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FULL PAPER

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Aspergillus bombycis genotypes (RFLP) from silkworm cultivation

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Abstract Eighteen isolates of *Aspergillus bombycis* from samples of dust, insect frass, and soil collected from eight silkworm rearing facilities in Japan, as well as single silkworm rearing facilities in Indonesia and Malaysia, were subjected to DNA fingerprinting. *Pst*I digests of total genomic DNA from each isolate were probed using the pAF 28 repetitive sequence. Among 18 isolates analyzed, 7 distinct DNA fingerprint groups were identified, including GTAb-2 isolated from rearing facilities in four prefectures of Japan. *Aspergillus bombycis* isolates share several features in common with domesticated yellow-green aspergilli, the koji molds used in traditional Oriental food fermentations, including a loose and deep colony texture, smooth-walled stipes, and the absence of sclerotia. Although aflatoxin is unknown from koji molds, all isolates of *A. bombycis* produced B and G aflatoxins. Aflatoxin has been linked to increased virulence in *Aspergillus* disease of silkworms, and there should be strong selection for aflatoxin production among clonal populations of *A. bombycis* adapted to silkworm cultivation. A hypothesis is offered that *A. bombycis* isolates from silkworm cultivation represent highly adapted forms of yet to be discovered "wild" populations that may infect *Bombyx mandarina*.

Key words Aflatoxin · *Aspergillus bombycis* · *Bombyx mori* · DNA fingerprints · Silkworm

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Introduction

Aspergillus bombycis Peterson et al. (*Aspergillus* section *Flavi*) is a recently described species known only from domesticated silkworm [*Bombyx mori* L. (Lepidoptera: Bombycidae)] culture in Indonesia and Japan (Peterson et al. 2001). In Japan, silkworm larvae hatched from eggs are grown in "cooperative rearing houses for young silkworms" for about 1 week, and then they are distributed to sericultural farmers for rearing to cocooning (Kawakami 1982). The larvae are fed chopped mulberry (*Morus alba* L.) leaves. The rearing houses are equipped to maintain a high temperature and humidity (28°–30°C, 85%–95% RH), conditions promoting optimal growth of the young larvae while preventing the mulberry leaves from withering (Yokoyama 1963; Kawakami 1982). This environment also favors the growth and sporulation of *Aspergillus* on silkworm feces (frass) and leaf litter, creating a high risk for *Aspergillus* disease among the younger larvae (Kawakami 1982).

This study contrasts the DNA fingerprints (restriction fragment length polymorphism, RFLP) of 18 cultures of *A. bombycis* that were isolated from eight silkworm rearing facilities in seven prefectures of Japan, as well as individual silkworm rearing facilities in both Indonesia and Malaysia (Table 1). These cultures were initially identified as isolates of *Aspergillus flavus* Link: Fr. Polymerase chain reaction (PCR) amplification (White et al. 1990) and sequencing of the internal transcribed spacer (ITS)1–5.8S rDNA–ITS2 regions, followed by comparisons to sequences from the ex-type culture of *A. bombycis* (Peterson et al. 2001), were used to make species-level identifications of all 18 *A. bombycis* isolates. None of the *A. bombycis* cultures were isolated directly from diseased or dead silkworms. Two cultures of *Aspergillus* section *Flavi*, IFO 8557 *Aspergillus oryzae* (Ahlb.) Cohn and IFO 8558 *A. flavus*, were isolated from diseased silkworms by Dr. Kiyoshi Aoki, Sericultural

Table 1. DNA fingerprint matches for *Aspergillus bombycis* isolates from silkworm rearing houses

