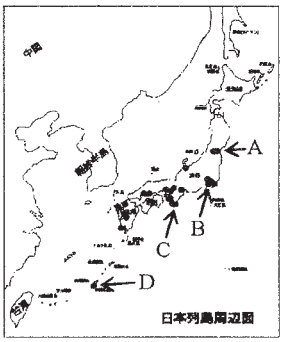

Fig.1 Distribution of *I. minor* in Japan.
● : Infested site.

Microsatellite genetic markers have been developed for several species of Isoptera, e.g. the higher termites (Termitidae) (Kaib et al., 2000, Harry et al., 2001) and, among the lower termites, mostly the Rhinotermitidae (Thompson et al., 2000, Vargo, 2000, Vargo and Henderson, 2000, Hayashi et al., 2002). For the lower termite family Kalotermitidae, six microsatellite loci were isolated for *Cryptotermes secundus* (Fuchs et al., 2003), no genetic markers were, however, available for *I. minor*. In this study, we developed polymorphic microsatellite markers to assess relationships among introduced (to Japan) and native (US) colonies (Indrayani et al., 2006).

Materials and methods

Insects: *I. minor* individuals were collected from a wild colony located in infested wood in Kozagawa Town, Wakayama Prefecture, Japan, and maintained with their nest materials at 26°C for a few months in our laboratory.

Preparation of genomic DNA from termites: Genomic DNA was extracted from the head of 20 worker-caste individuals of *I. minor* using a DNeasy tissue kit (Qiagen), according to the

manufacturer's recommended conditions. Termite head tissue was chosen to avoid contamination by residential gut microorganisms.

RAPD-PCR: Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was performed in a Gene Amp PCR system 2700 thermal cycler (Applied biosystems) with one of 24 random sequence primers (DNA Oligomer 10 set I and III, Wako, Japan). Each RAPD-PCR (100 μl) contained 2.5 U of *Ex Taq* DNA polymerase hot start version (Takara Bio, Japan), 2 mM Mg^{2+}, 0.2 mM dNTP, 1 μl of the extracted genomic DNA, and 20 pmol of one of the random sequence primers. The thermal cycler profile consisted of 40 cycles of 30 sec at 94°C, 30 sec at 30°C, and 1 min at 72°C.

Southern blot and DIG luminescent detection: Aliquots of 10 μl of PAPD-PCR products were electrophoresed on 1% agarose gels with 0.5 x TBE buffer. DNA bands on agarose gel were transferred to a positively charged nylon membrane (Roche diagnostics) by the capillary transfer method. DNA on the membrane was hybridized with digoxigenin (DIG)-labeled nucleotide probes $(GT)_{10}(GA)_{10}(AT)_{10}(GC)_{10}$-DIG (80 mer, in length) or $(AGC)_7(AGT)_7(ACG)_7(ATG)_7$-DIG (84 mer, in length) at 50°C for 2h. DIG-labeled probes combined with RAPD-PCR products on the membrane were detected using a DIG luminescent detection kit (Roche diagnostics) with a Gene Gnome 50000 bio imaging system (Syngene). Ten of 24 primers worked, producing a total of 43 positive DNA bands on the membrane. Southern blot and DIG luminescent detection were performed according to the manufacturer's protocols.

Sequencing of positive DNA: Aliquots of 90 μl of the remaining PAPD-PCR products containing the positive DNA were electrophoresed on 1% agarose gels, followed by staining with ethidium bromide. DNA bands positioned at the same R_f value of the positive bands on the nylon membrane detected with DIG-labeled probes were purified by Min Elute gel extraction kit (Qiagen) and cloned into the pGEM-T vector (Promega) with the JM 109 bacterial host according to manufacturer's recommended conditions. The constructed plasmids were extracted from the host bacterial cells using the Wizard Plus Minipreps DNA purification system (Promega) and were used as sequencing templates. The sequences were determined in both orientations with SP6 and T7 oligonucleotides as sequencing primers using a DYEnamic ET terminator cycle-sequencing kit (GE healthcare) with an ABI PRISM 3100 automated DNA sequencer (Applied biosystems).

Primer design: After elimination of overlapping sequences, 23 primer pairs were designed for amplification of the target regions using the WEB-based Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Among the designed 23 primer pairs, 10 primer pairs shown in Table 1 successfully amplified the target regions.

