Landscape-scale genetic differentiation of a mycangial fungus associated with the ambrosia beetle, *Xylosandrus germanus* (Blandford) (Curculionidae:Scolytinae) in Japan

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**Abstract**
In this study, we examined the genetic structures of the ambrosia fungus isolated from mycangia of the scolytine beetle, *Xylosandrus germanus* to understand their co-evolutionary relationships. We analyzed datasets of three ambrosia fungus loci (18S rDNA, 28S rDNA, and the β-tubulin gene) and a *X. germanus* locus dataset (cytochrome c oxidase subunit 1 (COI) mitochondrial DNA). The ambrosia fungi were separated into three cultural morphotypes, and their haplotypes were distinguished by phylogenetic analysis on the basis of the three loci. The COI phylogenetic analysis revealed three distinct genetic lineages (clades A, B, and C) within *X. germanus*, each of which corresponded to specific ambrosia fungus cultural morphotypes. The fungal symbiont phylogeny was not concordant with that of the beetle. Our results suggest that *X. germanus* may be unable to exchange its mycangial fungi, but extraordinary horizontal transmission of symbiotic fungi between the beetle’s lineages occurred at least once during the evolutionary history of this symbiosis.

**KEYWORDS**
*Ambrosiella hartigii*, coevolution, mycangium, phylogeography, *Xylosandrus germanus*

**1 | INTRODUCTION**

The associations among insects and fungi are highly diverse (Vega & Blackwell, 2005). Fungivorous insects include more than 150 families in 33 orders and reach 10% in the species of Insecta (Hammond & Lawrence, 1989). Insects also show a vast array of symbiotic relationships with a wide diversity of fungi. These relationships may confer a variety of benefits to the host insects, such as direct or indirect nutrition, the ability to counter the defenses of plant or animal hosts, protection from natural enemies, improved development and reproduction, and communication (Klepzig, Adams, Handelsman, & Raffa, 2009). Some insects, particularly fungus-growing ants, fungus-growing termites, and ambrosia beetles, rely on the cultivation of fungi for food, and these cultivated fungi are a nutrition source for larvae and adults (Mueller, Gerardo, Aanen, Six, & Schultz, 2005).

Ambrosia beetles have a close association with symbiotic fungi and carry the inoculum of symbiotic fungi in special structures (mycangia; Beaver, 1989). Ambrosia beetles bore into the xylem of woody plants and feed on fungi that they culture on the walls of the tunnels in the wood. The fungi may supply sterols and B-group vitamins important for beetle development (Kok, Norris, & Chu, 1970). Approximately 3,400 species of ambrosia beetles are found in 10 tribes of two subfamilies of Curculionidae, Scolytinae, and Platypodinae (Farrell et al., 2001). The xylomycetophagous habit is considered to have evolved at least seven times in Scolytinae (Farrell et al., 2001). Different beetle species in Scolytinae have different symbiotic associations with their own specific fungi (Kajimura & Hiji, 1994). Symbiotic fungi associated with scolytine beetles are polyphyletic and comprise two primary ophiostomatoid clades that include *Ceratocystis* and *Ophiostoma* (Farrell et al., 2001). The two genera are not closely related, and their
ancestors may have diverged more than 170 million years ago (Farrell et al., 2001). Thus, ambrosia beetles strongly depend on polyphyletic fungal groups.

*Xylosandrus germanus* (Blandford) is a common ambrosia beetle in the subfamily Scolytinae. This species was originally distributed in eastern and southeastern Asia (Nobuchi, 1981; Wood & Bright, 1993) but has now invaded central Europe, North America, and Hawaii (Bouget & Noblecourt, 2005; Cognato & Rubinson, 2008; Grégoire, Piel, Proft, & Gilbert, 2001; Henin & Versteirt, 2004; Lakatos & Kajimura, 2007; López, Iturondonbietia, & Goldarazena, 2007; Rabaglia, 2003; Rabaglia, Dole, & Cognato, 2006; Wanat & Mokrzycki, 2005; Weber & McPherson, 1983a; Wood & Bright, 1993). The beetle has at least 220–264 host species worldwide (Weber & McPherson, 1983b) and 146 species in Japan (Nobuchi, 1981). *X. germanus* has a much wider range of hosts than that other ambrosia beetles, and thus, it can colonize a range of forest types (Henin & Versteirt, 2004). It has become a serious forest pest in many countries, regardless of whether it is native or exotic in those countries (Grégoire et al., 2001; Kaneko, Tamaki, & Takagi, 1965; Nobuchi, 1981 Weber & McPherson, 1983a, 1984).

*Xylosandrus germanus* biology was well-documented by Kaneko and Takagi (1966). Only females disperse; bore into stems, twigs, and roots of susceptible woody plants; excavate a gallery system in the wood or pith; introduce symbiotic fungi; and produce a brood. The female parent remains with her brood until they are mature. Adults and larvae feed on the ambrosia fungus introduced by the female parent. The sex ratio is strongly female-biased; males are rare, reduced in size, and flightless. Young females mate with their brothers (inbreeding) before emerging to attack a new host. Males are haploid, and females are diploid (Takagi & Kaneko, 1966). *Ambrosiella hartigii* Batra, the symbiotic fungi of *X. germanus*, is common to Japan, China, the United States, and Germany (Batra, 1967; Weber & McPherson, 1984; Yang, Ye, & Zhang, 2008). *A. hartigii* has been isolated from adult female mycangia, except for the callow adult (Kaneko & Takagi, 1966; Yang et al., 2008). *Ambrosiella hartigii* was isolated from *X. germanus* and *Anisandrus dispar* (Fabricius) mycangia (Batra, 1967). Mayers et al. (2015) showed fungal symbiont isolated from *A. dispar* and *X. germanus* was *A. hartigii* and *Ambrosiella grossmanniae* Mayers, McNew & Harr., respectively, using molecular methods and morphology.