Genotype	Strain	Isolated		Source
		Year	By	
GTAb-1	NRRL 28900	1997	H. Takahashi	Frass, silkworm rearing house, SW 1, Chiba Pref., Japan
	NRRL 28901	1997	H. Takahashi	Frass, silkworm rearing house, SW 2, Chiba Pref., Japan
	NRRL 29236	1983	K. Tanaka	Dust, silkworm breeding room, Tsukuba, Ibaraki Pref., Japan (=MAFF 235213)
	NRRL 29237	1983	K. Tanaka	Soil, silkworm breeding room, Tsukuba, Ibaraki Pref., Japan (=MAFF 235218)
	NRRL 31137	1983	K. Tanaka	Dust, silkworm breeding room, Tsukuba, Ibaraki Pref., Japan (=MAFF 111304)
GTAb-2	NRRL 26010	1987	T. Goto	Frass, silkworm rearing house No. 2, Tokushima Pref., Japan
	NRRL 29241	1983	K. Tanaka	Dust, silkworm breeding room, Ooita Pref., Japan (=MAFF 111301)
	NRRL 31407	1984	K. Tanaka	Dust, silkworm breeding room, Kumamoto Pref., Japan (=MAFF 235224)
	NRRL 31408	1984	K. Tanaka	Dust, silkworm breeding room, Kumamoto Pref., Japan (=MAFF 235225)
	NRRL 31409	1984	K. Tanaka	Dust, silkworm breeding room, Kumamoto Pref., Japan (=MAFF 235228)
	NRRL 31405	1983	K. Tanaka	Dust, silkworm breeding room, Tsukuba, Ibaraki Pref., Japan (=MAFF 111548)
	NRRL 31410	1984	K. Tanaka	Dust, silkworm breeding room, Kumamoto Pref., Japan (=MAFF 235229)
GTAb-3	NRRL 31134	2000	T. Goto	Frass, silkworm rearing house, W. Sarawaku, Karimantan, Malaysia
	NRRL 31135	2000	T. Goto	Frass, silkworm rearing house, W. Sarawaku, Karimantan, Malaysia
GTAb-4	NRRL 29235	1983	K. Tanaka	Dust, silkworm breeding room, Yamagata Pref., Japan (=MAFF 235209)
GTAb-5	NRRL 25593	1987	T. Goto	Frass, silkworm rearing house No. 1, Kagawa Pref., Japan
GTAb-6	NRRL 31132	2000	T. Goto	Frass, silkworm rearing company, W. Sarawaku, Karimantan, Malaysia
GTAb-7	NRRL 29253	1998	T. Goto	Frass, silkworm rearing house, East Java, Indonesia
A. nomius	NRRL 31105	?	K. Aoki	Diseased silkworm: "*Aspergillus oryzae* disease" (=IFO 8557)
A. flavus	NRRL 31106	?	K. Aoki	Diseased silkworm: "brown muscardine disease" (=IFO 8558)

Experiment Station, Tokyo (Institute for Fermentatation Osaka, 1992). IFO 8557 was isolated from a silkworm showing symptoms of "*Aspergillus oryzae* disease," a direct translation of the Japanese name for the disease (Dr. Akira Nakagiri, personal communication). DNA sequencing revealed that NRRL 31105 (=IFO 8557) is *Aspergillus nomius* Kurtzman et al. IFO 8558 *Aspergillus flavus* was isolated from a silkworm showing symptoms of brown muscardine disease. DNA sequencing revealed that NRRL 31106 (=IFO 8558) is *A. flavus* (see Table 1). We find it both interesting and significant that the name *Aspergillus oryzae* has been applied to a disease of silkworms. *Aspergillus bombycis* is also described with a colony texture being floccose and pale greenish-yellow in color, the absence of sclerotia, and smooth stipes (Peterson et al. 2001). This common cultural appearance could suggest some degree of morphological convergence among isolates of *Aspergillus* section *Flavi* from diseased silkworms and the *Aspergillus* section *Flavi* cultures used as koji for traditional oriental food fermentations (Wicklow 1984; Wicklow et al. 2002). At the same time, isolates of *Aspergillus* section *Flavi* from silkworm cultivation produce aflatoxins whereas domesticated koji molds do not (Wang and Hesseltine 1982).

