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FULL PAPER

Donald T. Wicklow · Cesaria E. McAlpin · Quee Lan Yeoh

Diversity of *Aspergillus oryzae* genotypes (RFLP) isolated from traditional soy sauce production within Malaysia and Southeast Asia

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Abstract DNA fingerprinting was performed on 64 strains of *Aspergillus oryzae* and 1 strain of *Aspergillus sojae* isolated from soy sauce factories within Malaysia and Southeast Asia that use traditional methods in producing "tamari-type" Cantonese soy sauce. *Pst*I digests of total genomic DNA from each isolate were probed using the pAF28 repetitive sequence. Strains of *A. oryzae* were distributed among 32 genotypes (30 DNA fingerprint groups). Ten genotypes were recorded among 17 *A. oryzae* isolates from a single soy sauce factory. Genotypes Ao-46 and GTAo-47, represented by 8 and 5 strains, respectively, were isolated from a soy sauce factory in Kuala Lumpur and factories in two Malaysian states. Four strains of GTAo-49, isolated from three soy sauce factories in Malaysia; each produced sclerotia. Two strains were found to be naturally occurring color mutants of NRRL 32623 (GTAo-49) and NRRL 32668 (GTAo-52). Only two fingerprint matches were produced with the 43 DNA fingerprint groups in our database, representing *A. oryzae* genotypes from Japan, China, and Taiwan. *Aspergillus sojae* NRRL 32650 produced a fingerprint matching GTAo-9, the only known genotype representing koji strains of *A. sojae*. No aflatoxin was detected in broth cultures of these koji strains as determined by TLC.

Key words *Aspergillus oryzae* · DNA fingerprints · Genotypic diversity · Koji · Soy sauce

D.T. Wicklow (✉) · C.E. McAlpin
Mycotoxin Research Unit, National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Agricultural Research Service, Peoria, IL 61604, USA
Tel. +1-309-685-4011; Fax +1-309-681-6686
e-mail: donald.wicklow@ncaur.usda.gov

Q.L. Yeoh
Biotechnology Research Centre, Malaysian Agricultural Research and Development Institute, Kuala Lumpur, Malaysia

Introduction

The selective cultivation of a yellow-green mold to convert soybeans into food seasonings, such as *chiang-yu* (soy sauce), originated in China and dates to the Chou dynasty, 1121–220 B.C. (Fukashima 1979; Wang and Fang 1986). The manufacture of soy sauce involves two stages of fermentation, the koji stage and the brine mash stage. During the koji stage of fermentation, mold enzymes bring about the saccharification of starch and decomposition of protein in the raw materials that support yeast and bacterial growth essential to the main brine fermentation (Yong and Wood 1974; Yokotsuka 1986). Soy sauce manufacture in Southeast Asia is carried out using a traditional method similar to that practiced in southern China (Bhumiratana et al. 1980; Steinkraus 1983; Lotong 1985; Mongkolwai et al. 1997). Whole steam-cooked soybeans are mixed with wheat flour and spread (3–5 cm thick) onto bamboo trays that are incubated (5–7 days) on open shelves in rooms without temperature or moisture controls. No starter inoculum (tane koji) is applied. The koji is then transferred to earthenware/porcelain jars, covered with a salt brine, and fermented for as long as 3 months. This traditional method relies on koji molds permeating the bamboo trays, which are not washed or disinfected between batch fermentations. In the open air environment of traditional koji fermentation, the superior competitive abilities of these "domesticated" koji strains are essential to their continued survival because koji inoculum from previous fermentations is transferred among locations and between generations of soy sauce producers.

The genetic and phenotypic diversity of microbial populations that have developed in indigenous soy sauce-making environments is largely uncharacterized. This study examines the genetic diversity (restriction fragment length polymorphism, RFLP) of 64 strains of *Aspergillus oryzae* and 1 strain of *Aspergillus sojae* isolated before 1980 from traditional soy sauce production facilities within Malaysia, Philippines, Singapore, and Thailand.