A phylogenetic analysis based on cytochrome oxidase I (COI) mitochondrial DNA (mtDNA) revealed three distinct lineages (clades A, B, and C) within *X. germanus* in Japan (Ito, Kajimura, Hamaguchi, Araya, & Lakatos, 2008). The rates of substitutions per site between the three lineages are 12.4%–15.0%, which are similar to those calculated as differences among scolytine beetle species in the genera *Ips*, *Tomicus*, and *Dendroctonus* (Cai, Cheng, Xu, Duan, & Kirkendall, 2008; Cognato & Sperling, 2000; Duan, Kerdelhué, & Lieutier, 2004; Lakatos, Grodzki, Zhang, & Stauffer, 2007; Maroja, Bogdanowicz, Wallin, Raffa, & Harrison, 2007). Thus, these different *X. germanus* lineages may have genetically different fungi in their mycangia.

In this study, we investigated the genetic structure of an ambrosia fungus isolated from *X. germanus* mycangia and adult females used for fungal isolation in order to elucidate the differentiated fungal and beetle lineage patterns. We also discuss evolutionary events that may have influenced the diversification of their mutualistic system.

### TABLE 1 Description of *Xylosandrus germanus* samples used for isolations of mycangial fungi

| Sampling site                  | Acronym | Regions          | Areas            | Latitude (N) | Longitude (E) | Altitude (m) | No. of samplesa |
|-------------------------------|---------|------------------|------------------|--------------|---------------|--------------|-----------------|
| Furano, Hokkaido Pref.        | HKF     | Northern Japan   | Hokkaido         | 43° 10’      | 142° 20’      | 500–700      | 29 (25)         |
| Sapporo, Hokkaido Pref.       | HKS     | Northern Japan   | Hokkaido         | 43° 00’      | 141° 23’      | 70           | 31 (11)         |
| Iwate-gun, Iwate Pref.        | IWI     | Northern Japan   | Tohoku           | 39° 53’      | 141° 10’      | 190          | 7 (7)           |
| Tsuruoka, Yamagata Pref.      | YMT     | Northern Japan   | Tohoku           | 39° 39’      | 139° 49’      | 210          | 23 (11)         |
| Chichibu, Saitama Pref.       | SIC     | Eastern Japan    | Kanto            | 35° 55’      | 138° 50’      | 500          | 21 (10)         |
| Shimominochi-gun, Nagano Pref.| NGM     | Central Japan    | Chubu            | 37° 00’      | 138° 32’      | 670          | 2 (2)           |
| Chisagata-gun, Nagano Pref.   | NGC     | Central Japan    | Chubu            | 36° 32’      | 138° 21’      | 1,300        | 22 (10)         |
| Shiojiri, Nagano Pref.        | NGS     | Central Japan    | Chubu            | 36° 08’      | 138° 00’      | 790          | 4 (4)           |
| Nakashinkawa-gun, Toyama Pref.| TYN     | Central Japan    | Hokuriku         | 36° 36’      | 137° 20’      | 260          | 23 (10)         |
| Toyota, Aichi Pref.           | AIT     | Central Japan    | Tokai            | 35° 12’      | 137°34’      | 930–1,070    | 25 (10)         |
| Nantan, Kyoto Pref.           | KTN     | Western Japan    | Kinki            | 35° 16’      | 135° 30’      | 250          | 23 (10)         |
| Tanabe, Wakayama Pref.        | WKT     | Western Japan    | Kinki, Kii peninsula | 33° 42’      | 135° 33’      | 500          | 1 (1)           |
| Miyoshi, Hiroshima Pref.      | HRM     | Western Japan    | Chugoku, Sanyo   | 34° 47’      | 132° 51’      | 200          | 27 (11)         |
| Hata-gun, Kouchi Pref.        | KUH     | Western Japan    | Shikoku          | 33° 12’      | 133° 02’      | 1,336        | 22 (10)         |

aThe numbers in bracket are the number of individuals used in the DNA analysis of both *X. germanus* and its symbiotic fungi.

2 | MATERIALS AND METHODS

2.1 | Insect collection and fungi isolation

We collected *X. germanus* samples from 14 sites in Japan (Table 1). To capture live adult females, in 2007, we set up Nagoya University (Meidai) traps (Ito & Kajimura, 2006) baited with 99.5% ethanol at...
all sites and used 1–31 mature females from each site (Table 1). We also trapped adult females of *Xylosandrus brevis* (Eichhoff) in Aichi Prefecture (AIT) and *Scolytotrupes mikado* (Blandford) in AIT and Wakayama Prefecture (WKT). Two species of *Xylosandrus* beetles and *S. mikado* were identified according to Nobuchi (1981, 1980), respectively.

We isolated fungal conidia from mycangia of *X. germanus*, *X. brevis*, and *S. mikado* living adult females. All collected beetles were preserved at −20°C in 99.5% ethanol after fungal isolation. Isolates from mycangia were directly placed on potato dextrose agar (PDA) plates in 90-mm sterile Petri dishes and incubated at 20°C for 5 days in the dark. The isolates were grouped by cultural characteristics and identified at the generic level using the ambrosia fungi keys of Batra (1967).

### 2.2 DNA extraction, polymerase chain reaction (PCR), and DNA sequencing

Total DNA was extracted using the methods of Walsh, Metzger, and Higuchi (1991) and Suzuki, Taketani, Kusumoto, and Kashiwagi (2006), with some modifications. A small amount of mycelium was scraped from the surface of cultures grown on PDA, and all muscle tissue from the abdomen of each adult female was sampled to extract DNA. The mycelium and muscle tissue were macerated in 200 μl of Chelex 100 sodium (0.26 g/5 ml, Bio-Rad Laboratories, Hercules, CA, USA) and 4 μl of Proteinase K (600 μg/ml, Qiagen, Valencia, CA, USA). The samples were incubated at 56°C for at least 10 hr. After incubation, the samples were vortexed for 10 s and then heated at 95°C for 3 min to inactivate the proteinase. The solutions were vortexed again for 10 s and centrifuged at 15,027 g for 2 min. The supernatant was adjusted to a standard mixture density (1 ng/μl) by adding Tris EDTA (pH 8.0) and used for PCR analysis.

