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Common genotypes (RFLP) within a diverse collection of yellow-green aspergilli used to produce traditional Oriental fermented foods

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Abstract DNA fingerprinting was performed on 72 strains of *Aspergillus oryzae* and 9 strains of *Aspergillus sojae* isolated from chu (China) or koji (Japan) mold inoculum used in the production of traditional Oriental fermented beverages or foods including soy sauce, miso, and sake. The cultures were deposited with the ARS Culture Collection (NRRL) between 1909 and 2001. *Pst*I digests of total genomic DNA from each isolate were probed using the pAF28 repetitive sequence. All strains of *A. sojae* that we examined produced an identical DNA fingerprint and belong to the same DNA fingerprint group (GTAo-9). Strains of *A. oryzae* were distributed among 41 DNA fingerprint groups, including GTAo-12 represented by 11 strains, GTAo-19 represented by 5 strains, GTAo-5 and GTAo-15 each represented by 4 strains, and GTAo-8, GTAo-17, and GTAo-24 each represented by 3 strains. Thirty-three single strain isolates of *A. oryzae* produced unique fingerprints. Our data offer evidence suggesting that (1) the successful domestication of *A. parasiticus* genotypes yielding *A. sojae* occurred far less frequently than among genotypes of *A. flavus* var. *oryzae*; and (2) some *Aspergillus* genotypes employed in different fermentations and regions were derived from a common ancestral clonal population.

Key words *Aspergillus oryzae* · *Aspergillus sojae* · Chu · DNA finger prints · Domestication · Koji

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Introduction

The use of mold fermentation to convert cereal grains into alcoholic beverages, "chiu", or beans into food seasonings, such as huang-chiang (soybean paste), chiang-yu (soy sauce), and tou-shi (fermented whole beans), originated in China (Fukushima 1979; Wang and Fang 1986). Mold enzymes bring about saccharification of starch and decomposition of protein that supports yeast and bacterial growth essential to the main fermentation (Abiose et al. 1982; Kodama 1986). Huang-chu (China) or koji (Japan) are names given to the mold inoculum used to produce hydrolyzing enzymes for fermentations (Wang and Fang 1986; Yokotsuka 1991). The selective cultivation of a yellow-green mold to produce a "desirable mold inoculum with pure quality" dates to the Chou dynasty, 1121–220 B.C. (Wang and Fang 1986). These "domesticated yellow-green aspergilli," *Aspergillus oryzae* (Ahlburg) Cohn and *Aspergillus sojae* Sakaguchi & Yamada ex Murakami, were likely derived from naturally occurring "wild" varieties of *Aspergillus flavus* Link: Fr. and *Aspergillus parasiticus* Speare (Wicklow 1983a,b; Kurtzman et al. 1986; Geiser et al. 2000).

Commercial koji starters are sold as a dried mold spore inoculum, typically consisting of two strains of *A. oryzae* or *A. sojae* (Hesseltine et al. 1976). Generally, *A. oryzae* koji strains used to produce sake and miso are selected for high amylolytic activity whereas strains of *A. sojae* used in soy sauce production are selected for high proteolytic activity (Shibasaki and Hesseltine 1962; Flegel 1988; Hara et al. 1992). A desirable mold inoculum would have been shared among neighbors, dispersed by migrating peoples, cultured on different cereal grains, and exposed to new fermentation environments. Given this scenario, one could expect to isolate phenotypically distinct strains of *A. oryzae* or *A. sojae* derived from the same ancestral clonal population, the domestic equivalent of adaptive radiation in nature. In modern times, a desirable mold inoculum would be distributed as a commercial product to food companies that produce traditional Oriental fermented foods (Hesseltine et al.

1976) or would have become the intellectual property of a fermentation company (Nakadai et al. 1975). Here, the selection of strains with improved proteolytic activities is guided by a microbiologist or fermentation biochemist (Nasuno et al. 1971; Nakadai and Nasuno 1977; Sekine et al. 1969; Ushijima 1994).

This study contrasts the DNA fingerprints (restriction fragment length polymorphism, RFLP) of 72 strains of *A. oryzae* and 9 strains of *A. sojae* that have been used to produce traditional Oriental fermented beverages, foods, and seasonings (Table 1). The cultures were acquired between 1909 and 2001 by Charles Thom, Allan K. Smith, Kenneth B. Raper, Dorothy I. Fennell, Clifford W. Hesseltine, and the present authors and deposited with the ARS Culture Collection (NRRL). Peterson (2000) demonstrated through the use of 28S rDNA that *A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. sojae* were very closely related. Kumeda and Asao (2001), in using the ITS1-5.8S rDNA-ITS2 region of the ribosomal repeat unit, showed that sequences of *A. flavus* and *A. oryzae* are identical and that the sequences of *A. parasiticus* and *A. sojae* are identical. The sequence differences in the ITS regions between *A. flavus*/*A. oryzae* and *A. parasiticus*/*A. sojae* (~8 NT) are sufficient to readily distinguish the two species regardless of phenotypic variations. We have used polymerase chain reaction (PCR) amplification (White et al. 1990) and sequenc-