Materials and methods

Fungal strains

All cultures were obtained from the Agricultural Research Culture Collection, Peoria, IL, USA. The cultures were received by Dr. C.W. Hesseltine from Ms. Zahara Merican, Food Technology Centre, Malaysian Agricultural Research and Development Institute, Selangor, Malaysia, in 1979 and Professor Ho Coy Choke, University of Malaya, Kuala Lumpur, Malaysia, in 1983. No outside source of koji starter inoculum was used by any of the soy sauce factories at the time these collections were made. Sixty-four strains were classified as *Aspergillus oryzae* (Ahlburg) Cohn [=*A. flavus* subsp. *flavus* var. *oryzae* (Ahlburg) Kurtzman et al.], 1 strain was classified as *Aspergillus sojae* Sakaguchi & Yamada: Murakami [=*A. flavus* subsp. *parasiticus* var. *sojae* (Sakaguchi & Yamada) Kurtzman et al.], and 1 strain was classified as *Aspergillus tamarii* Kita (Table 1).

DNA extraction

Fungal mycelia were grown from *Aspergillus* conidial or cell suspensions in 500-ml flasks containing 200 ml yeast extract-peptone-dextrose (YEPD) broth. 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 E. Raeder and P. Broda as modified by McAlpin and Mannarelli (1995).

DNA hybridization and detection

*Pst*I-digested DNA (8 µg) was dispensed in each lane on 0.8% agarose gel in TAE buffer [0.04 M Tris-acetate, pH 8.00; 0.001 M ethylenediaminetetraacetic acid (EDTA)], run

Table 1. DNA fingerprint matches for *Aspergillus* strains isolated from traditional soy sauce production in Malaysia/Southeast Asian region

Genotype	Strain	Received as	Soy sauce producer	Location
Aspergillus tamarii				
GTAo-74	NRRL 26326	FTCC 5011	Koji; Pemandu	Alor Setar, Kedah, Malaysia
Aspergillus sojae				
GTAo-9*	NRRL 32650	FTCC 5146	Koji; Kan Chong	Penang, Malaysia
Aspergillus oryzae				
GTAo-46	NRRL 32604	FTCC 5148	Koji; Kan Chong	Penang
	NRRL 32605	FTCC 5152	Koji; Kwong Heng	Penang
	NRRL 32606	FTCC 5155	Koji; Soon Hin	Penang
	NRRL 32607	H-3022	Koji; unreported	Selangor, Malaysia
	NRRL 32608	H-3114	Koji; unreported	Selangor
	NRRL 32609	H-3087	Koji; unreported	Selangor
	NRRL 32614	FTCC 5178	Koji tray[a]; Yuen Chun	Kuala Lumpur, Malaysia
	NRRL 32617	FTCC 5164	Koji; Yuen Chun	Kuala Lumpur
GTAo-47	NRRL 32610	FTCC 5008	Koji; Yuen Chun	Kuala Lumpur
	NRRL 32611	FTCC 5009	Koji; Yuen Chun	Kuala Lumpur
	NRRL 32612	FTCC 5168	Koji tray[a]; Yuen Chun	Kuala Lumpur
	NRRL 32613	FTCC 5174	Koji tray[a]; Yuen Chun	Kuala Lumpur
	NRRL 32615	H-3201	Koji; unreported	Perak, Malaysia
	NRRL 32616	FTCC 5154	Koji; Soon Hin	Penang
GTAo-49	NRRL 32620[b]	FTCC 5010	Koji; Pemandu	Alor Setar, Kedah
	NRRL 32621[b]	FTCC 5013	Koji; Pemandu	Alor Setar, Kedah
	NRRL 32622[b]	FTCC 5157	Koji; Thye Wah	Penang
	NRRL 32623[b,c]	FTCC 5179	Koji tray[a]; Yuen Chun	Kuala Lumpur
GTAo-53	NRRL 32627	FTCC 5015	Koji; Kong Guan	Penang
	NRRL 32628	FTCC 5144	Koji; Woh Chun	Penang
	NRRL 32629	FTCC 5150	Koji; Kwan Loong	Penang
	NRRL 32630	FTCC 5149	Koji; Kan Chong	Penang
GTAo-15*	NRRL 32624	H-3232	Koji; unreported	Singapore
	NRRL 32625	H-3263	Koji; unreported	Thailand
	NRRL 32626	H-3241	Koji; unreported	Philippines
GTAo-50	NRRL 32631	FTCC 5171	Koji tray[a]; Yuen Chun	Kuala Lumpur
	NRRL 32632	FTCC 5175	Koji tray[a]; Yuen Chun	Kuala Lumpur
	NRRL 32633	H-3103	Koji; unreported	Malacca, Malaysia
GTAo-51	NRRL 32634	FTCC 5172	Koji tray[a]; Yuen Chun	Kuala Lumpur
	NRRL 32635	H-3138	Koji; unreported	Penang
	NRRL 32636	H-3184	Koji; unreported	Kedah
GTAo-52	NRRL 32637	H-3144	Koji; unreported	Penang
	NRRL 32638	FTCC 5141	Koji; Woh Chun	Penang
	NRRL 32639[d]	FTCC 5140	Koji; Woh Chun	Penang
GTAo-76	NRRL 32666	FTCC 5133	Koji; unreported	Seremban, Negerei Sembilan, Malaysia
	NRRL 32667	FTCC 5137	Koji; unreported	Butterworth, Penang
	NRRL 32668[b]	FTCC 5138	Koji; unreported	Butterworth, Penang

