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Symbiotic Fungi Associated with Ambrosia Beetles

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Abstract
This paper describes the species composition and the relative dominance of the associated fungi in the gallery system and in the mycangium of three ambrosia beetles (Scolytophathpus mikado Blandford, *Xylosandrus crassiusculus* (Motschulsky), and X. brevis (Eichhoff)) by analyzing the changes with the developmental stages of the beetle. Both isolation experiments and scanning electron microscopy were employed. The isolated fungi were classified into three groups: *Ambrosiella* spp., yeast-like fungi, and other fungi (mainly *Paecilomyces sp.*). *Ambrosiella* spp. were highly species-specific among scolytid species; Ambrosiella sp. 1, sp. 2, and sp. 3 were isolated from Scolytoplatypus mikado, *Xylosandrus crassiusculus*, and X. brevis, respectively. *Ambrosiella* spp. were generally predominant among the fungal groups in the egg/larval periods in the galleries and mycangia of each beetle species. Yeast-like fungi were constantly isolated from the galleries. These results suggested that the symbiotic fungi associated with the three ambrosia beetles were species-specific *Ambrosiella* spp., and associated yeast-like fungi were likely to be auxiliary ambrosia fungi.

Discipline: Insect pest
Additional key words: *Scolytoplatypus mikado* Blandford, *Xylosandrus crassiusculus* (Motschulsky), *X. brevis* (Eichhoff), mycangia, gallery

Introduction

It is generally recognized that ambrosia beetles (Scolytidae and Platypodidae) are forest insects that have mutualistic associations with specific fungal species. They also are considered to be forest pests that often severely impair timber production because their galleries penetrate deeply into the sapwood and fungi produce black staining in the wood tissues surrounding their galleries.

In contrast to most bark beetles (Scolytidae), ambrosia beetles cannot utilize phloem and sapwood as primary nutritive substances to complete their development. Instead, both adults and larvae of the beetles feed on fungi. The beetles transport the ectosymbiotic "ambrosia" fungi in highly specialized saclike organs termed mycangia (mycetangia)[2,6] and inoculate the fungal spores from the mycangia into the walls of galleries excavated in the wood. These fungal spores germinate to produce abundant mycelia and new spores in the galleries[7,8].

Early studies indicated that the symbiotic beetle-fungus association was highly species-specific[2]. Many subsequent studies, however, have suggested that in addition to the single associated species, other yeast-like and filamentous fungi, yeasts, and even bacteria present in the beetles and their galleries, are partially involved in the symbiotic association, although the proportional distribution of the microbial components may vary with the developmental stages of the beetle[1,3,8].

In this paper, I clarify the species composition and the relative dominance of the associated fungi in the gallery system and in the mycangium of three ambrosia beetles (*Scolytoplatypus mikado* Blandford, *Xylosandrus crassiusculus* (Motschulsky), and *X. brevis* (Eichhoff) by describing changes with the developmental stages of the beetle. Both isolation experiments and SEM (scanning electron microscopy) were employed.

Materials and methods

1) Study area and field collections

Field collections were performed in a mixed stand of deciduous trees and shrubs in the Experimental Forest of Nagoya University, Aichi Prefecture, in central Japan (980 m a.s.l.; 35°11′N, 137°33′E).

The beetles and their galleries at various developmental stages were obtained from the sampled trees (2–7 cm in diameter) of *Parabenzoin trilobum* Nakai (Lauraceae). Five living trees were felled almost every week during April–August in 1989 and 1990, and laid on the forest floor, being thus exposed to attack by beetles. After colonization and at regular intervals, five to ten of the log samples (about 1.5 m in length) were brought to the laboratory for dissection and microbial isolation. Some sampled logs were stored in an outdoor cage. During the dispersal flight, the new adult beetles were collected in the cage for isolation of mycangial microbes.

2) Isolation of microorganisms from galleries

Each sampled log was cut into 3–5 cm long sections, each of which contained one entrance hole. The surface of each section was rinsed with 99% ethanol and then heated over an open flame. The process was repeated three times. The surface-disinfected wood sections were split aseptically to expose the brood tunnels, from which eggs, young/mature larvae, pupae, and/or callow/sclerotized adults of the beetle were removed.