*Pst*I digests of total genomic DNA from each isolate were probed using the pAF28 repetitive sequence (McAlpin and Mannarelli 1995). The pAF28 DNA probe has proven reliable in classifying *A. flavus* strains according to their previously determined vegetative compatibility group (VCG) (McAlpin and Mannarelli 1995; McAlpin et al. 2002). The probe has also been used to estimate the genotypic diversity of *A. flavus* and *A. parasiticus* populations isolated from corn fields in Iowa and Illinois (Wicklow et al. 1998; McAlpin et al. 1998; D.T. Wicklow, C.E. McAlpin, and L.H. Tiffany, unpublished data) and that of koji molds used in traditional Oriental food fermentations (Wicklow et al. 2002), and to type clinical isolates of *A. flavus* from human sources (James et al. 2000). We wanted to examine the genetic diversity (RFLP) of this collection of *A. bombycis* isolates to determine if a few "domesticated" forms of *A . bombycis* are common to silkworm cultivation.

Materials and methods

Fungal strains

All cultures were obtained from the Agricultural Research Service Culture Collection, Peoria, Illinois, USA. Isolation data, permanent accession numbers, and source of the fungi examined in this study are listed in Table 1. Cultures were grown on slants of Czapek's agar (Cz) (Raper and Fennell 1965) for 10 days (25°C). A conidial suspension (density, 10^6/ml sterile distilled water containing 0.01% Triton X-100) prepared from the Cz slants served as inoculum for the procedures and analyses presented herein.

Mycotoxin production

Aflatoxin production was measured using glucose yeast extract (GY) broth (Wongurai et al. 1990), followed by high performance liquid chromatography (Manabe et al. 1978). Cyclopiazonic acid production was assayed from isolates grown on modified Czapek–Dox broth (Goto et al. 1996) using high performance liquid chromatography (Goto et al. 1987). Kojic acid production was determined by growing the fungi in glucose-peptone broth, and detection was performed using high performance liquid chromatography (Manabe et al. 1984).

DNA extraction

Fungal mycelia used for DNA extractions were grown from *Aspergillus* conidial or cell suspensions [1×10^5 colony-forming units (CFU)/ml culture medium] in 500-ml flasks containing 200 ml yeast extract-peptone-dextrose (YEPD) broth (3 g yeast extract, 10 g peptone, and 20 g dextrose in 1 l distilled water). Inoculum was obtained by harvesting conidia from 10-day slant cultures of Czapek agar that had been incubated at 25°C. Following incubation at 200 rpm on a rotary shaker for 22–24 h at 32°C, the mycelium was harvested by filtering through a Whatman no. 1 filter paper in a Buchner funnel and rinsed two or three times with sterile distilled water. The mycelial mat was placed in Sarstedt tubes, frozen overnight, and lyophilized for 24 h. DNA from the harvested mycelial mat was isolated and purified using the method of Raeder and Broda (1985) as modified by McAlpin and Mannarelli (1995).

DNA hybridization and detection

Total fungal DNA was digested to completion with the restriction endonuclease *Pst*I (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's recommendations. Then, 8 μg digested DNA was dispensed in each lane on 0.8% agarose gel in TAE buffer [0.04 M Tris-acetate, pH 8.0; 0.001 M ethylenediamine tetraacetic acid (EDTA)], run at $1.5\,V\,cm^{-1}$ for 22 h, and visualized with UV light after staining with ethidium bromide. Southern blots were performed according to the manufacturer's protocol by transferring restriction fragments from agarose gels to nylon membranes (Nytran N; Schleicher and Schuell, Keene, NH, USA) using a vacuum blotter (model 785; BioRad, Hercules, CA, USA). Probes were labeled by random primed labeling with digoxigenin using the Nucleic Acid Non-radioactive Hybridization System (Roche). Membranes were prehybridized, hybridized with labeled probes, and washed. DNA fingerprints were detected by CSPD (disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro) tricyclo [3.3.1^{3,7}] decan}-4-yl) phenyl phosphate) and exposed to Biomax MR X-ray film (Kodak Rochester, NY, USA) at room temperature for 1–2 h. Several film exposures were made to identify bands of varying intensity. The entire procedure involving DNA isolation, hybridization, detection, and fingerprint analyses was frequently repeated to confirm the DNA profiles of individual fungal cultures.