Table 1. *Continued*

Genotype	Strain	Received as	Soy sauce producer	Location
GTAo-35*	NRRL 32618	H-3240	Koji; unreported	Kedah
	NRRL 32619	H-3054	Koji; unreported	Perak
GTAo-48	NRRL 32640	FTCC 5005	Koji; Cheong Chan	Petaling Jaya, Selangor
	NRRL 32641	FTCC 5006	Koji; Cheong Chan	Petaling Jaya, Selangor
GTAo-54	NRRL 32642	FTCC 5012	Koji; Pemandu	Alor Setar, Kedah
	NRRL 32643	FTCC 5014	Koji; Chun Weng	Seremban, Negerei Sembilan
GTAo-56	NRRL 32644	FTCC 5151	Koji; Kwan Loong	Penang
	NRRL 32645	FTCC 5170	Koji tray[a]; Yuen Chun	Kuala Lumpur
GTAo-57	NRRL 32646	FTCC 5163	Koji tray[a]; Yuen Chun	Kuala Lumpur
	NRRL 32647	FTCC 5165	Koji tray[a]; Yuen Chun	Kuala Lumpur
GTAo-58	NRRL 32648	FTCC 5166	Koji tray[a]; Yuen Chun	Kuala Lumpur
	NRRL 32649	FTCC 5167	Koji tray[a]; Yuen Chun	Kuala Lumpur
GTAo-63	NRRL 32654	FTCC 5160	Koji tray[a]; Yuen Chun	Kuala Lumpur
GTAo-64	NRRL 32655	FTCC 5180	Koji tray[a]; Yuen Chun	Kuala Lumpur
GTAo-60	NRRL 32651	FTCC 5016	Koji; Man Sang	Penang
GTAo-61	NRRL 32652	FTCC 5142	Koji; Woh Chun	Penang
GTAo-62	NRRL 32653	FTCC 5153	Koji; Kwong Heng	Penang
GTAo-67	NRRL 32658	H-3173	Koji; unreported	Penang
GTAo-66	NRRL 32657	H-3174	Koji; unreported	Penang
GTAo-68	NRRL 32659	H-3199	Koji; unreported	Perak
GTAo-65	NRRL 32656	H-3033	Koji; unreported	Selangor
GTAo-72	NRRL 32663[b]	H-3246	Koji; unreported	Philippines
GTAo-73	NRRL 32664	H-3242	Koji; unreported	Philippines
GTAo-69	NRRL 32660	H-3225	Koji; unreported	Singapore
GTAo-70	NRRL 32661	H-3220	Koji; unreported	Singapore
GTAo-71	NRRL 32662	H-3262	Koji; unreported	Thailand
GTAo-75	NRRL 32665	H-3264	Koji; unreported	Thailand

[a] *Aspergillus*-infested bamboo koji trays
[b] Sclerotia produced on potato dextrose agar (PDA) + 1% yeast extract (dark incubation; 25°C)
[c] Naturally occurring color mutant, conidial heads light cadmium to yellow ocher [R., plates IV; XV: R, Ridgway R (1912) Color standards and color nomenclature. Published by the author, Washington, D.C.]
[d] Naturally occurring color mutant, conidial heads buckthorn brown to ochraceus buff (R., Plate XV)
* DNA fingerprints GTAO-09 = NRRL 1988, etc.; GTAo-15 = NRRL 3483, etc.; GTAo-35 = NRRL 2218 (Wicklow et al. 2002)

at 1.8 V/cm for 22 h, and visualized with UV light after staining with ethidium bromide. Southern blots were performed by transferring restriction fragments from agarose gels to nylon membranes (Nytran N; Schleicher and Schuell) using a vacuum blotter (model 785; BioRad Laboratories). Probes were labeled by random primed labeling with digoxigenin using the Nucleic Acid Non-radioactive Hybridization System (Roche Molecular Biochemicals). 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) at room temperature for 1–2 h.