PCR amplification for the symbiotic fungi was performed using the primer pairs NS1 (5’-GTA GTC ATA TGC TTG TCT C-3’; White, Bruns, Lee, & Taylor, 1990) and NS4 (5’-CTT CCG TCA ATT CCT TTA AG-3’; White et al., 1990), NL1 (5’-GCA TAT CAA TAA GCG GAG GAA AAG-3’; O’Donnell, 1993) and NL4 (5’-GGT CCG TGT TTC AAG ACG G-3’; O’Donnell, 1993), and Bt2a (5’-GGT AAC CAA ATC GGT GCT GCT TTC-3’; Glass & Donaldson, 1995) and Bt2b (5’-ACC CTC AGT GTA GTG ACC CTT GCC-3’; Glass & Donaldson, 1995) to amplify a portion of the small subunit (18S) rDNA, large subunit (28S) rDNA, and partial β-tubulin genes, respectively. PCR amplification for the insects was performed using the primer pairs C1-J-2183 (5’-CAA CAT TTA TTT TGA TTT GTG-3’; Simon et al., 1994) and TL-2-N-3014-ANT (5’-TGA AGT TTA AGT TCA ATG CAC-3’; Ito et al., 2008) to amplify a portion of the COI mtDNA gene. For the PCR analysis, we mixed 1 μl of extracted DNA, 2 μl of each primer (5 pmol/μl), 0.8 μl of dNTPs (Takara, Otsu City, Shiga, Japan), 1 μl of 10× PCR buffer (Takara), 0.1 μl of Taq DNA polymerase (5 units/μl, Takara), and 3 μl of distilled water in a total volume of 10 μl. The PCR conditions for NS1/NS4 were as follows: one cycle of denaturation at 94°C for 2 min; followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min; and one final cycle of extension at 72°C for 2 min. The PCR conditions for NL1/NL4 and Bt2a/Bt2b were as follows: one cycle of denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 2 min and annealing at 54°C for 1 min, and one final cycle of extension at 72°C for 10 min. The PCR conditions for C1-J-2183/TL-2-N-3014-ANT were as follows: one cycle of denaturation at 94°C for 1 min; followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 2 min; and one final cycle of extension at 72°C for 2 min. The samples were refrigerated at 4°C until the reaction tubes were removed from the PCR machine.

The PCR products were purified using a QIAquick PCR purification kit (Qiagen). Direct sequencing was performed using the ABI Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the same primer sets as used for the PCR reactions. Sequencing was performed using an ABI PRISM-3100 Genetic Analyzer (Applied Biosystems).

### 2.3 Data analysis

Sequences were aligned using the BioEdit v.7.0.2 software (Hall, 1999). BLAST searches were performed with sequences of each isolate in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov), and published sequences of relevant and related species were incorporated into the datasets (Tables 2 and 3). Calculations for the G+C composition were performed using the MEGA 4 software (Tamura, Dudley, Nei, & Kumar, 2007). For the phylogenetic analysis, we chose maximum-parsimony (MP) method (Nei & Kumar, 2000), using the MEGA 4 software. The MP analysis also used 1,000 bootstrap replicates. A phylogenetic analysis was also performed for the three loci (18S, 28S, and β-tubulin) using MP methods. Concordance among the three different gene datasets was evaluated by the incongruence length difference (ILD) test (Farris, Källersjö, Kluge, & Bult, 1993) implemented with PAUP*4.0b10 (Swofford, 2003), using 1,000 replicates.

### 3 RESULTS

#### 3.1 Morphological characters of symbiotic fungi isolated from X. germanus mycangia

Based on the color and growth pattern of colonies (mycelia tuft), isolates obtained from *X. germanus* mycangia were separated into three cultural types (Types I, II, and III) (Figure 1). Five days after inoculation, colony characteristics of Types I and II were similar to those of *A. hartigii* and *A. grosmanniae* shown in Batra (1967) and Mayers et al. (2015), respectively, but aerial mycelia were observed only in Type I colonies. Five day old Type III colonies were tinged with white on agar media. Type III colonies did not have cottony aerial mycelia as the Type I colonies.