Microbes were isolated from the wall of larval cradles of *S. mikado* and from the wall of cave-tunnels of *X. crassiusculus* (overwintering generation), and from the wall of vertical tunnels of *X. brevis*, in which the new brood of each species developed (Fig. 1). Several pieces of galleries were sampled to include various growing stages of the beetle. Fungal samples were taken directly from the surfaces of the walls using sterile needles and scalpels and transferred to YM and CM agar plates (Table 1). A small amount of 100 ppm streptomycin sulphate was added to inhibit bacterial contamination. The cultures were incubated at 25°C for 3–4 weeks until colonies were formed, and then purified by further replating.

3) Isolation of microorganisms from the mycangium of the beetles

Adult females of each species collected in the cage and from galleries were subjected to the following procedures[5]: 1) for sterilization of the body surface, the adult females were allowed to crawl on sterile, moist filter-paper in a petri dish for 2 h; 2) they were transferred to another moist petri dish for 12 h and subsequently were incubated on a dry petri dish for 12 h, this alternating cycle of incubation was repeated three times; 3) the sterile beetles were dissected aseptically in potato-dextrose agar (PDA) under a stereo-microscope; 4) the mycangia (located under the female pronotum in *S. mikado*; extending below the female mesonotum in *X. crassiusculus* and *X. brevis*) were separated from other body sections and placed on YM and CM agar plates. The cultures were incubated under the same conditions as those employed in the isolation from galleries.

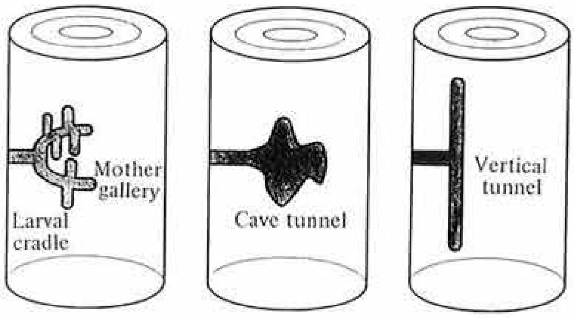

Fig. 1. Gallery system

Table 1. Composition of media

Substances	YM agar (g)	CM agar (g)
Glucose	10	20
Peptone	5	10
Yeast extract	3	5
Malt extract	3	–
$MgSO_4 \cdot 7H_2O$	–	2
K_2HPO_4	–	5
Agar	20	20
Distilled water	1,000 ml	1,000 ml

4) Scanning electron microscopy

A few wood-blocks (eq. 5 × 5 × 3 mm), each containing a piece of the gallery wall, were sampled aseptically at each developmental stage of the beetles. The gallery samples were fixed with 2% OsO_4 vapor in an airtight bottle. After 24 h the OsO_4 gas was removed by a suction pump during a period of 24 h, and the samples were subjected to desiccation for 3 days. Samples were then coated with carbon and gold. Observations were made with a SEM (Type JSM–T20, JEOL Inc.).

Results

1) Fungal flora associated with S. mikado

(1) Galleries

Isolation experiments: Species composition of the associated fungi in the larval cradles in relation to

Table 2. Fungi isolated from larval cradles of *S. mikado*

Stages of the beetles	Eggs	Young larvae	Mature larvae	Pupae	New adults	Overwintering	Dispersal
Number tested (A)	23	37	31	20	20	17	26
Number of isolates (B)	18	46	39	24	20	21	26
(B/A)	0.78	1.24	1.26	1.20	1.00	1.24	1.00
Ambrosiella sp.1	11(61%)	27(59%)	23(59%)	13(54%)	0	0	0
Yeast-like fungi	6(33%)	13(28%)	10(26%)	10(42%)	0	5(24%)	0
Paecilomyces sp.	1(6%)	5(11%)	3(8%)	1(4%)	20(100%)	14(67%)	26(100%)
Ceratocystis sp.	0	0	2(5%)	0	0	0	0
Unidentified sp.	0	1(2%)	1(3%)	0	0	2(10%)	0