Table 1. DNA fingerprint matches for *Aspergillus oryzae* and *Aspergillus sojae* strains used in Oriental food fermentations[a]

Genotype	Strain	Received	From	Source
GTAo-12	NRRL 467	1920	K. Oshima	Takamine Labs. Ao 5c "Ueda, Osaka" (= Thom 290-4429-Ao 5c)
	NRRL 1999	1949	P.-S. King	N.B.I.R. 2015; koji for soy sauce, Nanking
	NRRL 3485	1960	C.W. Hesseltine	Koji for miso
	NRRL 3486	1960	C.W. Hesseltine	Koji for miso
	NRRL 3487	1960	C.W. Hesseltine	Koji for miso
	NRRL 3488	1960	C.W. Hesseltine	Koji for miso
	NRRL 5588	1973	S. Hara	RIB 23; "from dust" at soy sauce and miso factory
	NRRL 5593	1973	S. Hara	RIB 331; koji for miso
	NRRL 6574	1966	C.W. Hesseltine	Hama-natto, Kyoto
	NRRL 6575	1966	C.W. Hesseltine	Hama-natto, Kyoto
	NRRL 6595	1981	S. Mitzutani	Hama-natto, T. Hasegawa Co.
GTAo-9	NRRL 1988	1947	P.-S. King	Koji for soy sauce, China
	NRRL 1989	1947	P.-S. King	Koji for soy sauce, Nanking
	NRRL 3351	1968	M. Mogi	Noda Institute for Scientific Research
	NRRL 5594	1973	S. Hara	RIB 1040
	NRRL 5595	1973	S. Hara	RIB 1041; koji for shoyu
	NRRL 5596	1973	S. Hara	RIB 1042; koji for shoyu
	NRRL 5597	1973	S. Hara	RIB 1046; koji for shoyu
	NRRL 5598	1973	S. Hara	RIB 1047; koji for shoyu
	NRRL 30039	1998	J. Dorner	S-12; Higuchi Matsunosuke Shoten Co.
GTAo-19	NRRL 454	1933	Y.K. Shih	China
	NRRL 466	1920	K. Oshima	Takamine Labs. Ao 5b "Ueda, Osaka" (= Thom 290-4429-Ao5b)
	NRRL 470	?	T. Takahashi	*A. oryzae* – D (= Thom Coll. = 290-Taka D)
	NRRL 4799	1969	D.I. Fennell	IFO 4134; Takahashi's *A. oryzae*-D; koji for sake
	NRRL 31119	2001	A. Nakagiri	IFO 30104; koji for amazake
GTAo-5	NRRL 460	1920	K. Oshima	Takamine Labs. Ao 1 "Hishiroku Co., Kyoto" (= Thom 290-4429-Ao 1)
	NRRL 468	?	T. Takahashi	*A. oryzae* – A (= Thom Coll. 290-Taka A); koji for sake
	NRRL 1911	1943	S.A. Waksman	"Takamine's original diastase strain"
	NRRL 5938[b]	1974	D.I. Fennell	Higuchi Matsunosuke Shoten Co., Osaka; koji
GTAo-15	NRRL 3483	1960	C.W. Hesseltine	Koji for miso
	NRRL 3484	1960	C.W. Hesseltine	Koji for miso
	NRRL 6270	1976	K.C. Lin	FR-1, soy sauce strain, Taiwan
	NRRL 6271	1976	K.C. Lin	FR-3, soy sauce strain, Taiwan
GTAo-17	NRRL 5592	1973	S. Hara	RIB 178; koji for sake
	NRRL 5937	1974	D.I. Fennell	Higuchi Matsunosuke Shoten Co., Osaka; koji
	NRRL 13765	1988	T. Mayo	Cedarlane Foods, CA; koji for amazake
GTAo-24	NRRL 461	1920	K. Oshima	Takamine Labs. Ao N "Japanese Brewing Station" (= Thom 290-4429-AoN)
	NRRL 478	?	T. Takahashi	*A. oryzae* – N (= Thom Coll. 290-Taka N); koji for tamari
	NRRL 479	?	T. Takahashi	*A. oryzae* – O (= Thom Coll. 290-Taka O); koji for tamari
GTAo-8	NRRL 1919	1943	?	Takamine Labs, "No. 42"
	NRRL 4803	1969	D.I. Fennell	IFO 4203; koji for miso
	NRRL 481	?	T. Takahashi	*A. oryzae* – Z (Thom Coll. 290-Taka Z)
GTAo-25	NRRL 462	1920	K. Oshima	Takamine Labs. Ao P "Japanese Brewing Station" (= Thom 290-4429-Ao P)
	NRRL 480	?	T. Takahashi	*A. oryzae* – P (= Thom Coll. 290-Taka P); koji for shoyu
GTAo-29	NRRL 471[c]	?	T. Takahashi	*A. oryzae* – E (= Thom Coll. 290-Taka E); koji for sake
	NRRL 472[c]	?	T. Takahashi	*A. oryzae* – F (= Thom Coll. 290-Taka F); koji for shoyu
GTAo-6	NRRL 477	?	T. Takahashi	*A. oryzae* – M (= Thom Coll. 290-Taka M)
	NRRL 552	?	T. Takahashi	*A. oryzae* – M (= Thom Coll. 290-51)