Type I was found in all 11 populations (HKF, HKS, IWI, YMT, NGM, NGS, TYN, AIT, KTN, WKT, and HRM), except for SIC, NGC, and KUH; Type II was found in six northern, eastern, central, and western populations (HKF, HKS, IWI, SIC, NGC, and KUH); and Type III was only
| Species                  | Source          | GenBank accession no. | References                                      |
|-------------------------|-----------------|-----------------------|-------------------------------------------------|
| Ambrosiella batrae      | C3130           | KR673881              | Mayers et al. (2015)                            |
| Ambrosiella beaveri     | CBS 121751      | –                     | Six, Stone, de Beer, and Woolfolk (2009)        |
|                         | CMW26179        | –                     | De Beer et al. (unpublished)                    |
|                         | PL5329          | –                     | Bateman et al. (unpublished)                    |
|                         | C2749           | KR673882              | Harrington, McNew, Mayers, Fraedich, and Reed (2014) |
| Ambrosiella grossmanniae| C3151           | KR673884              | Mayers et al. (2015)                            |
|                         | 1002HH51        | LC175288              | Lin et al. (unpublished)                        |
| Ambrosiella nakashimae  | C3445           | KR673883              | Mayers et al. (2015)                            |
|                         | 0414XX7         | LC175285              | Lin et al. (unpublished)                        |
| Ambrosiella hartigii    | TUB F4276       | AY858656              | Gebhardt, Weiss, and Oberwinkler (2005)          |
|                         | CBS 403.82      | AF275506              | Paulin-Mahady, Harrington, and McNew (2002)     |
|                         | CBS 404.82      | EU984256              | Alamouti, Tsubi, and Breuil (2009)              |
|                         | CMW20920        | –                     | Six et al. (2009)                               |
|                         | CMW25525        | –                     | De Beer et al. (unpublished)                    |
|                         | C1573           | KR673885              | Mayers et al. (2015)                            |
| Ambrosiella roeperi     | C2448           | KR673886              | Mayers et al. (2015)                            |
|                         | B239U1          | LC175297              | Lin et al. (unpublished)                        |
| Ambrosiella xylebori    | CBS 110.61      | AY858659              | Gebhardt et al. (2005)                          |
|                         | CBS 110.61      | –                     | EU984294                                    |
|                         | AFTOL-ID 1285   | DQ471031              | Spatafora et al. (2006)                         |
|                         | C3051           | –                     | Mayers et al. (2015)                            |
|                          | Hulcr5114       | KU961668              | Bateman, Sigut, Skelton, Smith, and Hulcr (2016) |
| Ceratocystis adiposa    | CMW2573         | –                     | De Beer et al. (unpublished)                    |
|                         | CCCF212707      | –                     | Seifert, Louis-Seize, and Sampson (2003)        |
|                         | CBS600.74       | EU984263              | Alamouti et al. (2009)                          |
|                         | VPCI 2818/12    | –                     | Agarwal et al. (2014)                          |
| Ceratocystis fagacearum| C999            | KR673891              | Mayers et al. (2015)                            |
| Ceratocystis major      | C927            | KR673892              | Mayers et al. (2015)                            |
| Ceratocystis fimbriata  | CMW3189         | –                     | Mayers et al. (2015)                            |
|                         | C1099           | KR673893              | Mayers et al. (2015)                            |
|                         | CBS 146.53      | U43777                | Issakainen, Jalava, Eerola, and Campbell (1997) |
| Ceratocystis norvegica  | WIN(M)87        | DQ318209              | Reid, Iranpour, Rudski, Loewen, and Hausner (2010) |
|                         | C3124           | KR673894              | Mayers et al. (2015)                            |
| Meredithiella norrisii  | C3152           | KR673888              | Mayers et al. (2015)                            |
| Phialophoropsis ferruginea | CBS 408.68    | –                     | Six et al. (2009)                               |
|                         | CBS 408.68      | –                     | Paulin-Mahady et al. (2002)                     |
|                         | CBS 378.68      | EU984254              | Alamouti et al. (2009)                          |
|                         | JB13            | EU984255              | Alamouti et al. (2009)                          |
|                         | CBS 460.82      | –                     | Six et al. (2009)                               |
|                         | M243            | KR673889              | Mayers et al. (2015)                            |
| Phialophoropsis sp.     | CBS460.82       | KR673890              | Mayers et al. (2015)                            |
found in two northern populations (HKF and YMT; Figure 2). Type I fungi were distributed throughout Japan, but the other two types were located in Japan.

3.2 | DNA sequencing and phylogenetic analyses

The amplicons obtained from the 18S regions of ambrosia fungi sequenced in this study were 997 bp in length. These fragments had 45.5% G/C content. Two haplotypes were defined from 132 isolates. Haplotype XgF28S01 was detected in all cultural types (Figure 3). Haplotype XgF28S02 and XgF28S03 were detected only in Type II (Figure 4). The X. germanus fungi haplotypes were clustered as a monophyletic group with those of A. grosmanniae (LC175288), A. roeperii (KU961669 and LC175297), A. hartigii (AF275506, EU984288, and KM495317), and A. xylebori (DQ470979, EU984294, and KU961669). Within this clade, three haplotypes of X. germanus fungi, XgF28S01–03, and A. hartigii and A. xylebori were clustered with high bootstrap value of 64, 63, and 63, respectively. Within XgF28S01–03 clade, two haplotypes, XgF28S02 and 03, were clustered with high bootstrap value of 73. Five haplotypes of X. germanus fungi were not clustered as a monophyletic group.

β-tubulin sequences of approximately 440 bp had 52.0% G/C content. These fragments varied from 436 to 442 nucleotides. Six haplotypes were defined from 125 isolates. Haplotype XgFBt01, XgFBt02, XgFBt03, and XgFBt04 were detected only in Type I (Figure 5). Haplotype XgFBt05 and XgFBt06 were detected only in Types II and III, respectively. The X. germanus fungi haplotypes were clustered as a monophyletic group with one strain of A. hartigii (EU825654). Type I and II haplotypes were also clustered with high bootstrap value of 65.

The ILD test indicated that the 18S, 28S, and β-tubulin datasets were concordant (p = .635). On the basis of the three combined loci, 11 multilocus haplotypes were defined from 129 isolates (Table 4). Haplotype 07 were detected only in Type I (Figure 6). Haplotypes XgF08–09 and XgF10–11 were detected only in Types II and III, respectively. Symbiotic fungi isolated from X. germanus mycangia clustered as a monophyletic group with one strain of A. hartigii, CBS 404.82, and A. xylebori, CBS 110.61. Within this clade, symbiotic fungi of X. germanus formed a subclade (clade XgF). Within clade XgF, Type I and II haplotypes formed subclades I–II, and Type III haplotypes were clustered as a monophyletic group. Within subclade I–II, only Type II

| TABLE 3 List of ambrosia beetles used in this study, including their sequences obtained from GenBank |
|-----------------------------------------------|
| Species | GenBank accession no. | COI mtDNA | References |
| Xylosandrus germanus | AB373682 | Ito et al. (2008) |  |
| | AB373683 | Ito et al. (2008) |  |
| | AB373684 | Ito et al. (2008) |  |
| | AB373703 | Ito et al. (2008) |  |
| | AB373704 | Ito et al. (2008) |  |
| | AB373711 | Ito et al. (2008) |  |
| | EF433438 | Lakatos & Kajimura (2007) |  |
| | EF433439 | Lakatos & Kajimura (2007) |  |
| Xylosandrus brevis | AB476316 | Ito & Kajimura (2009a) |  |
| Xylosandrus crassiusculus | AB462579 | Ito & Kajimura (2009b) |  |

**FIGURE 1** Colonies of symbiotic fungi isolated from Xylosandrus germanus mycangia. (a) Type I. (b) Type II. (c) Type III. Fungal cultures were held at 20°C for 5 days on potato dextrose agar in the dark.
haplotypes were clustered in a subclade (subclade II) and five Type I haplotypes, XgF02, and XgF04–07, were clustered in a subclade. We compared geographical distribution of seven Type I haplotypes using chi-square test. Seven Type I haplotypes were not uniformly distributed in Japan (χ²-test, p < .05; Figure 7). XgF01 was only found in three northern populations (HKF, HKS, and YMT). XgF02 was distributed in four northern populations (HKF, HKS, IWI, and YMT) and in two other populations along the Japan Sea (TYN and KTM). Both XgF03 and XgF04 were found in two other populations, HKS and AIT, and IWI and NGM, respectively. XgF05 was detected from seven northern to western populations (YMT, NGM, TYN, AIT, WKT, and HRM). XgF06 and XgF07 were only found in TYN and HRM, respectively.

