Fungi Associated With *Scolytogenes birosimensis* (Coleoptera: Curculionidae) Infesting *Pittosporum tobira*

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ABSTRACT The bark beetle *Scolytogenes birosimensis* Niijima is suspected to be involved in the decline of *Pittosporum tobira* (Thunb. ex Murray) Aiton in the coastal areas of Japan. We isolated fungi from adult *S. birosimensis* in nine different localities in Japan to assess their potential association and predict their contribution to the success of the beetle. Results from morphological identification of associated fungi showed that the beetle was associated with *Fusarium solani* and *Candida* spp. Furthermore, molecular analysis showed that *F. solani* was most closely related to the plant pathogenic fungus *F. solani* f. sp. *mori*. Fungal isolation from surface-sterilized, dissected beetles and scanning electron miscroscope (SEM) observation of the body surface suggested that the associated fungi were carried in the pits on the beetles' elytra. These findings contribute to the understanding of the relationships between *S. birosimensis* and its associated fungi.

KEY WORDS beetle–fungi association, *Fusarium solani*, phoretic, *Scolytogenes birosimensis*, tree mortality

Pittosporum tobira (Thunb. ex Murray) Aiton (Japanese cheesewood) is an indeciduate shrub distributed in the warm–temperate coastal zones of China, Korea, and Japan (Kitamura and Okamoto 1979). This shrub is one of the principal components of coastal forests and plays an important ecological role. Furthermore, because of its tolerance to drought and restricted water conditions (Kurauchi 1956), this shrub often is planted in gardens as well as on roadsides and afforested areas, and thus is economically important (Stamps 1987).

P. tobira populations have been declining in Chita Peninsula, Aichi Prefecture, in Japan. Observations have suggested that these shrubs are attacked by *Scolytogenes birosimensis* Niijima (Coleoptera: Curculionidae: Scolytinae). Although no reports have confirmed mortality of *P. tobira* by *S. birosimensis* (Murayama 1958), the beetle appears to be contributing to the decline given that no evidence of any other pest, environmental stress, or both exists in this area. Moreover, field observations indicated that the damage caused by this insect was not always restricted to the Aichi Prefecture.

Bark beetles invariably are associated with symbiotic fungi, which are thought to contribute to the success of the beetle. Some bark beetles are known to act as vectors for fungi that can kill their host trees. For example, *Ophiostoma ulmi* and *O. novo-ulmi*, the pathogens causing Dutch elm disease, are carried by species of *Scolytus* and *Hylesinus* (Stipes and Campana 1981). These fungi can kill the host trees, thus increasing the availability of dead trees for beetle breeding habitats. *Dendroctonus* spp. beetles, with their many fungal symbionts, have caused widespread mortality of pine trees in North America (Paine et al. 1997). Fungi associated with bark beetles also may serve as food for the developing brood (Francke-Grosmann 1967, Harrington 2005, Bleiker and Six 2007). Beetles may carry their fungal associates phoretically on the exoskeleton or in structures of the integument called mycangia, which are specialized for transporting fungi (Livingston and Berryman 1972, Furniss et al. 1990, Six 2003b, Harrington 2005). To date, no report has described the fungi and mycangia associated with *Scolytogenes* spp.

We isolated fungi from *S. birosimensis* and its galleries, and identified them through morphological observations to examine whether the beetle carries fungi that are pathogenic to host trees or that provide food for their larvae. In addition, we clarified the phylogenetic position of the main fungal associate by molecular analysis. We also examined the fungal transport structures of the beetles.

Materials and Methods

Sampling Site. Adult beetles of S. *birosimensis* and the infesting *P. tobira* were collected from nine sites along the seashore in Japan (Fig. 1; Table 1). Among

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Fig. 1. Sampling sites. The names of the localities and the collection dates are provided in Table 1.

the nine sites, notable declines in the *S. birosimensis* populations were observed at Aichi (site 4) and Fukuoka (site 8). Samples were collected from April 2003 to December 2005.

Sampling and Isolation. Twigs and trunks of P. *tobira* attacked by *S. birosimensis* were chosen randomly from damaged but living trees (Fig. 2a, b, c). Samples were stored at room temperature in tetron bags, and emerged adults from the samples were captured and used for fungal isolation. Fifty adults in 3–5 twigs (0.5–1 cm in diameter) in a tree from each locality except for Aichi and Fukuoka were collected. Also, in total, 250 and 150 adult beetles in each five twigs (≈1 cm in diameter) were obtained from each a tree in Aichi and Fukuoka, respectively. In addition, attacking adults, adults breeding under the bark, and their galleries from the Aichi were used for isolation and examined to compare the fungal flora on the beetle in different stages of its life cycle. Attacking adults means that the adult beetles were excavating egg galleries at premating stage. Breeding adults is that they are laying eggs at postmating stage.