Characterization of microsatellite loci: Genomic DNA was extracted from each head of 11 workers per colony of *I. minor* collected from Kozagawa Town, Wakayama Prefecture and Nishinomiya City, Hyogo Prefecture, Japan and Burbanks, California, LA, USA. PCR was conducted in a reaction volume of 100 μl containing 2.5 U of *Ex Taq* DNA polymerase hot start version, 2 mM Mg^{2+}, 0.2 mM dNTP, 1 μl of the genomic DNA extracted from *I. minor*, and 50 pmol of each of the forward and reverse primers. The thermal cycler profile consisted of 30 cycles of 30 sec at 94°C, 30 sec at 64°C, and 1 min at 72°C. PCR products were run on 6% acrylamide gels (100 V, 6 h, 13 cm of gel length) and visualized with ethidium bromide. The number of alleles was manually counted. The expected and observed heterozygosities were calculated using the Genetic Data Analysis (GDA) program (http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php, version 1.1). Colony genetic structures were also investigated by estimating F-statistics using the GDA program.

Results and discussion

Thirty-three individuals from 3 different colonies were screened for variability. Of the 23 primer pairs tested, 10 showed variation among the entire population, with 2-7 alleles per locus (Table 1). There were significant deviations of Hardy-Weinberg equilibrium ($P < 0.05$, exact tests for linkage and Hardy-Weinberg disequilibrium in GDA program) in one locus (07S-20) in the samples from Hyogo, three loci (07S-20, 28T-12, and 28T18.1) in the samples from Wakayama, and five loci (07S-10, 07S-20, 28T-10, 28T-18.1, and 28T-18.2) in the samples from California. No pairwise linkage disequilibrium was shown among loci in the samples from Hyogo, Wakayama, and California, when the effects of the within-locus disequilibrium had been removed by the GDA program to preserve genotypes.

Among 10 polymorphic loci, observed heterozygosity was less than expected heterozygosity in 8, except for two loci (07S-10 and 28T-18.1). Reduced heterozygosity is expected in inbred colonies (Thorne et al., 1999). The standard inbreeding coefficient (F_{IT}) value in the present study was 0.40 (95% CI = 0.25-0.52) and thus significantly higher than zero. Although the sample is too small to draw a conclusion, the higher F_{IT} value indicates that inbreeding occurs in this population of *I. minor*. This is consistent with the result of the drywood termite *Cryptotermes secundus* (Fuchs et al., 2003).

The results of the present study suggest that these microsatellite markers provide a sensitive tool for investigating the colony and population genetic structure of both native and introduced populations of *I. minor*.

Table 1 Characterization of 10 microsatellite loci in *Incisitermes minor* (Hagen)

Locus	Sequenced repeat motif	Primer sequence (5'-3')	*Ta* (°C)	Size (bp)	N_a	H_E	H_O	GenBank No.
07S-10	$(GA)_6$	F: AAATCCAGCCAACAGGAATG R: GCTGCTTTCAACCAGACACA	64	209	3	0.59	0.83	AB253316
07S-5	$(TC)_3T(TC)_2$	F: AACCAGGTGAACCAGTCGAG R: GTCGCCTTGTTATGGAGCAAT	64	310	6	0.73	0.36	AB253317
07S-20	$(CA)_4GACA$	F: TGGGCTCCAGTTTCGTAATC R: CGATCCATGTTCAGCTTCACT	64	304	7	0.78	0.65	AB253318
07S-13	$(CA)_2TA(CA)_2$	F: CGGCACGATCTTAATACACG R: CCACCGCTGTTCATTCAGTA	64	234	3	0.51	0.16	AB253319
07S-7	$ACAG(AC)_3$	F: TCGACGGTAGGGGAATACAG R: AGCCTACTTTAGGAAAGTGGATCTC	64	341	7	0.68	0.42	AB253320
28S-10	$(CT)_6G(CT)_3$	F: GGACAGCATCAGCATGGTT R: TGTGAACCTCGGTAGTGACCT	64	231	4	0.70	0.63	AB253321
28T-10	$(CT)_5$	F: TGCGTTAAGGTCAAAATGGA R: AAAGATAGTTTGGCCCCATAGA	64	281	6	0.68	0.52	AB253322
28T-12	$(TA)_5$	F: GCATCATATCCGGGCATTAG R: ACATGGGTGACGGTTTCTGT	64	327	7	0.84	0.45	AB253323
28T-18.1	$(TG)_4TT(TG)_2$	F: ACGTCACACCTGAGACATGG R: TGTTCTTCCGACTCAGCTTG	64	338	2	0.43	0.61	AB253324
28T-18.2	$CATTAT(CAT)_{10}$ $CAC(CAT)_2$	F: TCCAAGCGACCATAAAATCAG R: AGCCGTCGTAATGTTGTATGC	64	290	5	0.45	0.29	AB253325

T_a, annealing temperature of the primer; N_a, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity, number of individuals used to screen for polymorphism = 33.