DNA fingerprint analyses

DNA fragments were compared by designating and recording 55 fragment positions, representing different molecular weights, with an equidistant marker. Fragments within and between gels could be distinguished using the reference isolate K.E. Papa strain *A. flavus* NRRL 19997 (McAlpin and Mannarelli 1995; Wicklow et al. 2002) and a lambda standard. Strains 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. *Aspergillus flavus* genotypes 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. 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 pAF28 DNA probe has proven reliable in classifiying *A. flavus* strains according to their previously determined vegetative compatibility groups (VCGs) (McAlpin and Mannarelli 1995; McAlpin et al. 2002).

Banding profiles of the different strains were compared, and similarity was calculated using the Dice coefficient, following the NTSYS Numerical Taxonomy and Multivariate Analysis (Rohlf 1997). The similarity matrix was obtained and the cluster analysis generated using the SIMQUAL and SAHN programs. The SAHN program enables the comparison of DNA banding patterns of *Aspergillus* strains to determine which strains share identical fingerprints or can be classified in the same fingerprint group but does not necessarily imply phylogenetic relationships. A phenogram

was generated using the unweighted-pair-group method with arithmetic average (UPGMA).

Aflatoxin analyses

The ability of *Aspergillus* strains to produce aflatoxins was determined using a procedure developed at the National Peanut Research Laboratory (Horn et al. 1996). Individual strains were cultured for 5 days at 28°C in 1.0 ml broth consisting of 190 g glucose, 20 g yeast extract, and 10 g Bactosoytone (Difco Laboratories, Detroit, MI, USA) per liter in 2.0-ml vials capped with a Teflon septum (no. 73005; Waters Chromatography Division, Millipore). Following incubation, 1 ml chloroform was added to each vial, and the vials were vortexed for 15–20 s and left standing for 1–2 min for solvent partitioning. The presence of aflatoxins was determined by thin-layer chromatography (TLC) (AOAC 1984: p. 477). A microliter syringe was inserted through the Teflon cap, and a 20-µl chloroform layer was removed and spotted on TLC plates (Silica Gel 60 precoated plates no. 5721-7; EM Science) along with aflatoxin B1, B2, G1, and G2 standards.

Results

DNA fingerprinting was performed on 64 cultures of *A. oryzae*, 1 culture of *A. sojae*, and 1 culture of *A. tamarii*, all isolated from koji or koji mold-infested bamboo koji trays used in traditional soy sauce production facilities within Malaysia and other Southeast Asian countries (see Table 1; Fig. 1). Thirty-two genotypes (30 fingerprint groups) were recorded among 64 strains of *A. oryzae* from Malaysia (55 strains), Singapore (3 strains), Thailand (3 strains), and Philippines (3 strains). These included GT Ao-46, represented by 8 strains isolated from soy sauce factories in three locations, GT Ao-47 represented by 6 strains isolated from factories in three locations, GTAo-49 and GTAo-53, each