Table 1. Localities and date that *S. birosimensis* were collected

Site no.[a]	Locality	Sampling date
1	Hitachi, Ibaraki	10 Dec. 2005
2	Odawara, Kanagawa	2 May 2003
3	Kaga, Ishikawa	26 Nov. 2005
4	Chita, Aichi	24 Apr. 2003
5	Iwami, Tottori	8 Dec. 2005
6	Naruto, Tokushima	28 Oct. 2005
7	Shirahama, Wakayama	25 Aug. 2005
8	Fukuoka, Fukuoka	31 May 2004, 30 June 2004
9	Iriomote, Okinawa	19 Nov. 2005

[a] Each site is shown on the map in Fig. 1.

The collected adult beetles were not separated by sex before isolation because no differences were observed in the associated fungal flora between them in our preliminary study (H. M., personal observation). All beetles were placed in petri dishes containing 1% malt extract agar (MEA): (malt extract 10 g [Difco, Detroit, MI]; agar 15 g; distilled water 1,000 ml); and allow to walk around for 6 hr. After that, they were squashed on the plates. Similarly, each four pieces of bark and xylem (≈2 mm^3) were cut from single beetle gallery and placed onto MEA. Fifty galleries were used for isolation. The plates were kept in the laboratory at room temperature. Growing hyphae were lifted from isolation plates by using flame-sterilized tungsten needles and inoculated onto 2% MEA. After a month or more, cultures were identified using morphological characteristics such as spores and spore-producing structures.

Examination of Beetles for Fungal Transport Structures. One hundred adults that emerged from a log of *P. tobira* samples collected in Aichi were used for dissection to identify structures and locations by which fungal spores may be transported. Fifty beetles were washed with 70% EtOH for 2 min, 1% sodium hypochlorite for 1 min, and then rinsed with sterilized water for 2 min. Other 50 adult beetles were used for dissection without washing. The head, prothorax, and

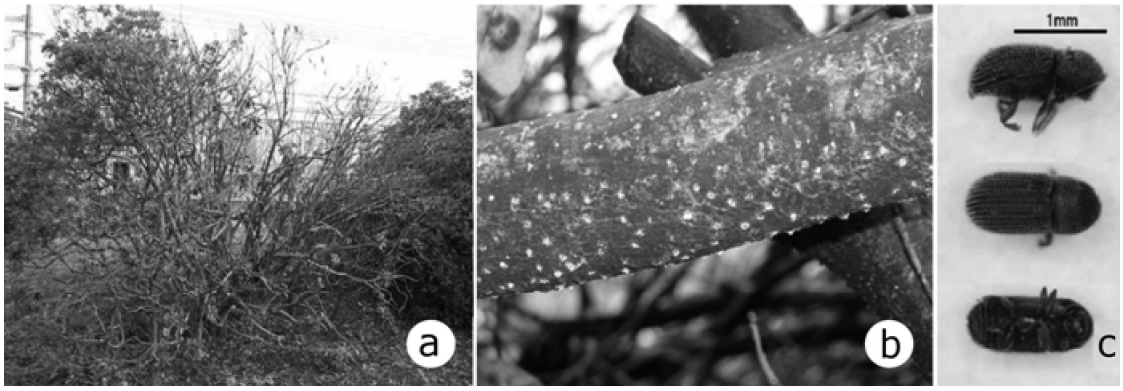

Fig. 2. *S. birosimensis* and decline of *Pittosporum tobira.* a. Decline of *P. tobira.* b. Mass attack of *S. birosimensis.* c. *S. birosimensis* adults.

abdomen were aseptically dissected and placed on 1% MEA in petri dishes. These parts were kept at room temperature, and the growing hyphae were picked and inoculated onto 2% MEA. Because the digestive tract is also a possible mode of transport for fungi, we also isolated the guts, which were aseptically removed from the abdomen and crushed on the 2% MEA. Cultures thus obtained were identified on the basis of morphology.

Five emerged beetles collected from Aichi were fixed with 70% EtOH and air-dried. These samples were observed using a three-dimensional real surface view microscope VE-9800 (Keyence Co. Ltd., Osaka, Japan).