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Molecular Phylogenetics of Asian *Coptotermes* (Isoptera: Rhinotermitidae)

by
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Abstract

The taxonomy of 11 species of *Coptotermes* from Asia was examined using molecular phylogenetic analyses and morphological measurements. Partial sequence of ribosomal RNA large subunit 16S was obtained from *Coptotermes formosanus* Shiraki, *C. gestroi* (Wasmann), *C. vastator* Light, *C. curvignathus* Holmgren, *C. kalshoveni* Kemner, *C. sepangensis* Krishna, *C. cochlearus* Xia & He, *C. dimorphus* Xia & He, *C. frenchi*, *C. lacteus* (Froggatt) and *C. acinaciformis* (Froggatt). *Globitermes sulphureus* (Haviland) was used as the outgroup. Phylogenetic trees were constructed using maximum parsimony, likelihood, and distance methods. Five distinct clades were obtained. The results suggested that: (i) *C. gestroi* and *C. vastator* are synonymous, (ii) *C. formosanus*, *C. cochlearus* and *C. dimorphus* are closely related species, with the latter two species possibly synonymous. The combination of molecular and morphological approaches was found to enable accurate species identification. The genetic evidence concerning the complex problem of *Coptotermes* taxonomy are discussed.

Keywords: *Coptotermes*, molecular phylogenetics, morphology, 16-S ribsomal DNA.

Introduction

Many invasive termite species from genus *Coptotermes*, believed to be originated in the Orient, have been transported from its native range to many parts of the world. This genus contained many highly destructive pests of timber, wooden structures and agricultural crops in most subtropical and tropical countries including United States and Malaysia (Su and Scheffrahn, 2000; Lee et al., 2003). World-wide damage caused by termites account for >USD$ 20 billion annually (Su 2002) where a substantial proportion of this amount were related to *Coptotermes* spp. In Malaysia, the management efforts of subterranean termite accounted an estimated US$ 8 to 10 million where *Coptotermes* species alone had caused >90% of the total infestation (Lee, 2002). Despite its economic importance, the phylogenetic relationships of species within this genus were poorly studied. Lately, molecular approach has been widely used in taxonomy studies (Miura et al. 1998; Jenkins et al. 2000; Ohkuma et al. 2004). Molecular phylogenetic studies are able to reveal the relationship among the populations and differentiate species regardless of caste (Szalanski et al. 2003). In this study, we used 16S mitochondrial gene coupled with morphological methods to elucidate the relationships among the *Coptotermes* spp.

Materials and methods

Morphology: *Coptotermes* samples and an outgroup species (*Globitermes sulphereus*) were obtained from various sources and kept in absolute ethanol (Table 1). Preserved specimens were identified using morphometric characters. Morphometric measurements on maximum head width, width of head at the base of mandibles and length of head to the base of mandibles were measured using Olympus SZ2-LGB stereo microscope attached with an Imaging Source Camera for the populations of the 11 species.