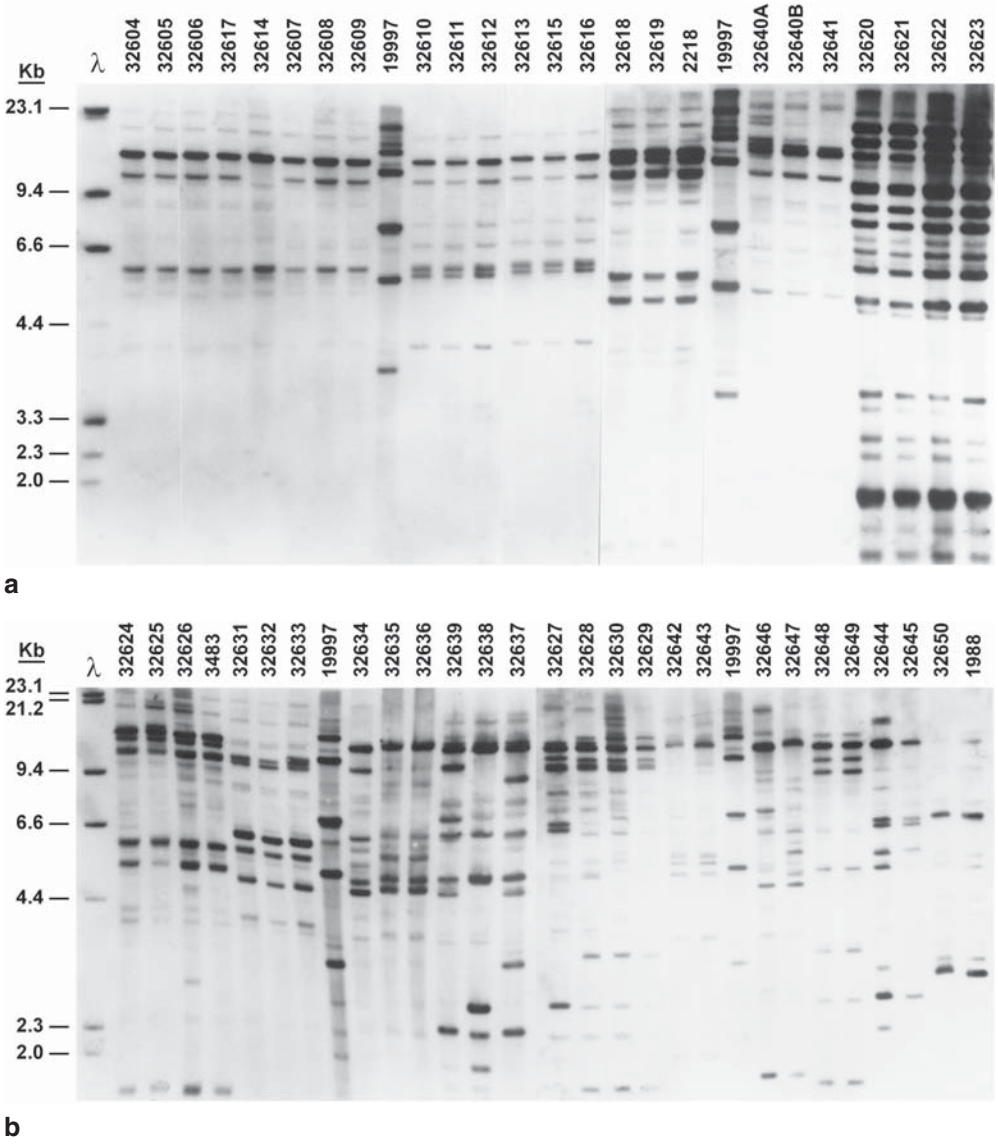

Fig. 1. DNA fingerprint matches among strains of *Aspergillus oryzae* and *A. sojae*. This figure includes one reference strain (*A. flavus* NRRL 19997) and size markers