[a] An additional 33 unique DNA fingerprints were produced by 33 single-strain isolates of *A. oryzae* listed in Materials and methods
[b] NRRL 5938 shares 84% band similarity with all strains in *GTAo-5*
[c] Strains share 93% band similarity

ing of the ITS1-5.8S rDNA-ITS2 regions, followed by comparisons to sequences from the *ex type* cultures of *A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. sojae*, to make species-level identifications of all 81 strains. It should be noted that none of the domesticated yellow-green aspergilli we examined showed sequences matching any other species in *Aspergillus* section *Flavi* (e.g., *Aspergillus bombycis* Peterson et al., *Aspergillus nomius* Kurtzman et al., *Aspergillus pseudotamarii* Ito et al.). *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 groups (VCGs) (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; Wicklow et al., unpublished data), and to type clinical isolates of *A. flavus* from human sources (James et al. 2000). We wanted to examine the genetic diversity (restriction fragment length polymorphism, RFLP) of this collection of domesticated aspergilli to determine if some of the strains might share a common ancestry.

Materials and methods

Fungal strains

All cultures were obtained from the Agricultural Research Service Culture Collection, Peoria, IL, USA. Seventy-two strains are classified as *Aspergillus oryzae* (Ahlburg) Cohn [= *A. flavus* var. *oryzae* (Ahlburg) Kurtzman et al.] and include the following: NRRL 447 (= Thom 290-2335-113; CBS 102.07) received in 1909 as *A. oryzae* from J. Westerdijk, Amsterdam. This fungus was isolated from koji (Murakami 1971); NRRL 448 (= Thom 113 L) source unknown. The first reference to "Thom 113 L" appears in a notebook listing the microbial cultures maintained by Mr. Lewis B. Lockwood, Curator of Microbial Cultures, Bureau of Chemistry, USDA 1931–1940, predating NRRL. Thom and Church (1921) would sometimes refer to a strain of *A. oryzae* as "resembling No. 113," as in Thom 290-147-113 (= NRRL 449) isolated from a Brazil nut, and therefore "Thom 113 L" was not necessarily derived from Thom 290-113; NRRL 451 (= Thom 290-4235.I5) received in 1917 from Dr. Round, Bureau of Chemisty, USDA. This fungus was isolated by Dr. Round from a sample of unfiltered soy sauce acquired for USDA at Tai Shem, Shemting Province, China; NRRL 454 (= Thom 290-5370-A409) received in 1933 from Y.K. Shih, National Wu-Han University, Wuchang, Huupeh, China. Several cultures were received in 1920 from Dr. Kokichi Oshima, Takamine Laboratories, Clifton, NJ (USA), including NRRL 455 (= Thom 290-4429-A) with the notation "Japanese Brewing Station"; NRRL 456 (= Thom 290-4429-Ao); NRRL 457 (= Thom 290-4429-Ao 6) "Nippon Jozo Kogyo Co., Tokyo"; NRRL 458 (= Thom 290-4429-Ao-Old); NRRL 459 (= Thom 290-4429-Ao K) "Japanese Brewing Station"; NRRL 460 (= Thom 290-4429-Ao 1) "Hishiroku Co., Kyoto"; NRRL 461 (= Thom 290-4429-Ao N) "Japanese Brewing Station"; NRRL 462 (= Thom 290-4429-Ao P) "Japanese Brewing Station"; NRRL 463 (= Thom 290-4429-Ao 2a) "Tsuboi, Osaka"; NRRL 464 (= Thom 290-4429-Ao 4a) "Higuchi"; NRRL 466 (= Thom 290-4429-Ao 5b) "Ueda, Osaka"; and NRRL 467 (= Thom 290-4429-Ao 5c) "Ueda, Osaka."