DNA fingerprint analyses

DNA fragments (pAF28 fingerprints) were compared by designating and recording 55 fragment positions, representing different molecular weights, with an equidistant marker. Each gel included three control lanes for the reference isolate, K.E. Papa strain *A. flavus* NRRL 19997 (McAlpin and Mannarelli 1995; Wicklow et al. 1998), and a lambda standard. Fragments within and between gels could be distinguished using the reference isolate. Cultures producing similar banding patterns in different gels were reprobed on the same gel to verify fragment positions. Some thicker and darker bands were difficult to decipher and these were reprobed, in addition to using different film exposures to identify bands of varying intensity. Fragments of more than 20 kb and less than 1.5 kb were not included in the analyses. *Aspergillus bombycis* genotypes (pAF28 fingerprints) were classified on the basis of the presence or absence of fragments, each of which is presumed to represent a single genetic locus. Isolates with identical fingerprints were recognized as belonging to the same genotype and may represent the same clonal population. Dice similarity coefficients (C) were used to calculate pairwise matching similarity values for each pair of isolates according to the equation $C = 2N_{xy}/(N_x + N_y)$, in which N_{xy} is the number of hybridizing DNA bands shared by the isolates x and y, and N_x and N_y refer to the number of DNA bands in isolates x and y, respectively (Nei and Li 1979). The similarity coefficients were used to generate the cluster analysis with SIMQUAL and SAHN programs (Rohlf 1993). DNA fingerprint groups were arbitrarily established to include any isolates with more than 80% similarity in numbers of hybridizing bands after Xia et al. (1993). The SAHN program determines which isolates share identical fingerprints or identifies those belonging to the same DNA fingerprint group (McAlpin et al. 1998), but it does not imply phylogenetic relationships.

Results

DNA fingerprint analyses

DNA fingerprinting was performed on 18 isolates of *A. bombycis* isolated from eight silkworm rearing facilities in Japan and individual rearing facilities in both Indonesia and Malaysia. DNA fingerprint matches are summarized in Table 1. Isolates of *A. bombycis* were distributed among seven DNA fingerprint groups, including GTAb-1 represented by 5 isolates, GTAb-2 represented by 7 strains, GTAb-3 represented by 2 isolates, and 4 isolates (NRRL 29235, NRRL 29253, NRRL 25593, NRRL 31132) producing DNA fingerprints that did not match any other isolate (<80% band similarity). *Aspergillus bombycis* strains belonging to GTAb-1 and GTAb-4 produced DNA fingerprints with bands of sufficient intensity to be readily distinguished when printed from a photographic negative (Fig. 1). The other strains produced faint bands that could be reproducibly detected on the original Southern blot film.

DNA fingerprinting revealed that genotypes GTAb-1 and GTAb-2 were distributed among three and four silkworm rearing facilities in Japan. GTAb-1 was isolated from samples of silkworm frass collected from rearing houses at two locations in Chiba Prefecture and a sample of dust collected from a silkworm breeding room within a cooperative rearing house near Tsukuba, Ibaraki Prefecture. GTAb-2 was isolated from samples of dust collected from silkworm breeding rooms at cooperative rearing facilities in Tsukuba (above), Kumamoto Prefecture, Ooita Prefecture,