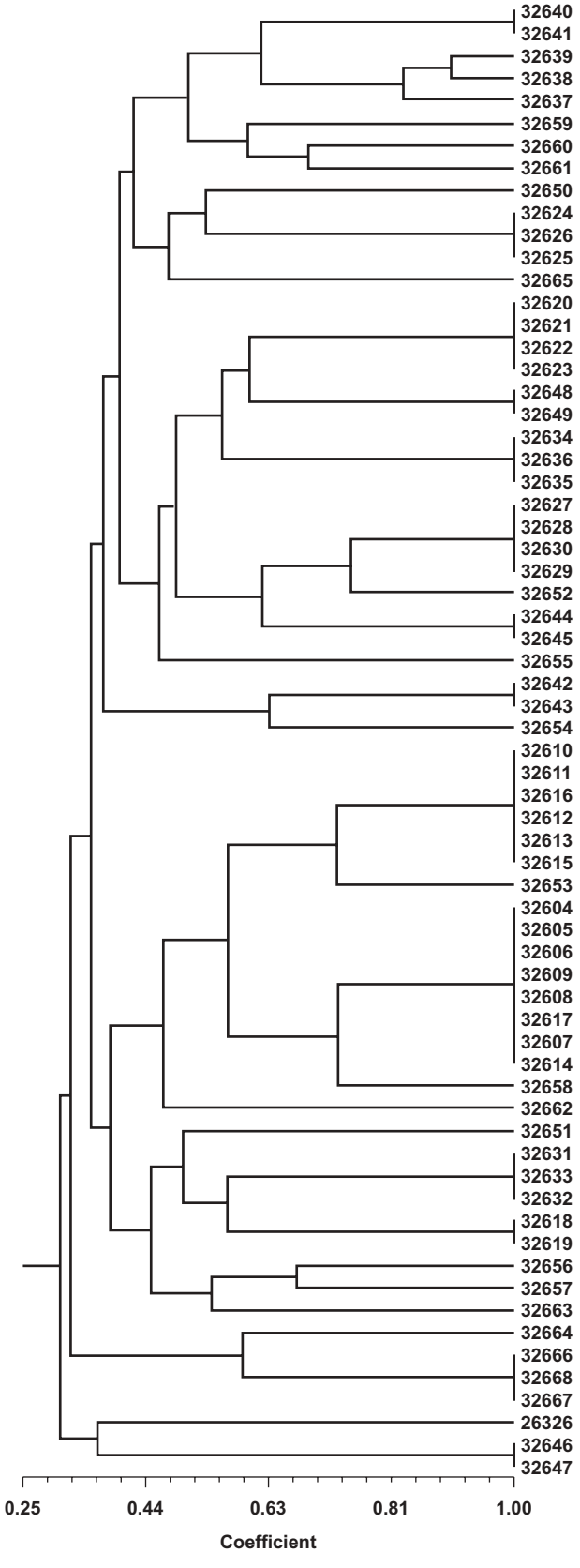

Fig. 2. Phenogram based on cluster analysis of the DNA fingerprints of *Aspergillus oryzae* (55 strains), *Aspergillus sojae* (NRRL 32650), and *Aspergillus tamarii* (NRRL 26326) using the Dice similarity coefficient of individual DNA bands produced by hybridization with pAF28 repetitive sequence. The similarity coefficients of the clustered strains correspond with the DNA fingerprints of the isolates belonging to the same DNA fingerprint group. The phenogram was generated using the NTSYS-pc, version 2.01

represented by 4 strains, and GT Ao-15, GT Ao-50, GT Ao-51, GT Ao-52, and GTAo-76, each represented by 3 strains. Six genotypes were represented by 2 strains, whereas 15 single-strain isolates of *A. oryzae* (not included in Fig. 1) produced DNA fingerprints that did not match any of the other isolates from Southeast Asia (<80% band similarity). The majority of the *A. oryzae* strains produced numerous bands, indicating a "moderate" to "highly repetitive" type of hybridization pattern (Wicklow et al. 1998). The phenogram, derived from the UPGMA analysis, revealed that the similarity coefficients of the *A. oryzae* strains corresponded with the DNA fingerprint patterns of clustered strains belonging to the same DNA fingerprint group (Fig. 2).

Only two fingerprint matches were produced with the 43 DNA fingerprint groups in our database representing *A. oryzae* cultures from Japan (63 strains), China (7 strains), and Taiwan (2 strains) (Wicklow et al. 2002). Genotype Ao-35, isolated from soy sauce factories in Kedah and Perak, included strain H-3054 received from C.C. Ho as "*Aspergillus wentii*." An isolate representing this DNA fingerprint group was earlier reported from koji used to produce sauce following traditional methods in Shanghai, China (NRRL 2218; Fig. 1) (Wicklow et al. 2002). Genotype Ao-15, isolated from koji used to produce sauce following traditional methods in Singapore, Thailand, and the Philippines, was reported from both soy sauce koji, Taiwan (NRRL 6270) and miso koji, Japan (NRRL 3483; Fig. 1) (Wicklow et al. 2002). *Aspergillus oryzae* GTAo-66 (NRRL 32657; H-3174), identified by C.C. Ho as a "superior strain for soy sauce" (ARS Culture Collection records), was isolated only once from a factory in Penang. Four cultures of GTAo-49, isolated from soy sauce factories in three Malaysian locations, produced sclerotia, including the naturally occurring color mutant (NRRL 36623). Two isolates were found to be color mutants of GTAo-49 (NRRL 36623 = conidial heads light cadmium yellow ocher; Ridgway 1912, plates IV, XV) and GTAo-52 (NRRL 32639 = conidial heads buckthorn brown to ochraceus buff; Ridgway 1912, plate XV). A single culture of *A. sojae* NRRL 32650 (FTCC 5146), from the Kan Chong factory in Penang, produced a DNA fingerprint matching *A. sojae* NRRL 1988 GTAo-9 (see Fig. 1), the only genotype found to represent *A. sojae* koji strains from Japan (7 strains) and China (2 strains) (Wicklow et al. 2002). A single culture of *A. tamari* NRRL 26326 (FTCC 5011) was isolated from the Pemandu factory that produces Malay-type soy sauce. No aflatoxin was detected in broth cultures of the Southeast Asian koji strains examined in this study (see Table 1) as determined by TLC.