Table 3. Fungi isolated from mycangia of *S. mikado*

Stages of the beetles	New adults		Overwintering	Dispersal	Boring
	Callow	Sclerotized			
Number tested (A)	9	28	39	20	31
Number of isolates (B)	5	28	49	21	28
(B/A)	0.56	1.00	1.26	1.05	0.90
Ambrosiella sp.1	2(40%)	0	1(2%)	14(67%)	12(43%)
Yeast-like fungi	2(40%)	2(7%)	18(37%)	2(10%)	6(21%)
Paecilomyces sp.	1(20%)	23(82%)	30(61%)	3(14%)	3(11%)
Ceratocystis sp.	0	2(7%)	0	2(10%)	2(7%)
Unidentified sp.1	0	1(4%)	0	0	2(7%)
Unidentified sp.2	0	0	0	0	2(7%)
Unidentified sp.3	0	0	0	0	1(4%)

the growing stages of the beetle is summarized in Table 2. The fungal species associated with *S. mikado* consisted mainly of *Ambrosiella* sp.1, yeast-like fungi (*Candida* spp., *Pichia* sp.), and *Paecilomyces* sp., but the degree of domination among them differed according to the growing stages of the beetles. From most of the samples tested, one or more fungal species were isolated, although fungi were less common in eggs than in the other stages. During the period when new broods were growing (between the egg and pupal stages), *Ambrosiella* sp.1, one of the most specific ambrosia fungi (Batra, 1967), occurred in about 60% of the isolations, followed by a yeast-like fungus which was found in 25–40% of the samples. However, during the period of occurrence of callow adults, overwintering adults, and dispersal flight adults *Ambrosiella* sp.1 was not isolated from the evacuated cradles. In contrast, a fungus imperfectus, *Paecilomyces* sp. predominated during these periods (Table 2).

SEM microscopy: During the egg stages, few fungal hyphae and spores were observed around the egg niche in which each egg had been deposited (Plate 1). After egg hatching, ambrosia fungi gradually extended their hyphae over the walls of the cradle and formed a thicker mycelial mass layer. During

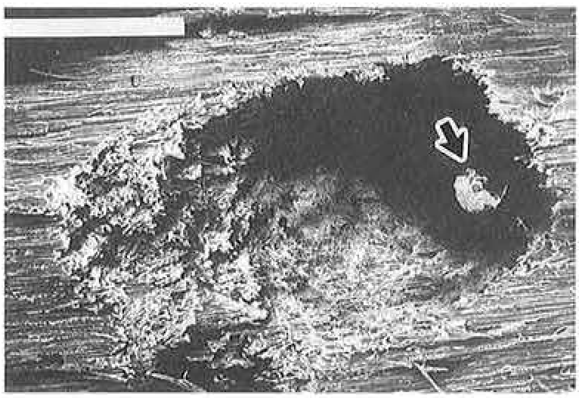

Plate 1. Egg niche
Arrow shows a desiccated egg.
Bar = 1 mm.

the early larval stages, a large number of ambrosia cells were found growing in the cradles, forming monilioid chains, a distinctive spore form characteristic of *Ambrosiella* sp.1 (Plate 2). During the period corresponding to larval maturation and pupal stage, the entrances and front portions of the larval cradles were filled with the ambrosia fungi, the cells of which formed continuous palisades. Neither mycelia nor ambrosial cells, however, could be observed at the bottom of the cradles where wood tissue was most exposed (Plate 3–a, b). As newly-emerging callow adults underwent a sclerotization process, the ambrosial cells and monilioid chains that had produced globose or oval-shaped spores, gradually became deformed or were replaced by mycelia (Plate 4) of a yeast-like fungus. When the adults were fully sclerotized, most of the ambrosial cells and monilioid chains on the walls of the cradles disappeared, and the intertwined mycelial layers became thinner, exposing wood tissue. The conidiophores and scattered conidia of *Paecilomyces* sp. were found alternatively on the walls (Plate 5). The conditions of the cradle walls during the periods of overwintering and dispersal were similar to those during the adult emergence (new adult) period.