Molecular Analysis. Molecular analysis of *Fusarium solani*, the fungus most frequently isolated, was conducted to clarify its taxonomic and phylogenetic position. Ten isolates of the fungus (deposited within the culture collection of the Ministry of Agriculture, Forestry and Fisheries, MAFF411034–411043) obtained from each area were incubated on 2% MA plates for 4 wk. Their DNA was directly amplified by polymerase chain reaction (PCR) on a GeneAmp 9700 thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, CA). A small amount of aerial mycelium was harvested and crushed in a 100-μm PCR tube with a pipette tip under an optical microscope. A 50-μl aliquot of the reaction mixture containing 25-μl Qiagen (Valencia, CA) GoTaq premix and 10 pmol of each primer and distilled water were added to the template. The ITS4 and ITS5 (White et al. 1990) primers were used. The reactions were initiated with 3 min of denaturation at 95°C, followed by 40 cycles of 2-step PCR consisting of 20 s at 95°C and 60 s at 54°C for the primers ITS5 and ITS4, with a final extension for 10 min at 72°C. Amplification products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and used for sequencing, which was conducted with the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit ver. 3.1 (Perkin Elmer Applied Biosystems) on the ABI PRISM 3100 Genetic Analyzer (Perkin Elmer Applied Biosystems). Both strands of the fragment were sequenced. The sequences obtained were deposited in DNA Data Bank of Japan (Accession Numbers: AB510592 and AB510593). Sequence data for the ITS1–5.8S rDNA-ITS2 (487 bp) were used for phylogenetic analyses.

The sequences were aligned using ClustalX version 1.81 (Thompson et al. 1997). Alignments were manually adjusted using the BioEdit software version 5.0.9 (Hall 1999), and the aligned data set was analyzed using PAUP*4.0 beta 10 (Swofford 2002). A parsimony analysis was carried out using a heuristic search with random stepwise addition and the tree-bisection reconnection option of the program. Gaps were treated as missing, all characters were equally weighted, and the MAXTREE option was set to auto-increase. Bootstrap values (1,000 replicates) also were calculated with the MAXTREE option set to 1,000.

Results

Species Composition of Isolated Fungi. We isolated >30 spp. of fungi from emerging adult beetles collected at nine sites in Japan (Table 2). Among them, *F. solani* and *Candida* spp. were the most commonly isolated species from the beetles at almost all sites. *F. solani* was isolated from the beetles at many localities at very high frequencies (90–98%), except in Kanagawa and Okinawa (20 and 24%, respectively). *Candida* spp. were consistently isolated from all localities at high frequencies (56–94%). *Cladosporium* spp. and *Penicillium* spp. also were isolated, although they are universally distributed and are likely to be incidental with this beetle. *Colletotrichum* spp., including the well-known plant pathogenic groups, were detected on the adults from two areas, Aichi and Wakayama (6 and 26%, respectively). A black yeast, *Aureobasidium pullulans,* was isolated from the adults sampled from five sites; it was particularly common in Fukuoka (64%), although less so at other sites (>10%). All other fungal species were present at low frequencies and thus likely to have been incidental.

We detected 11 fungal species from the attacking and breeding stages of *S. birosimensis* adults and its galleries collected from Aichi Prefecture (Table 3).

Table 2. Numbers of emerging adult *S. birosimensis* beetles that each fungus was isolated, and frequencies of occurrences (%) of the fungi at each locality

	Aichi	Kanagawa	Fukuoka	Wakayama	Tokushima	Okinawa	Ishikawa	Tottori	Ibaraki
Total no. of beetles examined	250	50	150	50	50	50	50	50	50
Fusarium solani	225 (90)[a]	10 (20)	141 (94)	47 (94)	47 (94)	12 (24)	47 (94)	49 (98)	49 (98)
Fusarium sp.1				6 (12)				2 (4)	
Fusarium sp.2				2 (4)	1 (2)				7 (14)
Fusarium sp.3									3 (6)
Candida spp.	180 (72)	36 (72)	111 (74)	42 (84)	31 (62)	47 (94)	30 (60)	35 (70)	28 (56)
Cladosporium cladosporioides	30 (12)	2 (4)	21 (14)	11 (22)	8 (16)	2 (4)	18 (36)	5 (10)	12 (24)
Cladosporium sphaerospermum	30 (12)		21 (14)		4 (8)			5 (10)	15 (30)
Cladosporium sp.1							5 (10)	3 (6)	
Colletotrichum	15 (6)			13 (26)					
Botrytis sp.								3 (6)	3 (6)
Penicillium sp.1	15 (6)	5 (10)	12 (8)	4 (8)	25 (50)	7 (14)	8 (16)	2 (4)	
Penicillium sp.2		3 (6)		15 (30)		27 (54)		1 (2)	5 (10)
Penicillium sp.3				5 (10)	40 (80)	10 (20)	15 (30)		3 (6)
Penicillium brevicompactum					6 (12)	1 (2)			
Penicillium sp.4					3 (6)				
Penicillium sp.5						20 (40)			
Paecilomyces variotii							20 (40)		
Gymnoascus sp.									4 (8)
Sterile mycelium sp. 1	60 (24)			3 (6)					
Sterile mycelium sp. 2	15 (6)			2 (4)					
Sterile mycelium sp.3									6 (12)
Aureobasidium pullulans		4 (8)	96 (64)		2 (4)		5 (10)	5 (10)	
Gliomastix sp.		3 (6)							
Sporothrix sp.		3 (6)							
Black yeast sp.						19 (38)			
Aspergillus nigrum				2 (4)					
Trichoderma harzianum				3 (6)					
Trichoderma sp.1									4 (8)
Basidiomycetes sp.1				4 (8)					
Others[b]	115 (46)	10 (20)	45 (30)	12 (24)	8 (16)	11 (22)	7 (14)	10 (20)	8 (16)