Discussion

Soy sauce factories in Southeast Asian countries were established by "master" soy sauce brewers who carried koji from southern China. Trained workers may have also started their own soy sauce factories, presumably using some of the same koji starter or *Aspergillus*-infested bamboo trays. This possibility could explain the common distribution of successful koji strains among soy sauce factories within Malaysia. Only the most competitive *Aspergillus* strains would influence the koji fermentation and infest the woven bamboo trays, thereby enabling the transfer of mycelium and spores. *Aspergillus oryzae* isolates from Southeast Asian soy sauce factories were represented by 30 DNA fingerprint groups (see Table 1) yet produced only two matches with the 43 DNA fingerprint groups representing *A. oryzae* strains obtained from sources of koji produced in Japan or China (Wicklow et al. 2002). Evidence of the genotypic diversity of *A. oryzae* in traditional soy sauce production is best demonstrated by the 10 genotypes recorded among 17 strains isolated from a single factory (Yuen Chun) in Kuala Lumpur (Table 1). Eleven genotypes were recorded among 17 *A. oryzae* strains isolated from koji sampled at nine soy sauce factories in Penang, Malaysia alone, including GT Ao-53 isolated from four factories (Table 1). A high genetic diversity [by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR)] was also found among native *Geotrichum candidum* strains isolated from fungus-ripened, soft and semihard cheeses collected from traditional cheese-making facilities from within the same regions of France (Marcellino et al. 2001).

Aspergillus genotypes isolated from different soy sauce factories within Malaysia were derived from common ancestral clonal populations. There is no better example of the distribution of a successful koji strain among traditional soy sauce factories than *A. oryzae* GTAo-49, which produces sclerotia in culture. Isolates of this genotype were obtained from koji sampled at different company factories in three Malaysian locations, compelling evidence that GTAo-49 is rooted in the early history of koji manufacture in Malaysia and represents a "successful" koji strain. Long-lived *Aspergillus* sclerotia (Wicklow et al. 1993) that form within the bamboo strips of koji trays could survive for years in the absence of koji making, as would occur when trays were transported over long distances or stored for extended periods. Sclerotia of *A. flavus* and *A. parasiticus* formed within the pith tissues of uninoculated corn cobs that overseasoned in the field (Zummo and Scott 1990). The discovery of a successful koji strain of *A. oryzae* that also produces sclerotia serves to highlight different pathways in *A. flavus* domestication (Wicklow 1983a,b; Kurtzman et al. 1986; Geiser et al. 1998; van den Broek et al. 2001). Microbiologists who isolated the koji strains examined in the present study emphasized differences in colony morphology and color in selecting for phenotypic diversity among koji mold isolates. Only two natural color mutants of *A. oryzae* were recorded from numerous koji samples, which would suggest that spontaneous color mutants are of recent origin and are not maintained in the populations. The isolation of *A. sojae* GTAo-9 (NRRL 32650; FTCC 5146) from the Kan Chong factory in Penang is of particular significance in that it offers further evidence that *A. sojae* is of Chinese origin. Wicklow et al. (2002) reported two strains classified as *A. sojae* GTAo-9 NRRL 1988 and NRRL 1989 (N.B.I.R. 2016) isolated from soy sauce koji in Nanking, China, and received in 1947 from Mr. P.-S. King. The seven other strains of *A. sojae* GTAo-9 were isolated from industrial sources of koji produced in Japan. No detectable aflatoxin was found in culture extracts of ten industrial strains of *A. sojae* (Matsushima et al. 2001). Wicklow et al. (2002) suggested that all strains of *A. sojae* used in the production of koji have originated from a common ancestral clonal population derived from *A. parasiticus*.