A series of cultures was received by Thom from Prof. Teizo Takahashi, University of Tokyo. Thom and Church (1921) identified these strains as lettered with the alphabet "A to P, then skip to Z" to correspond with Dr. Takahashi's usage in his own papers (Takahashi 1909; Takahashi and Yamamoto 1913). The situation is confused because Takahashi and Yamamoto (1913) reported 13 varieties (A, E, F, G, H, I, J, K, L, M, N, O, P) in addition to 3 varieties isolated from sake koji in a preliminary study, which are identified using letters from the Greek alphabet "alpha, beta, gamma" (Takahashi 1909). There is no reference in these publications to varieties B, C, D, or Z (Thom and Church 1921). Each culture represents an industrial strain used in Japan to manufacture fermented products such as miso, shoyu, or sake, as recorded by Takahashi and Yamamoto (1913). NRRL 468 (= Thom 290-Taka A) from koji for sake; NRRL 469 (= Thom 290-Taka C); NRRL 470 (= Thom 290-Taka D); NRRL 471 (= Thom 290-Taka E) from koji for sake; NRRL 472 (= Thom 290-Taka F) from koji for shoyu, Choshi City; NRRL 473 (= Thom 290-Taka G); NRRL 474 (= Thom 290-Taka H) from koji for shoyu, Choshi City; NRRL 475 (= Thom 290-Taka J) from koji from shoyu, Noda City; NRRL 476 (= Thom 290-Taka L) from koji for tamari, Handa City; NRRL 477 (= Thom 290-Taka M; NRRL 552) from koji for tamari, Mie Prefecture; NRRL 478 (= Thom 290-Taka N) from same sample of tamarii koji as variety "M"; NRRL 479 (= Thom 290-Taka O) from koji for tamari, Mie Prefecture; NRRL 480 (= Thom 290-Taka P) from koji for shoyu, Noda City; NRRL 481 (= Thom 290-Taka Z). NRRL 552 (= Thom 290-51; NRRL 477), a second source of "Thom 290-Taka M," was deposited with NRRL as "Thom 290-51." In L.B. Lockwood's numerical listing of cultures maintained by the Bureau of Chemistry, No. 51 is the accession number for "Thom 290-Taka M"; NRRL 1911 was received in 1943 from Dr. S.A. Waksman, Rutgers University, as "No. 35 – Takamine's original diastase strain"; NRRL 1919 was received in 1943 as "No. 42" from Takamine Laboratories.

Three cultures were received from Mr. Pei-Sung King, National Bureau of Industrial Research, Chungking, China, including NRRL 1997 (= N.B.I.R. 2082) from a "soya sauce shop" in Japan; NRRL 1998 (= N.B.I.R. 2001) from "koji obtained by Prof. Wei"; and NRRL 1999 (= N.B.I.R. 2015) from "soy sauce, Nanking." Four cultures were received in 1949 from Dr. Allan K. Smith, NRRL, who isolated them from samples of koji for soy sauce that he obtained in China (Smith 1949); these include NRRL 2217 (= A.K. Smith 56b) from partially fermented soybean–wheat flour mix, Chang Plant, Shanghai, China. The Chang Plant followed traditional methods for making soy sauce in approximately

50-gal earthenware jars and had been continuously operated by the Chang family for over 500 years (Smith 1949); NRRL 2218 (= A.K. Smith 56c) and NRRL 2220 (= A.K. Smith 73f), from samples of soy sauce mold used in the "old fashion method" at Shanghai; NRRL 2219 (= A.K. Smith 66a), from a sample of Tou-Si fermented soybeans provided by L.T. Cheng. Six cultures, NRRL 3483, NRRL 3484, NRRL 3485, NRRL 3486, NRRL 3487, and NRRL 3488, were isolated from a commercial koji starter for miso by Clifford W. Hesseltine in 1960. ARS Culture Collection records do not give the source of the koji or indicate if the individual strains were derived from more than one koji product. It was during this same time frame that Dr. Kazuo Shibasaki, Tohoku University, was conducting experiments on miso production with Hesseltine at NRRL (Shibasaki and Hesseltine 1962).

In 1969, Dorothy I. Fennell deposited the following cultures with NRRL as "Wisconsin Bacteriology numbers in lyophilization, ex ATCC." The cultures were obtained from IFO by K.B. Raper, University of Wisconsin (Raper and Fennell 1965). NRRL 4787 (= WB 4787; IFO 4083) is one of K. Saito's strains upon which I. Ohara (1953) based his recognition of *A. oryzae* var. *pseudoflavus* (Saito) Ohara; NRRL 4799 (= WB 4799; IFO 4134) is from T. Takahashi's "*A. oryzae* – D," isolated from koji for sake, and is the basis for *A. oryzae* var. *tenuis* Ohara; NRRL 4803 (= WB 4803; IFO 4203; K. Sakaguchi M 1–2) isolated from koji for miso, Kumamoto Prefecture by Prof. Kin-ichiro Sakaguchi, The University of Tokyo, is the basis for *A. oryzae* var. *microvesiculosus* Ohara; NRRL 4806 (= WB 4806; IFO 4294) was isolated from katsuobushi (dried bonito) and is the basis for I. Ohara's recognition of *A. sojae* var. *gymnosardae* (M. Yukawa) Ohara; NRRL 4894 (= WB 4894; OUT 5141) from Faculty of Engineering, Osaka University as *A. oryzae* var. *fulvus* Yamamoto; NRRL 5003 (= WB 5003; IFO 4233; K. Sakaguchi A5-1), *A. oryzae* var. *microsporous* Sakaguchi & Yamada; NRRL 5004 (= WB 5004; IFO 4242; K. Sakaguchi SH 10-5) *A. oryzae* var. *globosus* Sakaguchi & Yamada, from koji for shoyu, Chiba Prefecture; NRRL 5030 (= WB 5030; IFO 5321) is the basis for I. Ohara's recognition of *A. oryzae* var. *effusus* (Tiraboschi) Ohara; NRRL 5032 (= WB 5032; IFO 6215), an albino strain of *A. oryzae* from Japan, is the basis for *A. candidus* var. *amylolyticus* K. Takaoka.