[a] Frequency of occurrence (percentage, shown in parentheses) was calculated as follows: NF/NT × 100, NF equals the number of beetles from which the particular fungus was isolated, NT equals the total number of the beetles used.
[b] Others include the fungi which were isolated at low frequencies (only from a beetle).

Among them, *F. solani* (Fig. 3a and b) as well as *Candida* spp. were isolated from both adult stages of the beetles. *F. solani* was isolated from adult beetles, that is, from the bark of the egg gallery walls and the xylem underneath the galleries of the attacking stage at low frequencies (3.6, 4.0, and 10.0%, respectively), and from the breeding stage at relatively high frequencies (16.7, 74.0, and 58%, respectively). In contrast, *Candida* spp. were more frequently isolated from the bark of the egg gallery walls and the xylem underneath the galleries in the attacking stage than those in the breeding stage (Table 3). *Candida* spp. fre-

Table 3. Numbers of *S. birosimensis* adult beetles and their galleries which each fungus was isolated, and the frequencies of occurrences (percentage) of the fungi from beetles and their galleries in different breeding stage

	Attacking stage[a]			Breeding stage[b]		
	Adult beetles[c]	Phloem[d]	Xylem[e]	Adult beetles	Phloem	Xylem
Total no. of samples	55	50	50	60	50	50
Fusarium solani	2 (4)[f]	2 (4)	5 (10)	10 (17)	37 (74)	29 (58)
Fusarium sp.		1 (2)	1 (2)			
Penicillium sp.1	6 (11)	4 (8)	4 (8)	5 (8)		
Penicillium sp.2				1 (2)		
Candida spp.	21 (38)	24 (48)	23 (46)	39 (65)	4 (8)	5 (10)
Acremonium sp.		1 (2)				
Cladosporium sp.			4 (8)			
Black yeast sp.		1 (2)	1 (2)		3 (6)	
Sterile mycelium sp.					7 (14)	4 (8)
Sporothrix sp.					6 (12)	6 (12)

[a] The stage that adult beetles are excavating egg galleries. Premating stage.
[b] The stage that adult beetles are laying eggs. Postmating stage.
[c] Integrated data from male and female. Numbers of adult beetles that each fungus was isolated.
[d] Each a piece of phloem was cut from the wall of each an egg gallery. Numbers of pieces that each fungus was isolated.
[e] Each a piece of xylem was cut from each underneath egg gallery. Numbers of pieces that each fungus was isolated.
[f] Frequencies of occurrences (percentage, shown in parentheses) were calculated as follows: NF/NT × 100, NF equals number of samples that each fungus was isolated, NT equals number of all samples.