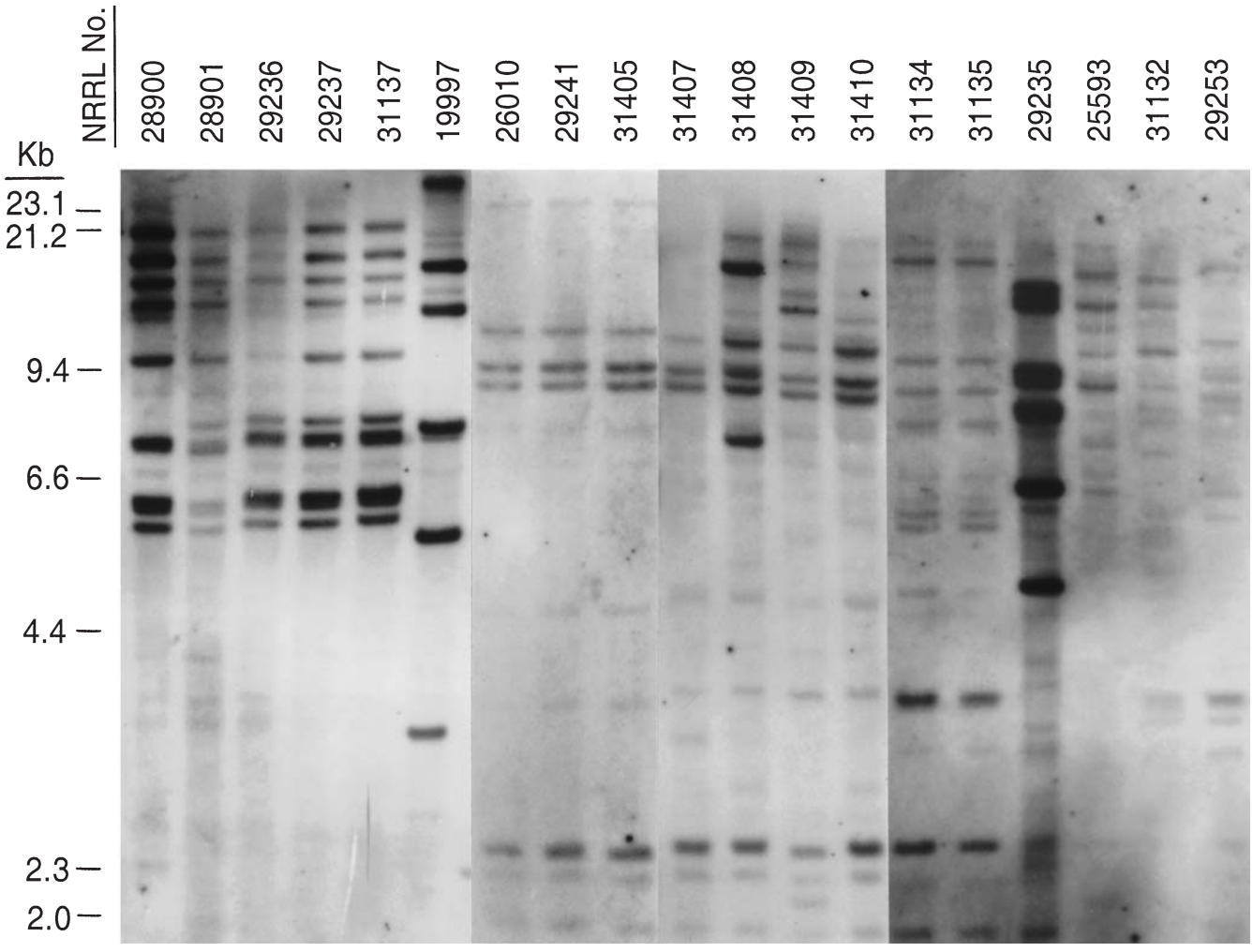

Fig. 1. DNA fingerprints of *Aspergillus bombycis* strains. The figure includes one reference strain (*Aspergillus flavus* NRRL 19997) and size markers

and from frass collected at a silkworm rearing house on Shikoku Island (Kagawa Prefecture). Four cultures of *A. bombycis* GTAb-2 from a rearing facility in Kumamoto Prefecture were each isolated from a different dust sample. *Aspergillus bombycis* isolates NRRL 31137 (GTAb-1) and NRRL 31405 (GTAb-2) were isolated from the same dust sample collected at Tsukuba. Two *A. bombycis* genotypes recorded from Japan were represented by a single isolate GTAb-4 (NRRL 29235) and GTAb-5 (NRRL 25593). Four isolates of *A. bombycis* from silkworm frass collected at rearing facilities in Indonesia and Malaysia were represented by genotypes not recorded from Japan; these included GTAb-3 (NRRL 31134, NRRL 31135), GTAb-6 (NRRL 31132) from Karimantan (W. Sarawaku), and GTAb-7 (NRRL 29253) from East Java.

Mycotoxin production

All isolates of *A. bombycis* as well as *A. nomius* NRRL 31105 produced aflatoxins B and G, in addition to kojic acid, but not cyclopiazonic acid (Table 2). *Aspergillus flavus* NRRL 31106 produced both cyclopiazonic acid, and kojic acid, but no aflatoxins were detected.