In 1973, several cultures were received from Dr. Shodo Hara, Research Institute for Brewing, Tokyo. Three of these cultures are listed in ARS Culture Collection records as having been isolated "from cereal." Dr. Osamu Akita, Director of Microbiology Division, Research Institute for Brewing, Hiroshima, Japan (personal communication), has informed us that these cultures were isolated by Dr. H. Murakami in 1950 from materials used in the manufacture of soysauce or miso. RIB 23 (= NRRL 5588) *A. oryzae* var. *brunneus* was isolated from "dusts"(waste) at a soysauce and miso factory, Nagano Prefecture; RIB 25 (= NRRL 5589) *A. oryzae* var. *brunneus* was isolated from defatted soybean meal, and RIB 40 (= NRRL 5590) *A. oryzae* var. *viridis* was isolated from broad bean at the same soy sauce factory in Kyoto Prefecture. Dr. Murakami notes on each of these records that "This strain is wild type not used for koji making"; NRRL 5592 (= RIB 178) isolated from koji for sake; NRRL 5593 (= RIB 331) isolated from koji from miso; NRRL 5599 (= RIB 1048; K. Sakaguchi SH 6-2) received as *A. oryzae* var. *brunneus* Murakami, isolated from koji for miso.

Two strains, NRRL 5937 and NRRL 5938, were isolated in 1974 by D.I. Fennell from a sample of koji provided by Dr. Matsuyama, Higuchi Matsunosuke Shoten, Osaka; NRRL 6270 (= FR-1) and NRRL 6271 (= FR-3) were received in 1976 from Dr. K.C. Lin, Food Industry Research and Development, Hainchu, Taiwan, who identified these cultures as two of the best soy sauce strains in Taiwan; NRRL 6574 and NRRL 6575 were isolated in 1966 by C.W. Hesseltine from a jar of hama-natto that was obtained from a temple in Kyoto; NRRL 6595 was received in 1981 from Mr. Shinichi Mizutani, T. Hasegawa Co., Kawasaki, Japan, as an isolate from hama-natto; NRRL 13765, received in 1988 from Terry Mayo, Cedarlane Foods, Glendale, CA (USA), is used for production of amazake, an alcoholic beverage from rice; NRRL 30038, received in 1998 from J. Dorner, National Peanut Research Laboratory, Dawson, GA (USA), is identifed in U.S. Patent No. 6027724 (Dorner et al. 2000) as a nontoxigenic strain of *Aspergillus* for biocontrol of toxigenic fungi; NRRL 31119 *Aspergillus oryzae* (= IFO 30104; RIB 430; RIB 1031) was received in 2001 from Dr. Akira Nakagiri, Institute for Fermentation, Osaka (IFO), isolated from koji for making amazake.

Nine strains are classified as *Aspergillus sojae* Sakaguchi & Yamada: Murakami and include the following: NRRL 1988 and NRRL 1989 (= N.B.I.R. 2016) were isolated from koji used in the manufacture of soy sauce in Nanking, by Mr. Pei-Sung King, National Bureau of Industrial Research, Chungking, China (Lockwood 1947); NRRL 3351 received in 1968 from M. Mogi, Noda Institute, Noda Shoya Co., Japan; five cultures received in 1973 from Dr. Shodo Hara, Research Institute for Brewing, Tokyo, included NRRL 5594 (= RIB 1040), which was received by the RIB Culture Collection in 1964 as IAM 2150 "*Aspergillus parasiticus* Speare"; NRRL 5595 (= RIB 1041; K. Sakaguchi SH 1-2) from koji for soy sauce, Tokyo; NRRL 5596 (= RIB 1042; K. Sakaguchi SH 21) from koji for soy sauce; NRRL 5597 (= RIB 1046; K. Sakaguchi SH 8-3), from koji for soy sauce, Kagawa Prefecture; NRRL 5598 (= RIB 1047; IAM 2677; K. Sakaguchi SH 25) from koji for soy sauce; NRRL 30039 received in 1998 from J. Dorner, National Peanut Research Laboratory, Dawson, GA (USA), is identifed in U.S. Patent No. 6027724 (Dorner et al. 2000) as a nontoxigenic strain of *Aspergillus* for biocontrol of toxigenic fungi.