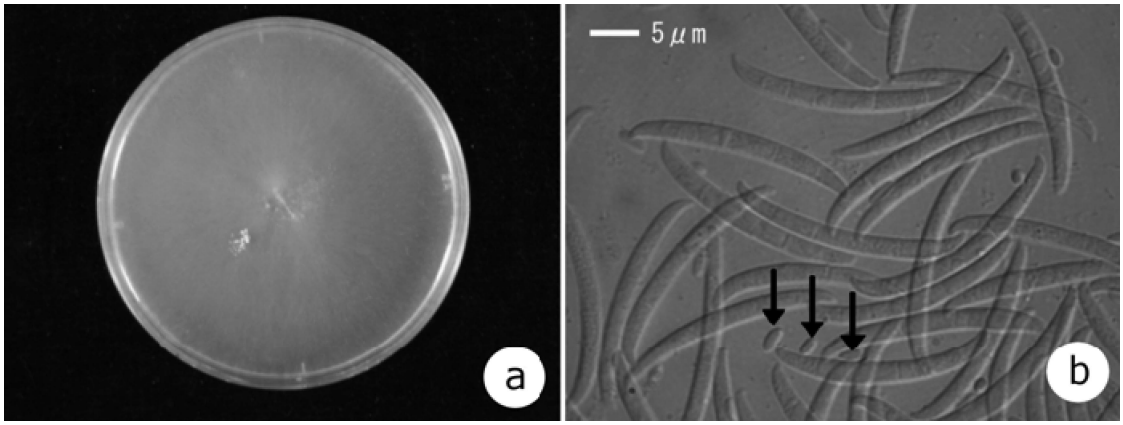

Fig. 3. *F. solani* isolated from *S. birosimensis*. a. Colony of *Fusarium solani* isolated from *S. birosimensis*. b. Falcated spores (macroconidia) and oblong-shaped spores (microconidia) of *F. solani*. Arrows show the microconidia.

quently were isolated from adult beetles of the breeding stage (65%) but at low frequencies from the bark of the egg gallery walls and the xylem underneath the galleries (8.0 and 10.0%, respectively). Other species of fungi were isolated from each stage at low frequencies and appeared to be incidental.

Phylogenetic Position of *F. solani* Associated with *S. birosimensis*. We amplified ≈500 bp of the ITS1–5.8s-ITS2 regions in ribosomal DNA for *F. solani* isolated in this study. The sequences aligned easily with those of other species in the *F. solani* complex.

Our molecular analysis showed that *F. solani* isolated in this study falls within the *F. solani* clade and is particularly closely related to *F. solani* f. sp. *mori*, which is an anamorph of the *Haematonectria haematococca* mating population III (Fig. 4). Furthermore, some of the *F. solani* isolates from the beetles captured from different localities in Japan were highly similar, differing by only one or 2 bp.

Fungal Transport Structures. *Fusarium solani*, *Penicillium* sp. 1, and *Candida* spp. were isolated from the surface-sterilized beetles at very low frequencies, although numerous fungi commonly were isolated from the nonsterilized beetles (Table 4). Several fungi were isolated from the dissected parts of the beetles, but they were not always detected from a specific part of the beetle (Table 5). All beetles appeared to carry saccharomycetous yeasts and no other fungi in their alimentary canals.

SEM often revealed fungal spores on the body surface of *S. birosimensis*, in particular within the punctures of the integument, especially those on the elytra (Fig. 5a and b, and c) and at the base of the asperites or setae on the prothorax (Fig. 6a and b). We observed several types of spores, which were globular, spherical, and oval-shaped, but we were unable to determine their identities. We did not observe falcated spores such as that of *Fusarium*.

Discussion

Scolytogenes birosimensis consistently was associated with numerous fungal species at all sampling localities. Among the isolated fungi, *F. solani* frequently was isolated from emerging adult beetles at most localities (>90%) with the exception of Kanagawa and Okinawa (20 and 24%, respectively). We lack sufficient information to determine why *F. solani* was relatively rare at these two sites. A likely explanation is the condition of the host trees. The samples collected in Kanagawa and Okinawa were somewhat dried and did not appear to be suitable hosts for the associated fungi (H. M., personal observation). Other factors may include behavioral differences associated with population density, differing environmental conditions, and the presence of competitors against the associated fungi. Additional research is needed to determine the factors influencing the species composition and the frequencies of the beetle-associated fungi.

Several *Fusarium* spp. are known to be plant pathogens that can cause severe damage to trees and crops (Nelson et al. 1981). Thus, *F. solani* has the potential to act as a pathogen when introduced to *P. tobira*. However, at least 50 species are reportedly classified under the name *F. solani* (O'Donnell, 2000), which complicates classification and nomenclature. Our molecular data showed that the *F. solani* associated with *S. birosimensis* was phylogenetically related, but distinct from *F. solani* f. sp. *mori*, a causal agent of bud mortality in mulberry trees (Kimura 1979). Thus, even though *F. solani* was low frequently isolated from adult beetles which are excavating egg galleries (Table 3), it may be relatively unimportant in terms of host declines. Conversely, the frequencies of *F. solani* from pieces of bark and xylem in the breeding stage were higher than those in the attacking stage, whereas those of yeasts from the body surfaces of the adult beetles in the breeding stage were lower than those in the attacking stage (Table 3). These data suggest that *F. solani* was introduced to a low proportion of the new egg galleries, grew rapidly, successfully established, but that yeasts do not grow well in bark and xylem during the breeding stage. Thus it appears that a succession of fungal flora occurs in the beetle gallery. In addition, emerging adult beetles are associated with *F.*