Table 1. Summary of the collected termites which were used in this study

Sample code	Species	Collecting sites	Collector
CG001MY	*C. gestroi*	Malaysia, Penang, USM.	BK Yeap
CG004MY	*C. gestroi*	Malaysia, Kuala Lumpur, Bangsar.	KT Koay
CG001SG	*C. gestroi*	Singapore, Serenity Terrace.	SPMA
CG002SG	*C. gestroi*	Singapore, Serangoon Avenue 3.	SPMA
CG001TH	*C. gestroi*	Thailand, Bangkok1.	V Charunee
CG002TH	*C. gestroi*	Thailand, Bangkok2.	V Charunee
CG001IN	*C. gestroi*	Indonesia, Cibinong.	S Yusuf
CG002IN	*C. gestroi*	Indonesia, Bogor,	S Yusuf
CF001JP	*C. formosanus*	Japan, Wakayama.	T Yoshimura
CF002JP	*C. formosanus*	Japan, Wakayama.	T Yoshimura
CF003JP	*C. formosanus*	Japan, Okayama.	T Yoshimura
CF004JP	*C. formosanus*	Japan, Kagoshima, colony A.	T Yoshimura
CF005JP	*C. formosanus*	Japan, Kagoshima, colony B.	T Yoshimura
CF006JP	*C. formosanus*	Japan, Kagoshima, colony C.	T Yoshimura
CF007JP	*C. formosanus*	Japan, Kagoshima, colony 1.	T Yoshimura
CF008JP	*C. formosanus*	Japan, Kagoshima, colony 3.	T Yoshimura
CF009JP	*C. formosanus*	Japan, Kagoshima, colony 5.	T Yoshimura
CF010JP	*C. formosanus*	Japan, Kagoshima, colony 6.	T Yoshimura
CF001CN	*C. formosanus*	China, Guangzhou, Guangdong Entomological Institute (insectarium).	JH Zhong
CF002CN	*C. formosanus*	China, Guangzhou, Sun Yat-sen University, Pu Garden.	JH Zhong
CF003CN	*C. formosanus*	China, Guangzhou, AVON Co.	JH Zhong
CF004CN	*C. formosanus*	China, Guangzhou, Guandong Entomological Institute (tree *Bauhinia blakeana*).	JH Zhong
CF001HW	*C. formosanus*	USA, Hawaii, Oahu.	J Yates III
CV001HW	*C. vastator*	USA, Hawaii, Oahu.	J Yates III
CV001PH	*C. vastator*	Philippines, Laguna Philippines, Los Banos, colony 1.	C Garcia
CV002PH	*C. vastator*	Philippines, Laguna Philippines, Los Banos, colony 2.	C Garcia
CV003PH	*C. vastator*	Philippines, Laguna Philippines, Los Banos, colony 3.	C Garcia
CK001MY	*C. kalshoveni*	Malaysia, Penang, USM.	BK Yeap
CK002MY	*C. kalshoveni*	Malaysia, Penang, Pantai Keracut.	YP Goh
CC001MY	*C. curvinagthus*	Malaysia, Penang, USM.	BK Yeap
CC001SG	*C. curvinagthus*	Singapore, Nim Road.	SPMA
CS001MY	*C. sepangensis*	Malaysia, Perak, Bagan Datoh estate	KM Lim
CCO001CN	*C. cochlearus*	China.	JH Zhong
CD001CN	*C. dimorphus*	China.	JH Zhong
CFR001AU	*C. frenchi*	Australia, Canberra.	T Evans
CL001AU	*C. lectues*	Australia, Canberra.	T Evans
CA001AU	*C. acinaciformis*	Australia, Darwin.	T Evans
CA002AU	*C. acinaciformis*	Australia, Griffith	T Evans
Unknown		Malaysia, Penang.	CY Lee
GS001MY	*G. sulphurues*	Malaysia, Penang, USM.	BK Yeap

Other studies			GeneBank accesion
	C. gestroi	Thailand, Bangkok.	AY302709
	C. gestroi	USA, Miama, Florida.	AY558907
	C. gestroi	Turks and Caicos Islands: Grand Turk.	AY558906
	C. gestroi	Antigua and Barbuda.	AY558905
	C. vastator	Philippines, Wedgewood.	AY302713
	C. vastator	Philippines, Manila.	AY302712
	C. vastator	USA, Honolulu, Hawaii.	AY302711
	C. acinaciformis	Australia.	AY558913
	C. lacteus	Australia, Beerburrum.	AY558912
	C. curvinagthus	Malaysia.	AY558909

DNA Extraction: The preserved specimen was washed with distilled water and dried on a filter paper. Total genomic DNA was extracted from single termite using DNeasy tissue kit manufactured by QIAGEN (Valencia, CA). Extracted genomic DNA from each sample was used as polymerase chain reaction (PCR) template. Amplification of 16S mtDNA gene was conducted using LR-J-13007 (TTA CGC TGT TAT CCC TAA) and LR-N-13398 (CGC CTG TTT ATC AAA AAC AT) primers. PCR was accomplished in a MJ Research PTC-200, Peltier Thermol Cycle, with a profile consisting of a precycle denaturation at 94°C for 2 min, a postcycle extension at 72°C for 10 min, and 35 cycles of a standard three-step PCR (53.1 °C annealing). PCR products were purified using SpinClean Gel Extraction Kit (column) and subjected for direct sequencing.

Data Analysis: BioEdit v7.0.5 software was used to edit individual electropherograms and to form contigs. Multiple consensus sequences were aligned using CLUSTAL X. The alignment results were adjusted manually for obvious alignment errors. The data were imported into PAUP4.0 (Swofford 2001)

and analyzed to generate maximum likelihood and parsimony bootstrapped trees. Using the heuristic search option, 1000 replicates were performed and 50% majority rule consensus trees were generated. A bootstrap test was used to test the reliability of trees (Felsenstein 1985). Gaps were treated as missing data.