Sequencing of COI from the mtDNA of X. germanus aligned 794 bp. Twenty haplotypes were defined from 133 individuals. These fragments had a 34.6% G/C content. The X. germanus haplotypes were clustered as a monophyletic group together with the haplotypes of the outgroups of X. brevis (AB476316) and Xyllosandrus crassiusculus (Motschulsky) (AB462579; Figure 8). The phylogenetic analysis revealed three distinct clades (A–C) with high bootstrap values. Clade A had 13 haplotypes (XgCOI01–13), clade B six haplotypes (XgCOI14–19), and clade C one haplotype (XgCOI20). X. germanus clades A, B, and C were unexceptionally associated with symbiotic fungi Types I, II, and III, respectively (Table 4, Appendix 1). However, no specific associations were observed between clade A and Type I at the haplotype level. For example, XgCOI03 of clade A had all haplotypes of Type I fungi in its mycangia, expect for XgF01. The numbers of Type II and III fungi haplotypes were too small to evaluate the relationships between the fungal and beetle haplotypes.

Nucleotide sequences obtained in this study were submitted to the DDBJ/EMBL/GenBank databases (accession numbers: LC140885–LC140924) (Table 5).

4 | DISCUSSION

Symbiotic fungi isolated from X. germanus mycangia in Japan had all three cultural types (Figures 1 and 2). The three types formed one clade with A. grosmaniae, A. roeperi, A. hartigi and A. xylebori in 18S and 28S (Figures 3 and 4). In β-tubulin gene, X. germanus fungi clustered as a monophyletic group together with A. beaveri, A. hartigi and A. xylebori clade (Figure 5). In combined three loci, X. germanus fungi clustered as a monophyletic group together with A. hartigi and A. xylebori clade (Figure 6). Therefore, all fungal isolates obtained in this study were identified as closely related species to four species, A. grosmaniae, A. roeperi, A. hartigi, and A. xylebori. Phylogenetic analyses based on 28S rDNA, β-tubulin, and the combined three loci revealed that Types I and II haplotypes formed subclade within X. germanus fungi clade with high bootstrap values (Figures 4–6). These results suggest that three types of X. germanus fungi, which are distinct from each other as per morphological and phylogenetic characters,
are distributed in Japan. These results also suggest that Type III first differentiated from ancestral members, common to all three types, and subsequently, ancestral members of Types I and II have differentiated into Types I and II.

The COI haplotypes of *X. germanus* were divided into three distinct lineages (Figure 8). This result was the same that of Ito et al. (2008). The beetles had a specific type of symbiotic fungi for each clade (Figure 8 and Table 4). These results suggest that *X. germanus* are unable to exchange mycangial fungi between clades of beetles. However, horizontal transmission of mycangial fungi may occur within the same beetle lineage, because no specific relationships were found between beetle and fungal haplotypes within same clade (Table 4). Some bark beetles likely exchange their fungi between neighboring nests in the same host tree (Six, 2003; Six & Bentz, 2007). Each Type I haplotype showed a nonrandom distribution on the Japanese archipelago (Figures 7 and 8). These distributions may be formed by *X. germanus*, because fungal dispersion depends on beetle migration. *X. germanus* cannot migrate between Hokkaido and other regions in Japan because of the Tsugaru Strait geographical barrier (Ito et al., 2008). However, scolytine beetles have a flying range of 10–15 km (Gries, 1985; Wood, 1982). Thus, the migration ability of the beetle may regulate fungal dispersion, resulting in the lack of random distribution in the Type I haplotypes. Additionally, COI clades of *X. germanus* can be distinguished by the cultural types of its symbiotic fungi, because the clades have strong correlations with the cultural types (Figure 8 and Table 4).

The phylogenetic divergence patterns of the symbiotic fungi did not coincide with those of *X. germanus* (Figures 6 and 8). In mycangial fungi, Type III lineage was sister to a clade containing Type I and Type II lineages (Figure 6). In contrast, ancestral members of *X. germanus* branched into clade A and clades B and C lineages first, and clade B and clade C lineages divided subsequently from the clades B and C (Figure 8). These results suggest that the first fungal differentiation may have occurred together with the first beetle differentiation. In particular, Types I and II and Type III may have concurrently diverged from fungal ancestors when clade A and clades B and C differentiated from beetle ancestors. Ito (2009) showed the differentiation between clade A and a clade composed of clades B and C, and clade B and clade C of *X. germanus* occurred six MYA and 5.2 MYA, respectively. Based on this molecular clock, first的不同
**FIGURE 4** Phylogram obtained from 607 bp of the 28S rDNA of symbiotic fungi isolated from mycangia of *Xylosandrus germanus* and related fungus species. One of 376 maximum parsimony (MP) trees. CI = 0.900, RI = 0.979, length = 22 steps. Bootstrap values (left) and branch support values (right) (>50%) are given above the branches. Bold letters indicate sequences obtained in this study. Cultural types (Type I–III) defined in Figure 1 are shown in bracket after haplotype codes (XgF28S01–05). XbF and SmF represent symbiotic fungi isolated from mycangia of *Xylosandrus brevis* and *Scolytoplatypus mikado*, respectively.