DNA extraction

Fungal mycelia used for DNA extractions were grown from *Aspergillus* conidial or cell suspensions [1×10^5 colony-forming units (CFU)/ml of 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

11 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 2–3 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, Indianapolis, IN, USA) according to the manufacturer's recommendations. Then, 8 μg of the digested DNA was dispensed in each lane on 0.8% agarose gel in TAE buffer [0.04 M Tris-acetate, pH 8.00; 0.001 M ethylene diaminetetracatic acid (EDTA)], run at 1.8 V cm 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; Bio-Rad, Hercules, CA, USA). Probes were labeled by random primed labeling with digoxigenin using the Nucleic Acid Nonradioactive 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) 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 strains.

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. 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. Fragments of more than 20 kb and less than 1.5 kb were not included in the analyses. *A. flavus* 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 $C = 2Nxy/(Nx + Ny)$, in which Nxy is the number of hybridizing DNA bands shared by the isolates x and y, and Nx and Ny 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 strains share identical fingerprints or identifies those belonging to the same DNA fingerprint group (McAlpin et al. 1998) but does not imply phylogenetic relationships.

Results and discussion

DNA fingerprinting was performed on 72 strains of *Aspergillus oryzae* and 9 strains of *Aspergillus sojae* isolated from chu (China) or koji (Japan), mold inoculum used in the production of traditional Oriental fermented beverages or foods including soy sauce, miso, and sake (see Table 1). Eleven genotypes were represented by 2 or more strains that produced identical fingerprints (Fig. 1). Genotype GTAo-29 consists of 2 strains, NRRL 471 and NRRL 472, which share 93% band similarity. NRRL 5938 is included in GT Ao-5 because it shared 84% band similarity with NRRL 460, NRRL 468, and NRRL 1911, 3 strains showing identical fingerprints. In our analyses of "wild" populations of *A. flavus* and *A. parasiticus* from crop fields, strains showing greater than 80% band similarity are recognized as belonging to the same "fingerprint group" (McAlpin et al. 1998, 2002). It has been our experience that all strains of *A. flavus* belonging to the same vegetative compatibility group (VCG) typically produced identical DNA fingerprints, with several vegetatively compatible strains producing fingerprints with 80%–99% band similarity (McAlpin and Mannarelli 1995; McAlpin et al. 2002). In the present study, 33 single strain isolates of *A. oryzae* produced DNA fingerprints that did not match any of the other isolates (<80% band similarity).

All strains of *A. sojae* that we examined produced identical DNA fingerprints and are included in GTAo-9 (Fig. 1). Each of these strains was isolated from koji used in the manufacture of soy sauce in both China and Japan. These results suggest that industrial strains of *A. sojae*, including the strains listed under GTAo-9, may have originated from a common ancestral clonal population, a domesticated form of *Aspergillus parasiticus*.

Strains of *A. oryzae* were distributed among 43 DNA fingerprint groups, including GTAo-12 represented by 11 strains, GTAo-19 represented by 5 strains; GTAo-5 and GTAo-15 represented by 4 strains, and GTAo-8, GTAo-17,