Results and discussion

Morphology: The soldier termites were used for morphological studies. Morphological differences were not visibly distinguished among the 11 species. Identification can only be based on non-robust morphological characters. Soldiers of *C. curvinagthus* were readily distinguished from the other taxa due to its large size and strongly incurved mandibles. *C. formosanus* was readily distinguished from *C. vastator* and *C. gestroi* with two pairs of setae projecting dorse-laterally from the base of the fontanelle, compared with only a pair of setae in the latter two species. However, the measurements on the maximum width of head, width of head at the base of mandibles, and length of head at the base of mandibles among the *Coptotermes* species were overlapping. As reported in Kirton and Brown (2003), there was a continuous variation in size and shape of a single species. The morphology of termites can be influenced by the age and state of the colony, the environment of the habitat and storage condition (Scheffrahn *et al.* 2005).

Nucleotide analyses: Average amplicon size of 16S gene resulting from DNA sequencing was approximately 428 basepairs (bp). The average nucleotide compositions among *Coptotermes* species gene for A, C, G and T are 42.82%, 24.61%, 10.57%, and 22.00%, respectively. The multiple sequences alignment for 16S gene, including the outgroup taxon resulted in a data matrix with 379 characters, of which 206 are constant and 52 parsimony-informative. Pairwise Tajima-Nei distances (Tajima and Nei, 1984) within *Coptotermes* spp. ranged from 0.57 % between *C. gestroi* and *C. vastator* to 13.6% between *C. formosanus* and *C. acinaciformis*.

Phylogenetic relationships: Only the tree from maximum parsimony analysis is shown here (Fig.1). The parsimony analysis using the heuristic search algorithm of the aligned sequences yielded a single maximally parsimonious tree with 219 tree length (CI = 0.932 and RI = 0.939). The tree topology of *Coptotermes* taxa was congruent with the Maximum Likelihood analysis (-In L 1566.57300). Bootstrap analyses of the aligned *Coptotermes* species and the outgroup taxon resulted in consensus trees with 5 distinct branches (Figure 1).

The clade I consisted of all the Australian *Coptotermes* including those sequences obtained from GenBank (*C. acinaciformis*, *C. frenchi*, and *C. lacteus*). They seem to be the endemic species to Australia. The clade II was composed of *C. curvinagthus* and an unknown species. The result suggests that the unknown species is closely related to *C. curvinagthus* although morphologically, it is very much smaller in size. There is noteworthy that *C. gestroi* and *C. vastator* formed a distinct clade III with numerous overlaps based on morphological characters (Kirton 2005). Tajima-Nei distance between *C. gestroi* and *C. vastator* (0.57 %) was fallen within intraspecific variation range. Therefore, we suggest that *C. gestroi* and *C. vastator* are synonymous. The more comprehensive results were published in Yeap *et al.* (2007). *C. sepangensis* branched out as a sister group to *C. kalshoveni*, forming the clade IV. Interestingly, the clade V was composed of *C. formosanus*, *C. cochlearus* and *C. dimorphus*. A similar Tajima-Nei distance as *C. gestroi/vastator* (0.57 %) was obtained for *C. cochlearus/dimorphus*, suggesting that both species are conspecific. These two species also found to be very close related to *C. formosanus*. The strong bootstrap support of this clade suggests that *C. cochlearus* and *C. dimorphus* may be evolved from *C. formosanus* or even conspecific. More studies are warranted to further substantiate this finding.

In summary, morphological taxonomy integrated with phylogenetic analysis could provide an accurate identification. Besides, the relationships among *Coptotermes* species could be revealed and the complex problem of *Coptotermes* taxonomy could be solved. The accurate identification and knowledge of *Coptotermes* phylogenetics is important for improvement of termite management strategies.