**FIGURE 5** Phylogram obtained from approximately 440 bp of the β-tubulin gene from symbiotic fungi isolated from mycangia of *Xylosandrus germanus* and related fungus species. One of 56 maximum-parsimony (MP) trees. CI = 0.840, RI = 0.898, length = 358 steps. Bootstrap values (left) and branch support values (right) (>50%) are given above the branches. Bold letters indicate sequences obtained in this study. Cultural types (Type I–III) defined in Figure 1 are shown in bracket after haplotype codes (XgFBt01–06). XbF and SmF represent symbiotic fungi isolated from mycangia of *Xylosandrus brevis* and *Scolytoplatypus mikado*, respectively.
### TABLE 4

Haplotype frequencies both mitochondrial DNA (COI) of *Xylosandrus germanus* and three loci DNA\(^a\) of symbiotic fungi isolated from its mycangia

| Insect clades | Insect haplotypes | Type I | Type II | Type III | Total |
|---------------|-------------------|--------|---------|----------|-------|
|               |                   | XgF01  | XgF02   | XgF03    | XgF04  | XgF05  | XgF06  | XgF07  | XgF08 | XgF09 | XgF10 | XgF11 | Total |
| A             | XgCOI01           | 6      | 12      | 1        | –       | –       | –      | –      | –     | –     | –     | –     | 19    |
|               | XgCOI02           | –      | –       | –        | –       | –       | –      | –      | –     | –     | –     | –     | –     |
|               | XgCOI03           | –      | 3       | 12       | 7       | 19      | 1      | 1      | –     | –     | –     | –     | 43    |
|               | XgCOI04           | –      | 1       | –        | –       | –       | –      | –      | –     | –     | –     | –     | 2     |
|               | XgCOI05           | 1      | 1       | –        | 1       | –       | –      | –      | –     | –     | –     | –     | 3     |
|               | XgCOI06           | –      | –       | –        | 1       | –       | –      | –      | –     | –     | –     | –     | 1     |
|               | XgCOI07           | –      | –       | 3        | –       | –       | –      | –      | –     | –     | –     | –     | 3     |
|               | XgCOI08           | –      | –       | –        | 1       | –       | –      | –      | –     | –     | –     | –     | 1     |
|               | XgCOI09           | –      | –       | 1        | –       | –       | –      | –      | –     | –     | –     | –     | 1     |
|               | XgCOI10           | –      | 1       | 2        | 1       | –       | –      | –      | –     | –     | –     | –     | 4     |
|               | XgCOI11           | –      | –       | –        | 1       | –       | –      | –      | –     | –     | –     | –     | 1     |
|               | XgCOI12           | –      | –       | –        | –       | 1       | –      | –      | –     | –     | –     | –     | 1     |
|               | XgCOI13           | –      | –       | –        | –       | –       | 1      | –      | –     | –     | –     | –     | 1     |
| B             | XgCOI14           | –      | –       | –        | –       | –       | –      | –      | –     | 2     | –     | –     | 2     |
|               | XgCOI15           | –      | –       | –        | –       | –       | –      | –      | 1     | 19    | –     | –     | 20    |
|               | XgCOI16           | –      | –       | –        | –       | –       | –      | –      | –     | 7     | –     | –     | 7     |
|               | XgCOI17           | –      | –       | –        | –       | –       | –      | –      | 2     | 6     | –     | –     | 8     |
|               | XgCOI18           | –      | –       | –        | –       | –       | –      | –      | 1     | –     | –     | –     | 1     |
| C             | XgCOI20           | –      | –       | –        | –       | –       | –      | –      | –     | –     | 10    | 1     | 11    |
|               | Total             | 7      | 19      | 19       | 11      | 22      | 1      | 1      | 4     | 34    | 10    | 1     | 129   |

\(a\) Combined 18S rDNA, 28S rDNA, and \(\beta\)-tubulin gene.
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mycangial fungi may have occurred six MYA. *X. germanus* had already developed into three lineages before colonization of the Japanese archipelago (Ito et al., 2008). After colonization, clade A and B beetles secondarily came into contact during the last glacial epoch in Japan (Ito et al., 2008). Type II was not differentiated within subclade I–II (Figure 6), suggesting that clade B ancestors may have symbiotically associated with Type II when clade B occurred and contacted to clade A.

We obtained two important results related to the phylogeny of *X. germanus* and its symbiotic fungi: a single beetle lineage is consistently associated with a single fungal type in the *X. germanus* fungal symbiont system, although more than two types of symbiotic fungi were found in northern populations (Figure 2), and exceptional horizontal transmission in symbiotic fungi between beetles lineages occurred at least once, sustaining novel beetle-fungus symbiotic relationships. Why are the beetles unable to exchange symbiotic fungi from the existing type to other types? In ambrosia beetles, glandular secretions into the mycangium can facilitate the growth of specific ambrosia fungi (Harrington, 2005; Norris, 1979). Some bark beetles such as the southern pine beetle (*Dendroctonus frontalis* Zimmermann) also have glandular cells in their mycangia and carry one specific fungal symbiont (Bridges, 1985). Thus, it is possible that specific ambrosia fungi lineages in *X. germanus* are selected by mycangia secretion. Colony growth rate on PDA and the competitive race of each fungal type vary according to thermal conditions (Ito & Kajimura, 2011). Some symbiotic fungi of bark beetles also have thermal traits in the field (Six & Bentz, 2007; Six & Paine, 1997; Solheim & Krokene, 1998). The fitness level of ambrosia and bark beetles decreases or increases depending on the symbiotic fungal species used for nutrition (Harrington, 2005; Kajimura, 2000; Six & Bentz, 2007). Therefore, *X. germanus* and their mycangial fungi mutual systems may experience constant

FIGURE 6  Phyllogram obtained from about 2,000 bp of the combined loci (the 18S, the 28S rDNA, and the β-tubulin gene) of symbiotic fungi isolated from mycangia of *Xylosandrus germanus* and related fungi species. One of 10 maximum-parsimony (MP) trees. CI = 0.863, RI = 0.913, length = 519 steps. Bootstrap values (left) and branch support values (right) (>50%) are given above the branches. Bold letters indicate sequences obtained in this study. Cultural types (Type I–III) defined in Figure 1 are shown in bracket after haplotype codes (XgF01-11). XbF and SmF represent symbiotic fungi isolated from mycangia of *Xylosandrus brevis* and *Scolytoplatypus mikado*, respectively