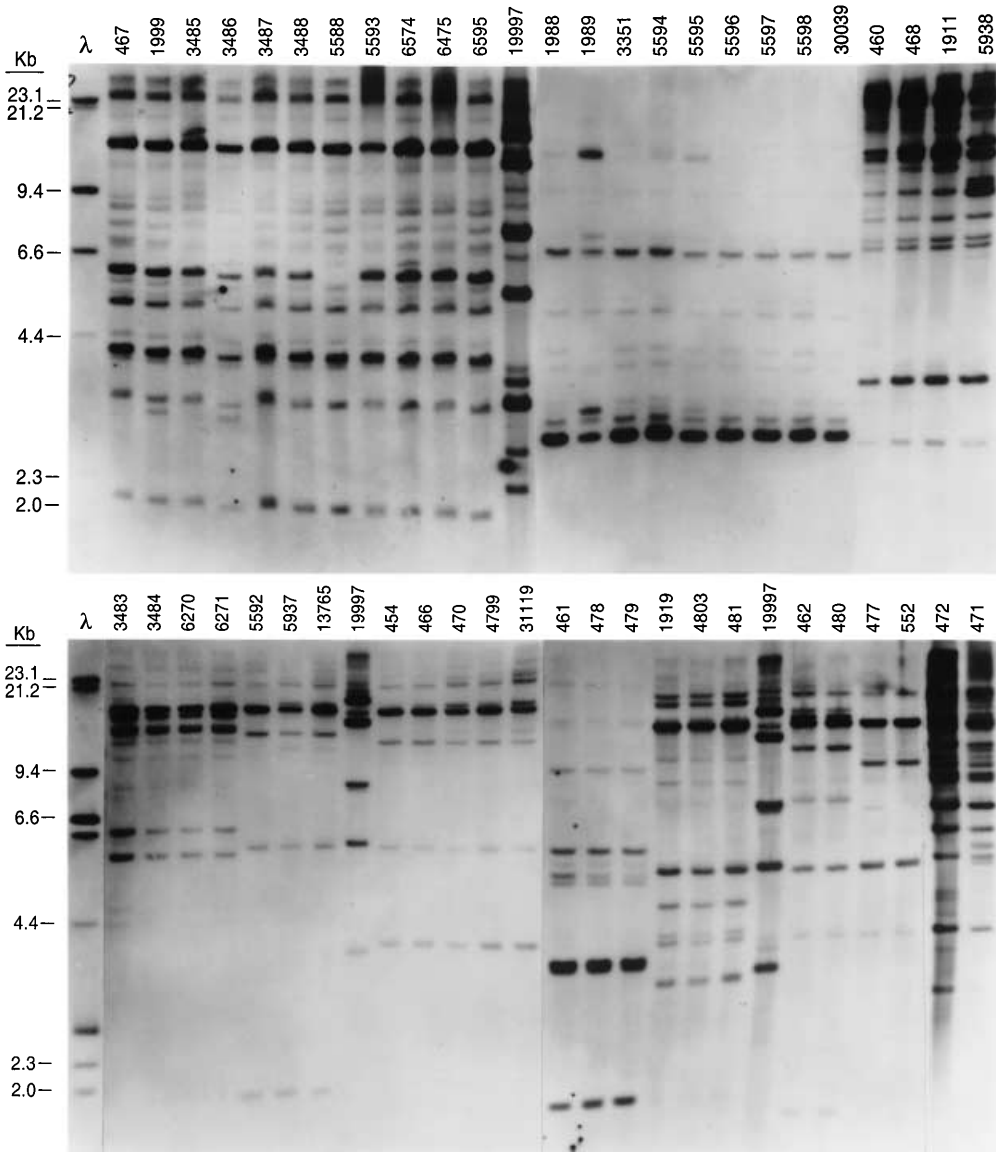

Fig. 1. DNA fingerprint matches among strains of *Aspergillus oryzae* and *Aspergillus sojae*. The figure includes one reference strain (*Aspergillus flavus* NRRL 19997) and size markers

and GTAo-24, each represented by 3 strains. *Aspergillus oryzae* GTAo-12 is apparently widely used as a koji mold, particularly in food fermentations using soybeans. DNA fingerprinting also reveals that NRRL 3485, NRRL 3486, NRRL 3487, and NRRL 3488, isolated from a sample(s) of koji for miso by dilution plating, may be isolates of the same fungus. It also appears likely that NRRL 6574 and NRRL 6575, isolated from a jar of hama-natto, represent the same fungus. Twenty-five years ago only 16 Japanese companies were involved in producing koji inoculum (Hesseltine et al. 1976). According to the authors, all sake and miso companies are believed to buy koji starter inoculum while the larger shoyu companies produce their own inoculum, which would explain why some strains of koji molds were more frequently recorded from koji used in these fermentations. The DNA fingerprint produced by NRRL 5588 (= RIB 23) is identical to those produced by domesticated strains grouped in GTAo-12 and probably represents a contaminant of the "cereal" from within the soy sauce plant.

Aspergillus oryzae GTAo-5 links NRRL 468, T. Takahashi's *A. oryzae* – "A," from koji used in the production of sake with NRRL 1911, received from S.A. Waksman in 1943 as "Takamine's original diastase strain." Takamine developed a fermentation process for the industrial production of fungal amylase (Takamine 1894). The process included the culture of *Aspergillus oryzae* on moist rice grains. NRRL 460 received from Takamine Labs. as "Ao1" may also represent Takamine's original strain (Takamine 1894). GTAo-15 is represented by two isolates from koji for miso, NRRL 3483 and NRRL 3484, as well as two strains recognized by K.C. Lin to represent the best soy sauce strains in Taiwan. GTAo-19 matches identical fingerprints for NRRL 470 and NRRL 4799, each derived from Takahashi's *A. oryzae* – "D" NRRL 470 was received before 1920 and examined by Thom and Church (1921), while NRRL 4799 (= IFO 4134) was received by the ARS Culture Collection in 1969 as WB 4799, a culture obtained from the IFO Culture Collection, Osaka, in 1962 by Raper and

Fennell (1965). We could find no information linking *A. oryzae* "D" to either NRRL 454 received from Y.K. Shih or NRRL 466 received from Takamine Labs as "Ao 5c." NRRL 31119 *Aspergillus oryzae* (= IFO 30104; RIB 430; RIB 1031) is the fungus that Murakami (1971) theorized was from the same source as Wehmer's *A. oryzae* NRRL 447. GTAo-17 includes strains of *A. oryzae* isolated from koji used in the production of alcoholic beverages, NRRL 5592, NRRL 5937, and NRRL 13765. GTAo-24 links NRRL 461 received by Thom from Takamine Labs. as "Ao N" with NRRL 478 received from Takahashi as *A. oryzae* "N." The match with Takahashi's *A. oryzae* "O" isolated from koji for tamari provides an example of a successful koji strain being used in different fermentations.