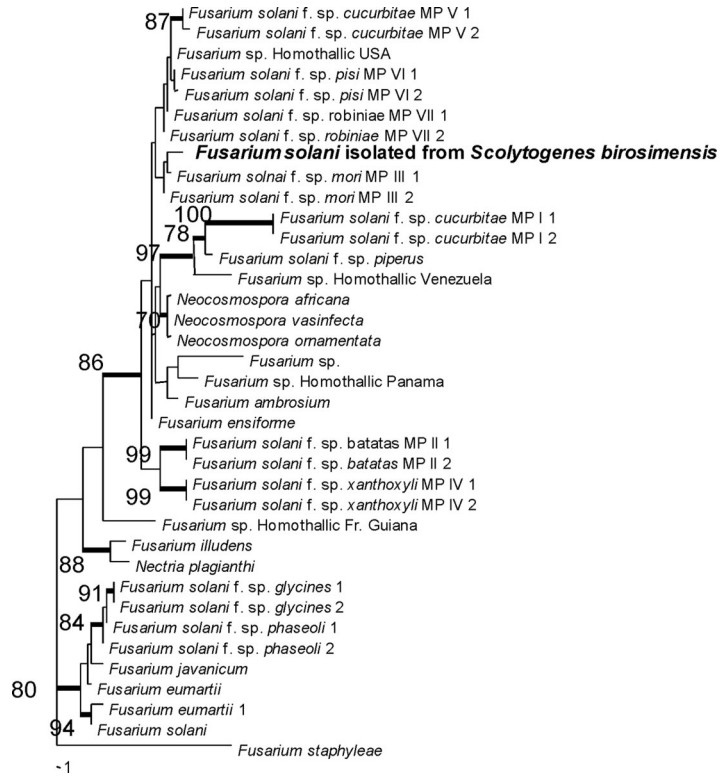

Fig. 4. One of the 238 most parsimonious trees of the *F. solani* complex based on 555 characters of the ITS regions and 5.8s ribosomal DNA. Tree length = 244; Consistency index (CI) = 0.7008; Homoplasy index (HI) = 0.2992; Retention index (RI) = 0.8119. All characters have equal weight; 421 characters are constant, 50 variable characters are parsimony-uninformative; number of parsimony-informative characters = 84; gaps are treated as "missing"; MaxTrees setting = 1,000. The bootstrap values were determined from 1,000 replications.

solani at high frequency (Table 2). Although further studies are required, the fungi on the surface of the insect bodies may be disappeared by dried condition, UV light or other factors during beetle flight. This pattern has been found in other bark beetles such as *Ips typographus* and *Tomicus piniperda* (Solheim, 1992; Masuya et al., 1998). In *T. piniperda*, the virulent species *Leptographium wingfieldii* was initially introduced at low frequencies, but was subsequently detected at high frequencies (Masuya et al. 1998). Because the *F. solani* isolated from *S. birosimensis* exhibited similar isolation trends to those of *L. wingfieldii*, strong virulence of *F. solani* is predicted. Its virulence is currently under investigation.

The fungal species primarily associated with bark beetles that attack conifers are *Ophiostoma* and their related species (Sallé et al. 2005). Several such asso-

Table 4. Numbers of *S. birosimensis* adult beetles that each fungus was isolated, and frequencies of occurrences (percentage) of fungi isolated from the beetles with or without surface-sterilization

	Not sterilized[a]	Sterilized[b]
Total no. of the beetles	50	50
Fusarium solani	39 (78)[c]	5 (10)
Candida spp.	38 (76)	5 (10)
Penicillium sp.1	10 (20)	3 (6)
Aureobasidium pullulans	8 (16)	
Cladosporium cladosporioides	12 (24)	
Ascochyta sp.	3 (6)	
Acremonium sp.	6 (12)	
Others[d]	39 (78)	

[a] Each beetle was squished without surface-sterilized onto MEA.
[b] Each beetle was placed onto the MEA after washing with 70% EtOH for 2 min, 1% sodium hypochlorite for 1 min, and then rinsed with sterilized water for 2 min.
[c] Frequencies of occurrences (percentage) are shown in parentheses.
[d] Others includes the fungi that were isolated at low frequencies (only from a beetle).

Table 5. Number of the beetles dissecting parts which each fungus was isolated, and frequencies of occurrences (percentage) of fungi from washed and dissected beetle from Aichi

	Head	Prothorax	Abdomen	Digestive tract
Total no. of dissecting parts	50	50	50	50
Fusarium solani	1 (2)[a]	3 (6)	4 (8)	1 (2)
Candida spp.	5 (10)	2 (4)	5 (10)	50 (100)
Penicillium sp.1		3 (6)	2 (4)	

[a] Frequencies of occurrences (percentages) were shown in parentheses.