| Sequence | Haplotype Code | Cultural Type |
|----------|---------------|---------------|
| Phialophoropsis ferruginea strain CBS 408.68 | XgF10 (Type III) | |
| Phialophoropsis ferruginea strain JB13 | XgF11 (Type III) | |
| Phialophoropsis ferruginea strain CBS 378.68 | XgF09 (Type II) | |
| Ceratocystis adiposa strain CBS600.74 | XgF08 (Type II) | |
| SmF01 | | |
| XbF01 | | |
| Ambrosiella xylebori strain CBS 110.61 | | |
| Ambrosiella hartigii strain CBS 404.82 | | |
| XgF03 (Type I) | XgF04 (Type I) | |
| XgF02 (Type I) | XgF05 (Type I) | |
| XgF07 (Type I) | XgF06 (Type I) | |
| 20 nucleotide change | 50/90 | |
| 99/100 | 100/100 | |
| 97/100 | 99/100 | |
| 93/100 | 99/100 | |
| 91/100 | 99/100 | |
| 94/100 | 100/100 | |
These selection pressures would help maintain the specific relationships between *X. germanus* and their mycangial fungi. Further investigations, particularly those focusing on glandular cells and thermal conditions, will clarify the factors involved in maintaining the *X. germanus*-fungal symbiosis.
TABLE 5 List of GenBank accession no. of nucleotide sequences obtained in this study

| Species                                           | Locus                  | Haplotype       | GenBank accession no. |
|---------------------------------------------------|------------------------|-----------------|-----------------------|
| Fungal symbiont isolated from X. germanus mycangium | 18S ribosomal RNA      | XgF18S01        | LC140885              |
|                                                   |                        | XgF18S02        | LC140886              |
|                                                   | 28S ribosomal RNA      | XgF28S01        | LC140889              |
|                                                   |                        | XgF28S02        | LC140890              |
|                                                   |                        | XgF28S03        | LC140891              |
|                                                   |                        | XgF28S04        | LC140892              |
|                                                   |                        | XgF28S05        | LC140893              |
|                                                   | β-tubulin gene         | XgFBt01         | LC140897              |
|                                                   |                        | XgFBt02         | LC140898              |
|                                                   |                        | XgFBt03         | LC140899              |
|                                                   |                        | XgFBt04         | LC140900              |
|                                                   |                        | XgFBt05         | LC140901              |
|                                                   |                        | XgFBt06         | LC140902              |
| Fungal symbiont isolated from X. brevis mycangium  | 18S ribosomal RNA      | XbF18S01        | LC140887              |
|                                                   |                        | XbF18S01        | LC140889              |
|                                                   | 28S ribosomal RNA      | XbF28S01        | LC140893              |
|                                                   | β-tubulin gene         | XbFBt01         | LC140903              |
| Fungal symbiont isolated from S. mikado mycangium | 18S ribosomal RNA      | SmF18S01        | LC140888              |
|                                                   |                        | SmF18S01        | LC140895              |
|                                                   | 28S ribosomal RNA      | SmF28S01        | LC140896              |
|                                                   | β-tubulin gene         | SmFBt01         | LC140904              |
| Xylosandrus germanus                               | COI mtDNA              | XgCOI01         | LC140905              |
|                                                   |                        | XgCOI02         | LC140906              |
|                                                   |                        | XgCOI03         | LC140907              |
|                                                   |                        | XgCOI04         | LC140908              |
|                                                   |                        | XgCOI05         | LC140909              |
|                                                   |                        | XgCOI06         | LC140910              |
|                                                   |                        | XgCOI07         | LC140911              |
|                                                   |                        | XgCOI08         | LC140912              |
|                                                   |                        | XgCOI09         | LC140913              |
|                                                   |                        | XgCOI10         | LC140914              |
|                                                   |                        | XgCOI11         | LC140915              |
|                                                   |                        | XgCOI12         | LC140916              |
|                                                   |                        | XgCOI13         | LC140917              |
|                                                   |                        | XgCOI14         | LC140918              |
|                                                   |                        | XgCOI15         | LC140919              |
|                                                   |                        | XgCOI16         | LC140920              |
|                                                   |                        | XgCOI17         | LC140921              |
|                                                   |                        | XgCOI18         | LC140922              |
|                                                   |                        | XgCOI19         | LC140923              |
|                                                   |                        | XgCOI20         | LC140924              |

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CONFLICT OF INTEREST
None declared.

AUTHOR CONTRIBUTIONS
M. Ito designed the study, wrote the initial draft of the manuscript, and analyzed and interpreted data in the study. H. Kajimura contributed to interpretation of data, assisted in the preparation of the manuscript, and critically reviewed the manuscript. All authors approved the final version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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**APPENDIX 1**  List of cultural types and haplotypes of symbiotic fungi isolated from mycangia of *Xylosandrus germanus* and haplotypes of *X. germanus* samples

| Acronym | ID   | Cultural types | Fungus haplotypes | Insect haplotypes |
|---------|------|----------------|-------------------|------------------|
| HKF     | HKF-01 | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-02  | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-03  | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-04  | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-05  | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-06  | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-07  | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-08  | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-09  | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-10  | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-11  | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-12  | Type-I        | 01 01 01 01 01 | 01 01 01 01 01 |
| HKF-13  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-14  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-15  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-16  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-17  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-18  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-19  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-20  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-21  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-22  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-23  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-24  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKF-25  | Type-II       | 02 01 01 02 02 | 01 01 01 01 01 |
| HKS     | HKS-01 | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| HKS-02  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| HKS-03  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| HKS-04  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| HKS-05  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| HKS-06  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| HKS-07  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| HKS-08  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| HKS-09  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| HKS-10  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| HKS-11  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| IWI     | IWI-01 | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| IWI-02  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| IWI-03  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| IWI-04  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| IWI-05  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| IWI-06  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |
| IWI-07  | Type-I        | 01 02 01 02 01 | 01 02 01 02 01 |