Discussion

The origin of silk production or sericulture, from the moth *Bombyx mori* (L.), and the weaving of silk began in China about 2700 B.C. Chinese legend states that Empress Si Ling Chi encouraged the mulberry culture, sericulture, and silk manufacturing and is credited with the invention of the loom to make garments (Cherry 1987). Techniques of sericulture were introduced to Japan by the third century B.C., and the silkworm industry was well established by the third century A.D. (Yoshitake 1988). *Aspergillus bombycis* and *Bombyx mori* would share a common history of "domestication" in sericulture that overlaps the domestication of yellow-green aspergilli (i.e., *A. oryzae*; *Aspergillus sojae* Sakaguchi & Yamada ex Murakami) used as chu (China) or koji (Japan) to produce alcoholic beverages, condiments, or foods (Wang and Fang 1986; Yokotsuka 1991). A desirable

Table 2. Production of aflatoxins, cyclopiazonic acid, and kojic acid by isolates of *Aspergillus bombycis* in laboratory culture[a]

Genotype	Strain	AFB1	AFB2	AFG1	AFG2	CPA	KA
GTAb-1	NRRL 28900	+	+	+	+	(−)	+
	NRRL 28901	+	+	+	+	(−)	+
	NRRL 29236	+	+	+	+	(−)	+
	NRRL 29237	+	+	+	+	(−)	+
	NRRL 31137	+	+	+	+	(−)	+
GTAb-2	NRRL 26010	+	+	+	+	(−)	+
	NRRL 29241	+	+	+	+	(−)	+
	NRRL 31407	+	+	+	+	(−)	+
	NRRL 31408	+	+	+	(−)	(−)	+
	NRRL 31409	+	+	+	+	(−)	+
	NRRL 31405	+	+	+	+	(−)	+
	NRRL 31410	+	+	+	+	(−)	+
GTAb-3	NRRL 31134	+	+	+	+	(−)	+
	NRRL 31135	+	+	+	+	(−)	+
GTAb-4	NRRL 29235	+	+	+	+	(−)	+
GTAb-5	NRRL 25593	+	+	+	+	(−)	+
GTAb-6	NRRL 31132	+	+	+	+	(−)	+
GTAb-7	NRRL 29253	+	+	+	+	(−)	+
A. nomius	NRRL 31105	+	+	+	+	(−)	+
A. flavus	NRRL 31106	(−)	(−)	(−)	(−)	+	+

[a] Data represent mycotoxins detected in a single culture extract: aflatoxin, glucose yeast (GY) extract broth (Wongurai et al. 1990); cyclopiazonic acid, modified Czapeks–Dox broth (Goto et al. 1996); kojic acid, glucose-peptone broth (Manabe et al. 1984)
Detection limits: aflatoxin B_1 (AFB_1), 2 μg/kg; AFB_2, 2 μg/kg; AFG_1, 1 μg/kg; AFG_2, 1 μg/kg; cyclopiazonic acid (CPA), 10 μg/kg; kojic acid (KA), 100 μg/kg

chu or koji starter inoculum would have been shared among neighbors and dispersed by migrating peoples (Wicklow et al. 2002), but ancestral populations of *A. bombycis* would be dispersed with infected silkworms or as contaminants of materials used in silkworm culture. In modern times, *A. bombycis*-contaminated eggs, larvae, or materials from silkworm breeding facilities (cooperatives) have the potential of being distributed to silkworm rearing houses or farms.