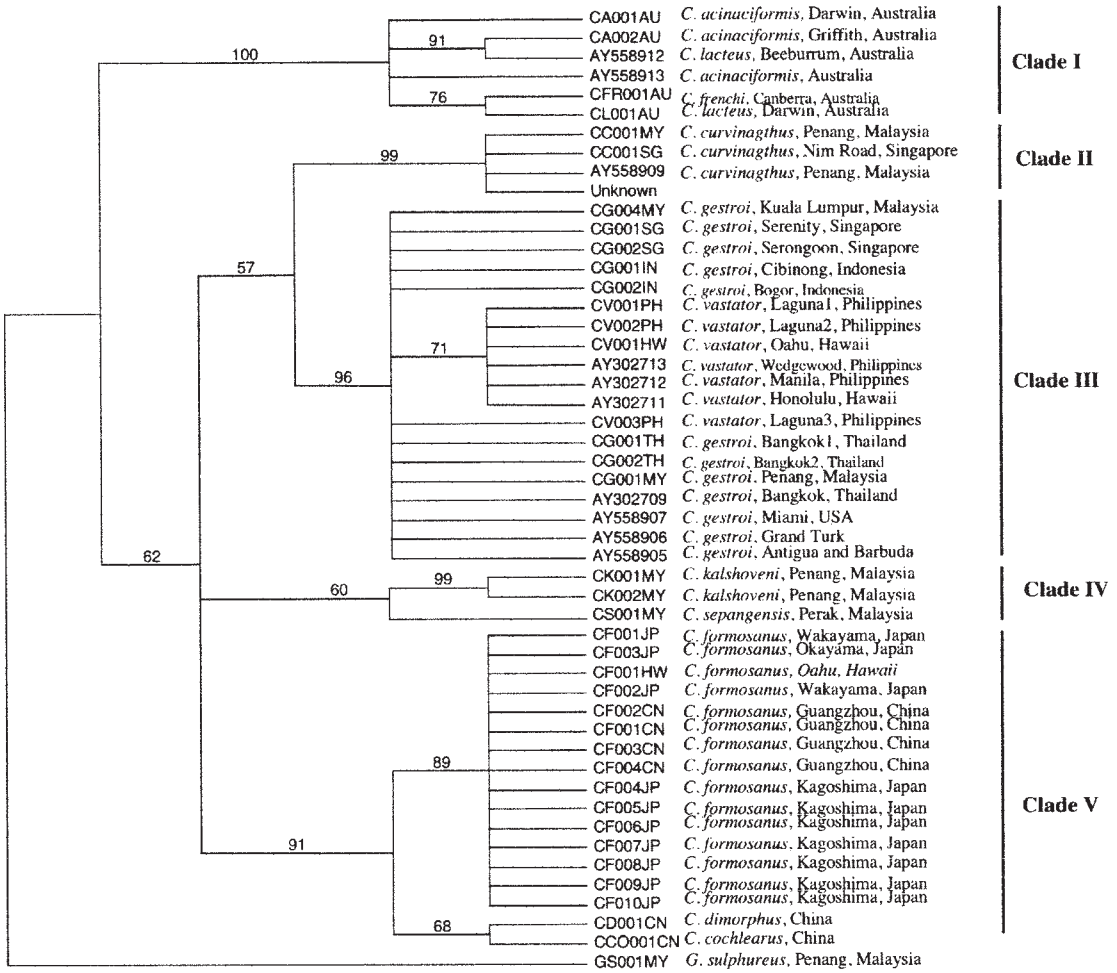

Fig. 1. A single most parsimonious tree obtained for 16S gene using a heuristic search option in PAUP4.0b10 (Swofford 2000). Bootstrap values for 1000 replicates are listed above the branches supported at ≥50%. GenBank accession numbers represent the samples that are pooled from the NCBI database.

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Efficiency Durability of Polymer-Composition from Vetiver Grass and Variable Ratio of Plasticizer and Filler to the Attack of Subterranean Termites

by
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Abstract

Study on the durability of polymer-composite board made from vetiver grass mixed with plasticizer and filler at different ratio to the attack of subterranean termites were conducted in laboratory against Thailand most economically subterranean termite *Coptotermes gestroi* Wasmann by no choice test method and field test by choice test method. Result revealed that products from polymer composites of all treatments were highly durable to termite attack both in laboratory and field test.

Keywords: subterranean termites, vetiver grass, polymer-composite, plasticizer, filler

Introduction

The study on the durability of polymer-composites from vetiver grass to the attack of subterranean termites reported here was part of the project on "The Inter-relationship between Vetiver Grass and Termites to Response to Royal Initiation of His Majesty King Bhumibol Aduldej" which was supported by National Research Council of Thailand.

Vetiver grass, widely known internationally as His Majesty the King's outstanding plant which help solving problems of soil conditions, is extensively cultivated throughout Thailand mainly to prevent soil erosion and to enhance land productivity. Similar to other cereal plants, straws of vetiver grass which compose of highly lignocelluloses have been used as materials for composites board generally called Biological Composites (Bio-Composites).

Biological composites or lignocelluloses composites are generally grouped into three categories:

1. Conventional panel made of lignocelluloses mix with resin.
2. Polymer composite board or reinforcing thermoplastic board made of biological fiber with thermoplastic as reinforcing material.
3. Inorganic bond composite board, which is the product of inorganic binder and biological fiber.