(Continues)
| Acronym | ID   | Cultural types | 18S | 28S | β-tubulin | Three loci<sup>b</sup> | COI |
|---------|------|----------------|-----|-----|-----------|------------------------|-----|
| YMT     | YMT-01 | Type-I         | 01  | 02  | 03        | 05                     | 03  |
|         | YMT-02 | Type-I         | 01  | 01  | 02        | 03                     | 03  |
|         | YMT-03 | Type-I         | 01  | 02  | 03        | 05                     | 04  |
|         | YMT-04 | Type-I         | 01  | 02  | 02        | 04                     | 03  |
|         | YMT-05 | Type-I         | 01  | 01  | 01        | 01                     | 05  |
|         | YMT-06 | Type-I         | 01  | 02  | 02        | 04                     | 03  |
|         | YMT-07 | Type-I         | 01  | 02  | 01        | 02                     | 05  |
|         | YMT-08 | Type-I         | 01  | 02  | 02        | 04                     | 05  |
|         | YMT-09 | Type-I         | 01  | 02  | 01        | 02                     | 04  |
|         | YMT-10 | Type-I         | 01  | 02  | 02        | 04                     | 03  |
|         | YMT-11 | Type-III       | 01  | 04  | 06        | 10                     | 20  |
| SIC     | SIC-01 | Type-II        | 01  | 01  | 05        | 08                     | 15  |
|         | SIC-02 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
|         | SIC-03 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
|         | SIC-04 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
|         | SIC-05 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
|         | SIC-06 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
|         | SIC-07 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
|         | SIC-08 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
|         | SIC-09 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
|         | SIC-10 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
| NGM     | NGM-01 | Type-I         | 01  | 02  | 03        | 05                     | 03  |
|         | NGM-02 | Type-I         | 01  | 02  | 03        | 04                     | 06  |
| NGC     | NGC-01 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
|         | NGC-02 | Type-II        | 02  | 01  | 05        | 09                     | 16  |
|         | NGC-03 | Type-II        | 02  | 01  | 05        | 09                     | 16  |
|         | NGC-04 | Type-II        | 02  | 01  | 05        | 09                     | 16  |
|         | NGC-05 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
|         | NGC-06 | Type-II        | 02  | 01  | 05        | 09                     | 16  |
|         | NGC-07 | Type-II        | 02  | 01  | 05        | 09                     | 16  |
|         | NGC-08 | Type-II        | 02  | 01  | 05        | 09                     | 15  |
|         | NGC-09 | Type-II        | 02  | 01  | 05        | 09                     | 16  |
|         | NGC-10 | Type-II        | 02  | 01  | 05        | 09                     | 16  |
| NGS     | NGS-01 | Type-I         | 01  | 01  | 02        | 03                     | 07  |
|         | NGS-02 | Type-I         | 01  | 02  | 02        | 03                     | 07  |
|         | NGS-03 | Type-I         | 01  | 01  | 02        | 04                     | 08  |
|         | NGS-04 | Type-I         | 01  | 01  | 02        | 03                     | 07  |

(Continues)
APPENDIX 1 (Continued)

| Acronym<sup>a</sup> | ID   | Cultural types | Fungus haplotypes | Insect haplotypes | 18S | 28S | β-tubulin | Three loci<sup>b</sup> | COI |
|---------------------|------|----------------|-------------------|-------------------|-----|-----|-----------|----------------|-----|
| TYN                 | TYN-01 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | TYN-02 | Type-I         | 01                | 02                | 02  | 04  | 03        | 03             | 03  |
|                     | TYN-03 | Type-I         | 01                | 02                | —   | —   | 10        | 11             | 11  |
|                     | TYN-04 | Type-I         | 01                | 02                | 02  | 04  | 11        | 11             | 11  |
|                     | TYN-05 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | TYN-06 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | TYN-07 | Type-I         | 01                | 02                | 01  | 02  | 03        | 11             | 11  |
|                     | TYN-08 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | TYN-09 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | TYN-10 | Type-I         | 01                | 02                | 04  | 06  | 03        | 03             | 03  |
| AIT                 | AIT-01 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | AIT-02 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | AIT-03 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | AIT-04 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | AIT-05 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | AIT-06 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | AIT-07 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | AIT-08 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | AIT-09 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | AIT-10 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
| KTN                 | KTN-01 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | KTN-02 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | KTN-03 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | KTN-04 | Type-I         | 01                | 02                | 01  | 02  | 11        | 11             | 11  |
|                     | KTN-05 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | KTN-06 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | KTN-07 | Type-I         | 01                | 02                | 03  | 05  | 12        | 05             | 05  |
|                     | KTN-08 | Type-I         | 01                | 01                | 02  | 03  | 03        | 03             | 03  |
|                     | KTN-09 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | KTN-10 | Type-I         | 01                | 02                | 02  | 04  | 03        | 03             | 03  |
| WKT                 | WKT-01 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
| HRM                 | HRM-01 | Type-I         | 01                | 03                | 03  | 07  | 03        | 03             | 03  |
|                     | HRM-02 | Type-I         | 01                | 02                | —   | —   | 03        | 03             | 03  |
|                     | HRM-03 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | HRM-04 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | HRM-05 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | HRM-06 | Type-I         | 01                | 02                | 03  | 05  | 13        | 03             | 03  |
|                     | HRM-07 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | HRM-08 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | HRM-09 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | HRM-10 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |
|                     | HRM-11 | Type-I         | 01                | 02                | 03  | 05  | 03        | 03             | 03  |

(Continues)
### APPENDIX 1 (Continued)

| Acronym | ID  | Cultural types | Fungus haplotypes | Insect haplotypes |
|---------|-----|----------------|-------------------|-------------------|
| KUH     | KUH-01 Type-II | 02 01 05 09 17 |                   |                   |
|         | KUH-02 Type-II | 02 01 05 09 17 |                   |                   |
|         | KUH-03 Type-II | 02 01 05 09 17 |                   |                   |
|         | KUH-04 Type-II | 02 01 — — 18   |                   |                   |
|         | KUH-05 Type-II | 02 01 05 09 17 |                   |                   |
|         | KUH-06 Type-II | 02 01 05 09 17 |                   |                   |
|         | KUH-07 Type-II | 01 01 05 08 19 |                   |                   |
|         | KUH-08 Type-II | 02 01 05 09 17 |                   |                   |
|         | KUH-09 Type-II | 01 01 05 08 17 |                   |                   |
|         | KUH-10 Type-II | 01 01 05 08 17 |                   |                   |

*a Site names are defined in Table 1.

*b Combined 18S rDNA, 28S rDNA and β-tubulin gene.