The "domesticated yellow-green aspergilli" *A. oryzae* and *A. sojae* were derived from naturally occurring "wild" varieties of *A. flavus* and *A. parasiticus* (Wicklow 1983a,b; Kurtzman et al. 1986; Geiser et al. 2000). Wicklow (1984) argues that strains of *A. oryzae* differ morphologically from wild strains of *A. flavus* primarily because long-term domestication would result in the loss of characters lacking adaptive value in a koji environment. Traditional sericultural farmers maintain populations of silkworms in all life stages at the same location, in addition to providing a constant source of substrate suitable for saprotrophic colonization in the form of dead insects, insect frass, and mulberry leaf litter. Temperatures and humidities necessary for silkworm rearing and cocoon production also favor *Aspergillus* growth. The cultures of *A. bombycis* and *A. nomius* (NRRL 31105) examined in the present study (Table 1) resemble *A. oryzae* by their loose colony morphology, absence of sclerotia, and presence of smooth conidiophores. All 18 isolates of *A. bombycis* as well as *A. nomius* (NRRL 31105) produce B and G aflatoxins. There is no better anecdotal evidence demonstrating the importance of aflatoxin-producing ability in insect pathogenicity (Wicklow 1988; Wicklow et al. 1994). There is no obvious need for sclerotia in the *Aspergillus* disease cycle in sericulture where the ability to persist for long periods in soil is not critical to *Aspergillus* survival. It is noteworthy that isolates of *A nomius* (NRRL 29234, NRRL 29238, and NRRL 29239) from silkworm rearing houses were not found to produce sclerotia (Peterson et al. 2001). *Aspergillus flavus* (NRRL 31106) produced sclerotia and cyclopiazonic acid, probably having lost the ability to produce aflatoxins following decades of periodic culture transfer in the laboratory.

Wicklow (1984) theorized that the lifting of natural environmental constraints may be linked to a greater variability in dimensions of conidia and conidiophores, as well as the production of a flocculent aerial mycelium of domesticated koji molds. *A. bombycis* colony texture is loose and deep, the conidiophore dimensions also showing variability [300–500 (–1000) × 10–20 μm] (Peterson et al. 2001). These authors also identified "smooth stipes" as characters that could be used, in the absence of DNA sequences, to distinguish *A. bombycis* from *A. nomius* or *A. flavus*, which are typically roughened. Domesticated koji strains also show smooth-walled conidiophores, and it has been argued that conidiophore wall roughenings in *A. flavus* and *A. parasiticus*, while serving to deter microarthropod predation in nature, would confer no selective advantage in a koji environment (Wicklow 1983b). The conidial heads produced by *A. bombycis* are 300–600 × 100–200 μm (L × W) with dry chains of conidia split into three or four smaller columns in mature heads (Peterson et al. 2001), whereas *A. oryzae* produces fewer, smaller, uniseriate conidial heads (Raper and Fennell 1965; Wicklow 1984). The dispersal of an infective conidial inoculum depends on airborne conidia reaching new substrate, including susceptible silkworm larvae.

In silkworm cultivation, as in nature, selection would favor large conidial heads and efficient aerial dispersal of an infective inoculum. The conidial dimensions of *A. bombycis* are variable in size [(3.5) 4–7 (8.5) μm diameter]. Domesticated yellow-green aspergilli also produce conidia that tend to be larger and more variable in size [*A. oryzae*, 4.5–7 up to 8–10 μm; *A. sojae*, (4–) 5–6 (–8) μm] when compared with wild isolates *A. flavus* and *A. parasiticus*. Unlike the conidia of *A. oryzae* or *A. sojae*, *A. bombycis* conidia are conspicuously roughened. The conidia of *A. bombycis* have an important role in fungal dispersal, attachment, and infection of silkworm larvae; therefore, conidial morphology is unlikely to differ from the wild state. No wild isolates of *A. bombycis* have been identified, apart from sericultural environments, that would allow us to compare wild forms of *A. bombycis* with populations adapted to silkworm cultivation. *Aspergillus* section *Flavi* cultures isolated from diseased silkworms, collections of silkworm frass, or samples of dust from silkworm rearing facilities have been identified as *A. flavus*, *A. flavus-oryzae* group Raper & Fennell (1965), and *A. oryzae* (IFO List of Cultures 2000; Kawakami 1982; MAFF Genebank 2002; Murakoshi et al. 1977; Ohtomo et al. 1975). Some of the *A. flavus* isolates from silkworm cultivation that were accessioned by MAFF Genebank (2002) were subsequently determined to be *A. bombycis* or *A. nomius* (Peterson et al. 2001). Unfortunately, *Aspergillus* cultures used in studies of silkworm pathogenicity by Kawakami (1982), Murakoshi et al. (1977), Kurisu and Manabe (1978), and Ohtomo et al. (1975) are no longer available for study, and we cannot determine if any of these cultures were *A. bombycis* or *A. nomius*.