It was not possible to make a meaningful evaluation of the diversity of koji populations among soy sauce factories. Although *Aspergillus* strains examined in the present study were selected on the basis of differences in cultural morphology and microscopic characteristics, the number of *Aspergillus* strains preserved from each factory is also related to the number of koji samples collected and this depended on the cooperation of factory owners. It is anticipated that further sampling and strain isolation would lead to the identification of additional *Aspergillus* genotypes among the koji mold populations in each of the soy sauce factories.

The prevalence of *A. oryzae* genotypes in traditional soy sauce production, and not *A. sojae*, may be attributed, in part, to the prolific aerial dispersal of *A. oryzae* conidia within the traditional open-air koji-making environment as contrasted with *A. sojae*. *Aspergillus parasiticus* is recognized as being better adapted to a soil environment than *A. flavus* because it is more prevalent in peanut seeds than in aboveground crops (Diener et al. 1987). Horn and Greene (1995) observed that populations of *A. parasiticus* in crop field soils are less diverse (vegetative compatibility group diversity) than those of *A. flavus* because conidal dispersal is more restricted than in *A. flavus*. *Aspergillus flavus* is more commonly recorded from infected maize grain at harvest and more frequently isolated from the air above crop fields where both *A. parasiticus* and *A. flavus* may be present in similar proportions in crop field soils and crop debris (Horn et al. 1995; Wicklow et al. 1998; McAlpin et al. 1998). Horn (2005) has shown that the incidence of peanut seed colonization by *A. parasiticus* was optimal at 22°C compared to an optimum of 30°–37°C for colonization by *A. flavus* and proposed that *A. flavus* would have a competitive growth advantage in colonizing seeds of maize and other crops (e.g., cotton, tree nuts) that develop above ground and are exposed to warmer temperatures. In Southeast Asia, the production of soy sauce koji is performed in an open-air environment at ambient temperatures ranging from 28° to 35°C (Lotong and Suwanarit 1983), which could favor domesticated forms of *A. oryzae* in the competitive colonization of steam-cooked soybeans mixed with wheat flour. In traditional koji production, domesticated strains of *A. oryzae* or *A. sojae* behave as a "biocontrol inoculum." *Aspergillus*-infested koji trays provide an immediate source

of koji inoculum for the solid substrate fermentation. Rapid conidial germination should further enable these domesticated koji strains to overwhelm most wild contaminants (Wicklow 1984). *Aspergillus oryzae* or *A. sojae* strains isolated from traditional koji making would offer a source of competitively superior koji strains for biocontrol applications in crop fields as contrasted with industrial strains selected for mechanized and highly controlled fermentations in a sanitized environment (Dorner et al. 2000).

Industrial strains of *A. oryzae* and *A. sojae* have been thoroughly tested for their ability to produce aflatoxin and found to be negative (Sugiyama 1984; Barbesgaard et al. 1992; Matsushima et al. 2001). Although the 48-h incubation time for industrial koji production may be too short for aflatoxin production (van den Broek et al. 2001), in traditional koji making incubation times can vary from 4 to 10 days. Malaysian soy sauce is reportedly free of aflatoxin (Steinkraus 1983), and in the present study no aflatoxin was detected in broth cultures inoculated with strains of *A. oryzae* or *A. sojae* isolated from Southeast Asian countries (see Table 1). A report of aflatoxin B1 contamination (6–15 ppb) for 15 of 32 samples of kecap, a traditional Indonesian soy sauce (Sardjono et al. 1992), was followed by the isolation of two aflatoxin-producing strains of *Aspergillus* from koji used in kecap production (Nikkuni et al. 2002). White mutant strains of industrial Japanese koji molds offer Indonesian soy sauce manufacturers a simple visual method for determining if their koji is contaminated with potentially toxigenic yellow-green aspergilli originating from within the factory environment (Nikkuni et al. 2002). The survival of native koji strains is at risk given an industry trend toward fewer, large-scale mechanized factories and the introduction of standardized koji inoculum and fermentation conditions, in an effort to provide a more consistent product quality (Lotong and Suwanarit 1983; Flegel 1988; Fukashima 1989; Yeoh 1995; Roling et al. 1996; Mongkolwai et al. 1998; Sulistyo and Nikkuni 2006). The collection and preservation of local native koji strains which over the centuries have been selected by master brewers as producing the "best" soy sauce becomes essential.

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