(2) Mycangia

The frequency of fungi isolated from the mycangia of females during the callow adult period was much lower than in the other stages of the beetles (Table 3). *Ambrosiella* sp.1 was most frequently isolated during the period of both dispersal and boring, accounting for about 50% of the total fungal strains isolated. *Candida* sp. and *Paecilomyces* sp., on the other hand, occurred mostly in sclerotized or over-

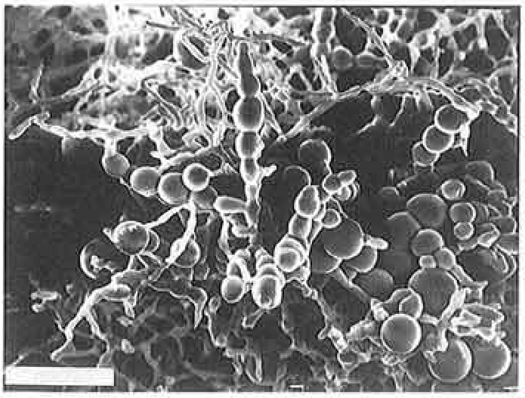

Plate 2. Monilioid chains of *Ambrosiella* sp.1 Bar = 50 μm.

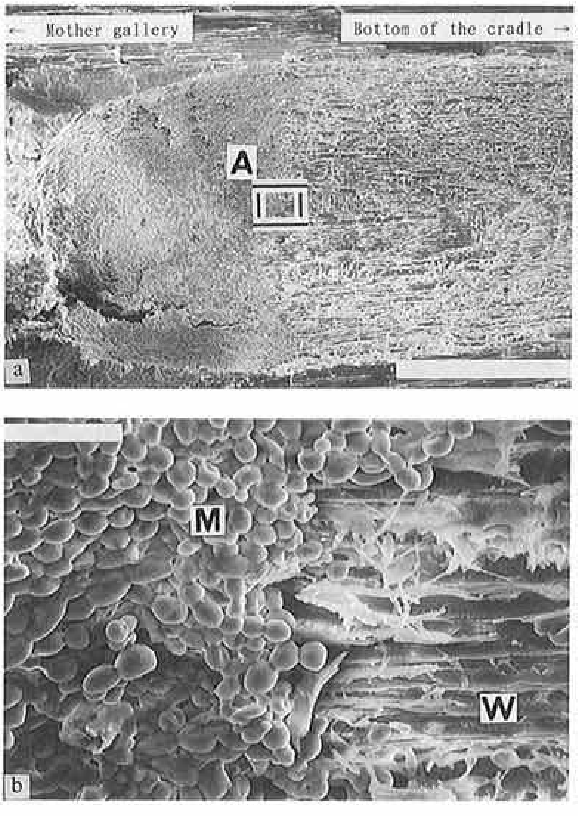

Plate 3. Larval cradle in the pupal period
a: Longitudinal section of the cradle (Bar = 1 mm),
b: Magnified detail of the area A in Plate 3–a (Bar = 50 μm).
M: Moniloid chains of *Ambrosiella* sp.1,
W: Exposed wood tissue.

Plate 4. Deformed ambrosial cells and mycelia on the walls of a cradle in the callow-adult period
Bar = 100 μm.

Plate 5. Conidiophores and scattered conidia of *Paecilomyces* sp. on the walls of a cradle in the sclerotized adult period
Bar = 50 μm.

wintering adults. The latter fungus species was the most common of all the fungal species.

2) Fungal flora associated with X. crassiusculus

(1) Galleries

Isolation experiments and SEM microscopy: Table 4 shows the species composition and relative dominance of the associated fungi in the cave-tunnels of *X. crassiusculus* during the overwintering generation. The fungal species associated with *X. crassiusculus* were mostly represented by the three fungal groups, *Ambrosiella* sp.2, yeast-like fungi (*Candida* spp., *Pichia* spp., *Saccharomycopsis* sp.), and the other fungi. *Ambrosiella* sp.2 showed similar morphological features to those of *Ambrosiella* sp.1 but they differed in the smell, growth rate, and color of colony. The degree of domination among these groups differed according to the growing stages of the beetles. Fungi were isolated at a lower frequency in the egg period than in the other periods. The dynamics of fungal flora in the galleries of *X. crassiusculus* showed a trend almost similar to that of *S. mikado*.