A knowledge of the mycotoxin profiles of *A. bombycis*, *A. nomius*, and other species in *Aspergillus* section *Flavi* enables us to reexamine the results of some of this earlier literature. Like *A. nomius* and *A. parasiticus*, strains of *A. bombycis* produce B and G aflatoxins but no cyclopiazonic acid (see Table 2) (Peterson et al. 2001). Murakoshi et al. (1977) reported the production of both B and G aflatoxins by isolates of "*A. flavus*" obtained from naturally infected cadavers of silkworm larvae, pupae, and feces. They also demonstrated the presence of aflatoxins B_1 and G_1 in living larvae of silkworms inoculated with one of the isolates, the first reported proof of the existence of aflatoxins in living insects infected with *A. flavus*. *Aspergillus flavus* does not produce aflatoxins G_1 or G_2, which could suggest that these isolates were either *A. bombycis* or *A. nomius*, species that resemble *A. flavus* in culture (Peterson et al. 2001).

Aflatoxin production among clonal populations of *A. bombycis, A. nomius*, or *A. flavus* would be under strong selection in silkworm rearing facilities because aflatoxin contributes to the virulence of this entomopathogenic fungus. Aflatoxin B_1 was detected in silkworm larvae that became parasitized and died following artificial inoculation by *A. flavus* (Ohtomo et al. 1975). Murakoshi et al. (1977) suggested a causal relation between the production of aflatoxins in diseased silkworm larvae and the process of their death. Formaldehyde is used as a fungicide in silkworm rearing rooms. *Aspergillus flavus* isolates with a greater tolerance to formaldehyde resistance were also more virulent to silkworms (Murakoshi et al. 1977; Kawakami 1982). The ability of *A. bombycis* populations to form heterokaryons (Horn and Greene 1995) would allow for the exchange of genes among *A. bombycis* isolates exposed to different forms of selection among silkworm production facilities that could increase pathogen virulence or resistance to disinfectants such as formaldehyde and mercuric fungicides. Such clonal populations would soon dominate silkworm houses and be spread from one silkworm rearing house to another during transfer of materials, *B. mori*, or sericultural workers. The isolation of *A. bombycis* genotypes GTAb-1 and GTAb-2 from three or more silkworm rearing facilities in different prefectures of Japan suggests that each genotype is derived from clonal populations that have become highly adapted to silkworm cultivation environments in Japan and have been spread among these facilities.

How does one explain the consistent differences in DNA fingerprint banding intensity among *A. bombycis* genotypes? Although we do not know the DNA sequence of the pAF28 probe, it was characterized as containing repetitive DNA (McAlpin and Mannarelli 1995). Hypothesized copy number changes in repetitive microsatellite DNAs (Bennett and Todd 1996), which are well known in eukaryotes (Schmidt and Heslop-Harrison 1996), would reduce the binding efficiency of the pAF28 probe, resulting in fainter banding patterns at the stringency employed here. In the absence of selection in the wild, such changes in copy number may accumulate in ancient clonal populations of *A. bombycis*. At the same time, *A. bombycis* genotypes displaying a strong banding intensity (GTAb-1; GTAb-4) may share a lesser history of adaptation to sericulture.

Cultures of *A. bombycis* sharing the same DNA fingerprint probably originated from the same wild clonal population of *A. bombycis* and have since become highly adapted phenotypically in association with silkworm cultivation. *Bombyx mandarina* Moore is presumed to be the wild ancestor of *B. mori* because fertile hybrid progeny are produced from a cross between these two species (Nakamura et al. 1999). *Bombyx mandarina* inhabits China, Korea, Japan, and far eastern Russia and is independent of humans, whereas the domesticated *B. mori* can no longer survive in nature. The search for a wild-type clonal population of *A. bombycis* might focus on *Aspergillus* section *Flavi* isolates that parasitize *B. mandarina*. We suggest that this wild population of *A. bombycis* may be found to produce a velvety colony, sclerotia, uniformly shorter stipes with roughened outerwalls, and less variable conidia.

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