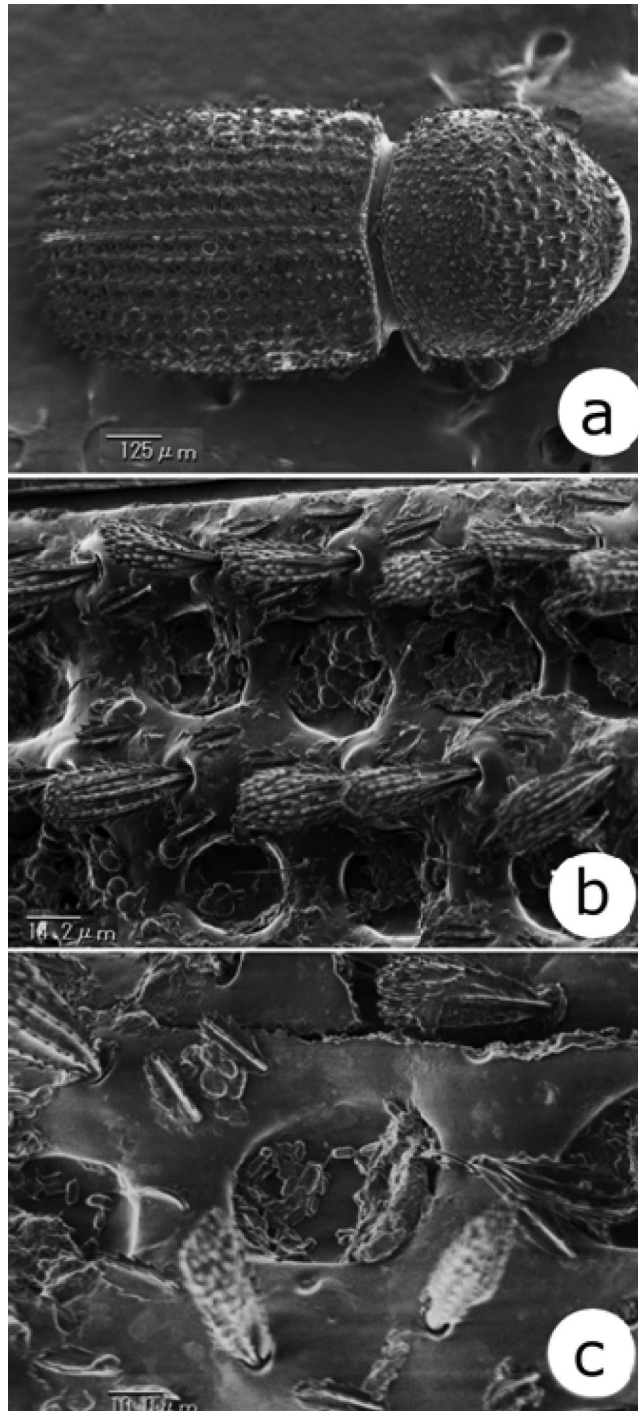

Fig. 5. Surface of *S. birosimensis* bodies. a. Dorsal view of *S. birosimensis*. b. Punctures of the integuments on the elytra. c. Fungal spores (arrows) aggregated in the puncture.

ciated fungal species are predicted to benefit the beetles by overcoming the resistance of host trees and/or improving the nutritional conditions of host trees for larval development (Harrington 2005). The coffee berry borer beetle *Hypothenemus hampei* (Ferrari) is a well-known scolytid pest affecting coffee plants. This insect is often associated with *F. solani* and other fungal species, including saccharomycetous yeasts such as *Candida* (Carrion and Bonet 2004; Perez et al. 2005), similar to the associations of *S. birosimensis* in