The results of SEM microscopy observations supported those of isolation experiments. Namely, the fungal spores and hyphae of *Ambrosiella* sp.2 were most frequently observed during the periods of both boring and eggs (Plate 6) but yeast-like fungi and other fungi were predominantly observed after overwintering periods.

(2) Mycangia

The dynamics of the fungal symbionts in the mycangia was very different from that of the symbionts in the galleries (Table 5). The mycangia of the female adults had a simple fungal flora, constantly accounting for 50–100% of the total number of isolates. Only a single fungal species *Ambrosiella* sp.2, was consistently stored in the mycangia in all the adult stages, except for the callow adults period during which yeast-like fungi predominated over *Ambrosiella* sp.2.

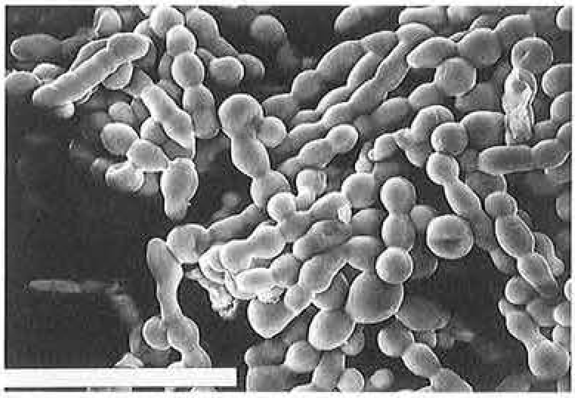

Plate 6. Monilioid chains of *Ambrosiella* sp.2 Bar = 50 μm.

Table 4. Fungi isolated from galleries of *X. crassiusculus*

Stages of the beetles	Boring	Eggs	Larvae	Overwintering (F)	Overwintering (L)	Pupae
Number tested (A)	36	24	42	27	54	27
Number of isolates (B)	36	29	84	54	101	48
(B/A)	1.00	1.21	2.00	2.00	1.87	1.78
Ambrosiella sp.2	23(64%)	23(79%)	13(15%)	0	0	0
Yeast-like fungi	13(36%)	4(14%)	33(39%)	27(50%)	48(48%)	27(56%)
Paecilomyces sp.	0	0	34(40%)	27(50%)	47(47%)	10(21%)
Ceratocystis sp.	0	2(7%)	4(5%)	0	0	0
Acremonium sp.	0	0	0	0	4(4%)	7(15%)
Verticillium sp.	0	0	0	0	2(2%)	0(8%)

Table 5. Fungi isolated from mycangia of *X. crassiusculus*

Stages of the beetles	New adults		Dispersal	Boring
	Callow	Sclerotized		
Number tested (A)	24	30	23	10
Number of isolates (B)	12	28	17	10
(B/A)	0.50	0.93	0.74	1.00
Ambrosiella sp.2	2(17%)	28(100%)	17(100%)	9(90%)
Yeast-like fungi	10(83%)	0	0	0
Unidentified sp.	0	0	0	1(10%)

Table 6. Fungi isolated from galleries of *X. brevis*

Stages of the beetles	Eggs	Larvae	New adults		Overwintering
			Callow	Sclerotized	
Number tested (A)	36	46	12	42	27
Number of isolates (B)	57	78	14	75	27
(B/A)	1.58	1.70	1.17	1.79	1.00
Ambrosiella sp.3	34(60%)	40(51%)	3(21%)	0	0
Yeast-like fungi	23(40%)	23(29%)	11(79%)	29(39%)	0
Paecilomyces sp.	0	12(15%)	0	42(56%)	27(100%)
Ceratocystis sp.	0	0	0	4(5%)	0
Penicillium sp.	0	3(4%)	0	0	0
Unidentified sp.	0	0	0	0	0