The composites used in the experiment were produced using vetiver grass as lignocellulosic source in the process of conventional panel and reinforcing thermoplastic panel.

Termites are known to be the most important pest causing damage to wooden construction and other products including bio-composites especially in tropical and subtropical region of the world. In spite of the economical importance, damage of construction by termites has been seldom investigated in Thailand.

Materials and methods

Termites

Coptotermes gestroi, the most important building-attacking species in urban area in Thailand. C. gestroi were collected from infested houses in Bangkok, Thailand, for used in laboratory test.

Polymer composites

Vetiver-plastic composites obtained from laboratory at the king Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand, prepared by using of vetiver leaf which had

been grinded into vetiver-leaf dust blended with polymer-composition such as Polypropylene (PP), Polyethylene (PE), Poly-vinyl chloride (PVC) by using the high-speed mixer, melt-blended in a single-screw extruder, and final panel formed by injection molding technique. The polymer composites used in this experiment contained plasticizer such as Diocthyl Phthalate (DOP) and other additives filler such as Talcum, $CaCO_3$ mixed with vetiver fiber.

Test Methods

Experimental Design

In both laboratory and field experiments, each treatment comprised of four replications arranged in randomized complete block design.

Laboratory

Plastic boxes 8x11x5 cm^3 were used to test in laboratory test. As shown in the figure, sandy soil (20 mesh) was use to provide test termites with easy access to the test particles. Polymer composites (2.5x2.5 cm^2, pre-weighed) were placed in the middle of plastic box and covered with sand. For the untreated control used rubber wood of the same size as the composites.

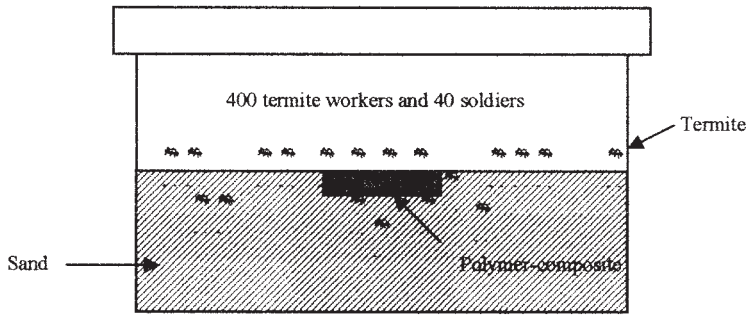

Fig. 1 An assembled test unit on durability of polymer-composite to termites attack (lab test).

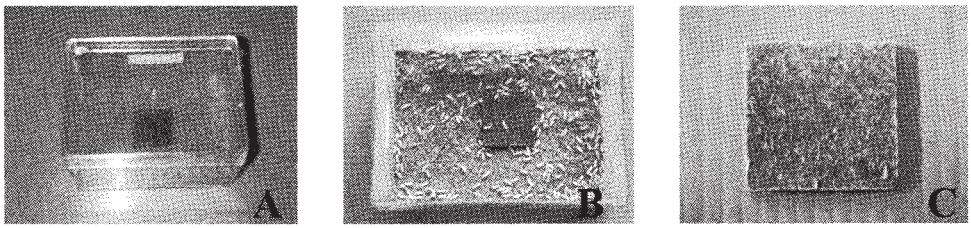

Fig. 2 Choice test in plastic box (a-b) polymer-composites (c)

After four weeks, Polymer composite were taken out from the plastic boxes, cleaned, and oven-dried, and re-weights to determine percentage weight loss from the equation:

$$\text{Weight loss (\%)} = (W_1 - W_2) / W_1 \times 100$$

Where, W_1 = weight of polymer composite before exposure to termite
W_2 = weight of polymer composite after exposure to termite

Durability of samples then subjected to five level of durability classification according to the rate as shown below.

Percent of damage	Visible attack on wood	Classified durability
0	None	Very durable (VD)
1-10	Hardly visible damage	Durable (D)
11-35	Superficial and slightly damage	Moderately durable (MD)
36-80	Moderately damage	Non durable (ND)
81-100	Heavy damage	Perishable (P)

Field

Polymer-composites size for field experiment was 5x10 cm^2; Samples were put into concrete circular tube (diameter 80 cm). On top of cement block which placed upright on ground in the center of the circular tube as shown in the figure. Control sample were rubber wood of the same size.