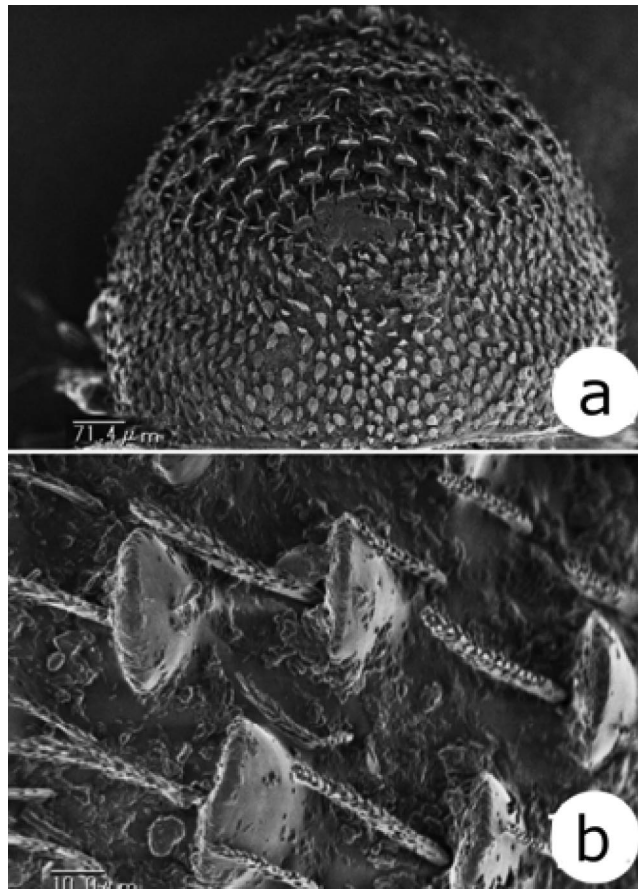

Fig. 6. Surface of *S. birosimensis* bodies. a. Dorsal view of the prothorax of *S. birosimensis*. b. Fungal spores (arrows) at the base of the asperites or setae on the pronotum.

this study. The association between *F. solani* and *H. hampei* has been suggested to contribute to larval development (Rojas et al. 1999, Morales-Ramos et al. 2000), whereas a recent study demonstrated that beetle-associated fungi have no such effect (Perez et al. 2005). Rather, the role of the associated fungi was suspected to be to detoxify host defense chemicals enabling beetle survival (Vega and Dowd 2005). *F. solani* and *Candida* spp. isolated in this study may play a similar role for *S. birosimensis.* Indeed, saponin, a substance that is toxic to many organisms, is contained in *P. tobira* (Yosioka et al. 1972). These predicted functions should be examined by future analyses.

If a given fungal species is frequently isolated from a specific part of the surface-sterilized beetle, the beetle may possess mycangia in that body part. In contrast, if specific or nonspecific fungi are isolated from several parts of the surface-sterilized beetles, no mycangia may exist, but rather transport may be incidental or limited to simple pits. In this study, nonspecific fungi were isolated from several parts of the surface-sterilized beetles (Table 4 and 5). Thus, mycangia such as those present in certain *Dendroctonus* or *Dryocoetes* spp. appeared to be absent in *S. birosimensis.* However, we found that fungal spores were abundantly present within the punctures of the integument, particularly the elytra. Furthermore, spores were sometimes found at the base of the asperites or setae on the prothorax. These observations suggest that *S. birosimensis* transports spores in these structures. Punctures on the elytra, asperites, and setae on the prothorax in several scolytid beetles, for example, *Scolytus ventralis* LeConte (Livingston and Berryman 1972), *Hylurgops palliatus* (Gyllenhal), *Hylastes ater* (Paykull), and *Hylastes cunicularius* Erichson (Francke-Grosmann 1967), are thought to similarly accumulate fungal spores and provide a mechanism for fungal transport. In these cases, the punctures on elytra possess pores connected to gland cells; these fit the concept of mycangia as defined by Farris and Funk (1965). However, similar organs that are less developed, such as those noted for *S. birosimensis* in this study, are termed pseudomycangia (Beaver 1989) or mycangia sensu Six (2003a).

We were unable to determine which spores on the elytra of the beetles represent those of the major fungal associates, *F. solani* and *Candida* spp. *F. solani* has two types of spores; one is falcated and other is oval or oblong-shaped (Fig. 3b). However, no falcated spores were observed on the beetles under the SEM

(Figs. 5b,c; 6a) indicating that the oval or oblong-shaped spores of *F. solani* were acquired by the beetles, and that the falcated spores of *F. solani* may not function on this beetle–fungus association. Indeed, the oval or oblong-shaped spores, rather than the falcate spores, seem to fit well within the punctures on the elytra. The data at present are limited, but the selectivity of spores as well as the specificity of the fungal associates with the beetles are subjects that should be addressed in the future.

Here, we identified the fungi that are closely associated with the bark beetle *S. birosimensis* in Japan. The beetle had been identified in Japan by 1958, but no reports of it causing damage existed at that time (Murayama 1958). Thus, the recently reported declines of *P. tobira* that are suspected to be because of the bark beetle may be reflective of environmental stresses such as climate change, air pollution effects, or both on *P. tobira* and the beetle population. To clarify whether the decline of *P. tobira* is definitely the result of its mutualistic association between *S. birosimensis* and their associated fungi, one must examine the virulence of the associated fungi and the physiological conditions of the host trees.

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