Table 7. Fungi isolated from mycangia of *X. brevis*

Stages of the beetles	New adults		Overwintering	Boring
	Callow	Sclerotized		
Number tested (A)	24	24	27	14
Number of isolates (B)	26	24	19	14
(B/A)	1.08	1.00	0.70	1.00
Ambrosiella sp.3	23(89%)	24(100%)	11(58%)	12(86%)
Yeast-like fungi	2(8%)	0	0	1(7%)
Paecilomyces sp.	1(4%)	0	0	1(7%)
Penicillium sp.	0	0	6(32%)	0
Unidentified sp.	0	0	2(11%)	0

3) Fungal flora associated with X. brevis

(1) Galleries

Isolation experiments and SEM microscopy: The fungal species associated with *X. brevis* were mostly represented by the same three fungal groups associated with the other two species (Table 6), although *Ambrosiella* sp.3 exhibited a different smell and color than the colonies of *Ambrosiella* sp.1 and sp.2. Also the dynamics of the fungal flora in the galleries of *X. brevis* showed a trend similar to that of *S. mikado* and *X. crassiusculus*, except that the frequency of fungal isolation in the egg period was higher.

The results of SEM microscopy observation showed that the characteristic fungal spores and hyphae of *Ambrosiella* sp.3 were frequently observed during the periods corresponding to both egg and larval stages (Plate 7). The conidiophores and scattered conidia of *Paecilomyces* sp. were found alternatively on the gallery walls in overwintering periods.

(2) Mycangia

The dynamics of the fungal flora in the mycangia of *X. brevis* was largely different from that of the flora in the galleries, and showed a trend similar to that of *X. crassiusculus* (Table 7). Only a single fungal species, *Ambrosiella* sp.3, was consistently stored in the mycangia in all the adult stages.

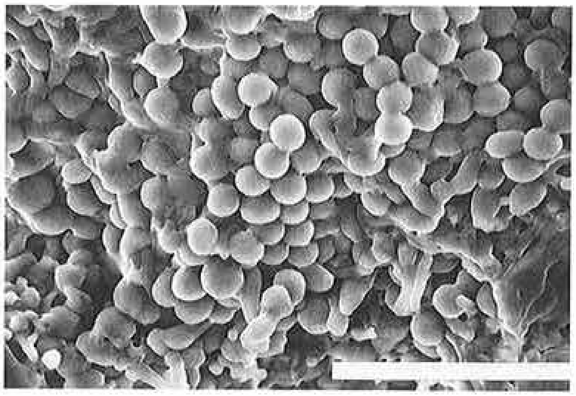

Plate 7. Monilioid chains of *Ambrosiella* sp.3 Bar = 50 μm.

Discussion

Since ambrosia beetles ingest most of their nutrients during the larval stage, the relative dominance and state of the associated fungal species in the galleries during the larval growth period are likely to be critical for the reproductive processes of the beetles[3,4]. The results of the isolation from galleries of three ambrosia beetle species showed that fungi of the genus *Ambrosiella* were predominant during the larval stages and a single species of *Ambrosiella* was isolated from each ambrosia beetle species. Direct observations by SEM also demonstrated that *Ambrosiella* sp.1 actively forms its conidiophores and conidia around the inner walls of the larval cradles near the mother galleries, where the larvae of *S. mikado* intensively graze on the fungal layers. During the beetle dispersal and boring periods, spores of *Ambrosiella* spp. exclusively occupied the mycangia. These results indicate that individual species of *Ambrosiella* sp. are the major symbiotic fungi associated with each species of ambrosia beetles in the beetle-fungus symbiosis.

Yeast-like fungi (*Candida* spp., *Pichia* spp., etc.), on the other hand, were less abundant but more constantly isolated from the galleries than were *Ambrosiella* spp. while the egg and larvae were growing. *Candida* sp. or another yeast have been classified as auxiliary associated fungi which are likely to be fed on secondarily by ambrosia beetles[3]. Yeast-like fungi are likely to be another key component of the microbial complex. However, their functional or nutritional role in the complex could not be clarified in the present study.

The other fungi, mainly *Paecilomyces* sp. were dominant in the galleries when the beetles completed their growing stages. Although the genus *Paecilomyces* contains a large number of entomopathogenic species, it remains to be determined whether the *Paecilomyces* sp. in this study were pathogenic. These species may essentially be unrelated to ambrosia beetles.

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