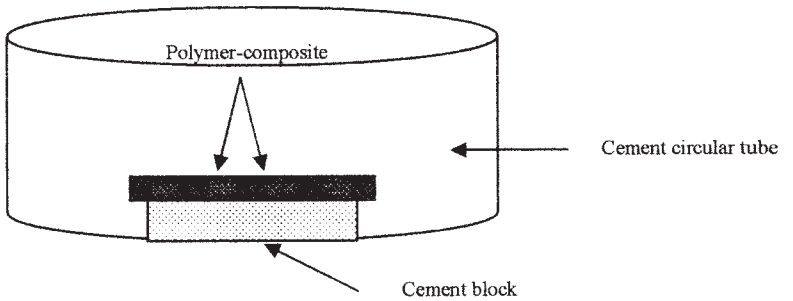

Fig. 3 An assembled test unit on durability of polymer-composite to termites attack (field test).

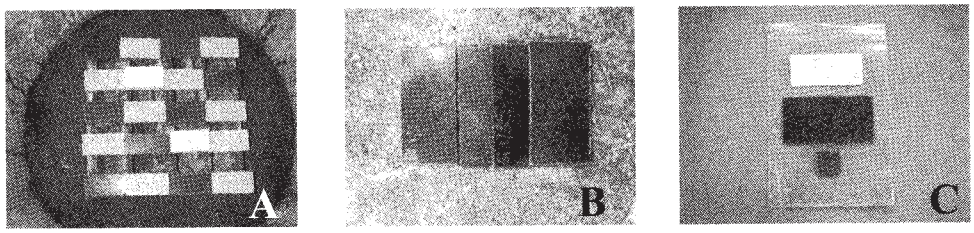

Fig. 4 No Choice test in cement circular tube (a) polymer-composites (b-c)

Polymer composites were rated using visual inspection on percentage of damage caused to samples by termites at six month after installation. Classification of durability was the same as for the one in laboratory tested.

Result and Discussion

Table 1-1 shows average percent loss weight of polymer composite by the attack of termites in laboratory tested after four weeks and average percentage of visual rating damage of polymer composites in field tested after six month, comparing between polymer-matrix (PP, PE and PVC).

Table 1-1 Average percentage of damage by termites in both of laboratory and field tests comparing between polymer matrixes.

Factor	Laboratory	Field
Type of matrix	average percent of loss weight of composites	average percent of visual rating damage
PP (Poly propylene)	1.21	4.31

Table 1-1 Average percentage of damage by termites in both of laboratory and field tests comparing between polymer matrixes. (cont.)

Factor	Laboratory	Field
Type of matrix	average percent of loss weight of composites	average percent of visual rating damage
PE (Poly ethylene)	1.20	3.59
PVC (Poly vinyl chloride)	1.63	1.54
Control (Rubber wood)	36.23	100.00

Table 1-2 shows average percent loss weight of polymer composite by the attack of termites in laboratory test and visual rating damage of polymer composites in field test, comparing between loadings of plasticizer, diocthyl phthalate (DOP), on mechanical properties of natural fibers-poly vinyl chloride composites.

Table 1-2 Average percentage of damage by termites in both of laboratory and field tests comparing between loading of plasticizer.

Factor		Laboratory	Field
Type of matrix	Plasticizer content (phr)	average percent of loss weight of composites	average percent of visual rating damage
PVC (Poly vinyl chloride) + natural fibers of vetiver grass	0.0	0.00	0.00
	5.0	0.35	2.00
	7.5	0.00	1.50
	10.0	0.12	2.50
	20.0	0.75	3.75
Control (Rubber wood)		42.88	100.00

Table 1-3 shows average percent loss weight of polymer composite by the attack of termites in laboratory test and visual rating damage of polymer composites in field test, comparing between comparing of fillers (Talcum and $CaCO_3$) on mechanical properties of natural fibers-poly vinyl chloride composites.

Table 1-3 Average percentage of damage by termites in both of laboratory and field tests comparing between loadings of plasticizer.

Factor		Laboratory	Field
Type of matrix	Filler	average percent of loss weight of composites	average percent of visual rating damage
PVC (Poly vinyl chloride) + natural fibers of vetiver grass	Talcum	1.94	2.19
	$CaCO_3$	0.78	0.90
Control (Rubber wood)		42.88	100.00

The results of the study on the durability of polymer-composites from vetiver grass to the attack of subterranean termites conducted in laboratory for 4 weeks against Thailand most economically subterranean termite *Coptotermes gestroi* Wasmann by no choice test method and more than 6 month field test by choice test method revealed that products from polymer composites which made of polymer matrix (PP, PE, PVC) mixed with vetiver grass and also product of vetiver grass fiber mixed with plasticizer (DOP contents) and filler (Talcum, $CaCO_3$) were highly resistant to termite attack both in laboratory and in field condition.

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