In another example, GTAo-29 matches (93% band similarity) NRRL 471 *A. oryzae* "E" from koji used for sake with NRRL 472 *A. oryzae* "F" from koji used for shoyu (Takahashi and Yamamoto 1913). GTAo-25 properly matches NRRL 480 *A. oryzae* "P" from koji for shoyu, Noda City with NRRL 462 received from Takamine Labs. as "Ao P." Likewise, GTAo-6 matches NRRL 477 and NRRL 552, both of which are cultures derived from Takahashi's *A. oryzae* "M". GTAo-8 pairs NRRL 481 (= Thom Coll. 290-Taka-Z) with NRRL 4803 (= IFO 4203; K. Sakaguchi M 1–2) isolated from koji for miso, and NRRL 1919 was received from Takamine Labs. in 1943 as "No. 42" with no other information.

One of the most encouraging results from this study was our ability to match fingerprints of cultures identified by letters D, M, N, and P (Takahashi and Yamamoto 1913; Thom and Church 1921) with other NRRL strains identified in culture collection records by those same letters. These successful matches can be viewed as "controls" for testing the efficacy of the pAF28 DNA probe for use in accurately distinguishing among genotypes of *Aspergillus oryzae*. The pAF 28 DNA probe might be used to identify strains of *A. oryzae* or *A. sojae* that have a greater potential for forming anastomoses between vegetative hyphae or "heterokaryons," important to the genetic improvement of industrial strains involving the parasexual life cycle (Ishitani and Sakaguchi 1956; Thorbek and Eplov 1974; Benkhemmar et al. 1985).

The acqusition of koji molds by the ARS Culture Collection was largely guided by mycologists who sought to examine strains that were used as the basis for establishing new species or varieties. This process would serve to emphasize diversity in the collection and explains, in part, why we recorded a large majority of *A. oryzae* genotypes (unique DNA fingerprints) represented by a single isolate (see Table 1). We find it interesting that the DNA fingerprint from *A. oryzae* NRRL 447 (= Thom 290-113; CBS 102.07) produced no matches with any other *A. oryzae* fingerprint. Murakami (1971) offers indirect evidence to support his hypothesis that *A. oryzae* Thom No. 113 may have been isolated from a sample of koji produced at the factory of Shichiro Fukuda in Tokyo, which in 1917 became the Nippon Jozo Kogyo Co. Furthermore, Murakami suggested that *Aspergillus oryzae* RIB 430 (= RIB 1031; IFO 30104; NRRL 31119), which was isolated from koji produced at the Fukuda Factory, dates back to 1890, before Wehmer or Takahashi, and may represent H. Ahlburg's isolate of *A. oryzae*. Results of DNA fingerprinting show that NRRL 31119 matches the fingerprints of *A. oryzae* isolates assigned to GTAo-19, which do not include *A. oryzae* NRRL 447.

Nontoxigenic strains of *Aspergillus oryzae* NRRL 30038 (= S-03) and *Aspergillus sojae* NRRL 30039 (= S-12) were identified as useful fungal biocontrol agents for preventing aflatoxin contamination in agricultural commodities, especially those for human consumption such as peanuts and corn (Dorner et al. 2000). The strains S-03 and S-12 do not produce aflatoxin, any bis-furan ring-containing intermediates of the aflatoxin biosynthetic pathway, or cyclopiazonic acid and were obtained commercially from a food manufacturer, Higuchi Matsunosuke Shoten Co., Osaka, Japan (Dorner et al. 2000). The DNA fingerprint produced by *A. sojae* NRRL 30039 was identical to fingerprints of *A. sojae* isolates in GTAo-9. Aspergillus *oryzae* NRRL 30038 did not match fingerprints produced by any other strain of *A. oryzae* (<80% band similarity).

It has been argued that *A. oryzae* and *A. sojae* are domesticated forms of *A. flavus* and *A. parasiticus*; their colony and microscopic characteristics in addition to physiological and chemical phenotypic attributes are the result of adaptation to a koji environment over hundreds of years of human culture (Wicklow 1983a,b, 1984). At the same time, there are numerous strains classified as *A. oryzae* or *A. flavus-oryzae* that were isolated from nature but may resemble *A. oryzae* in culture. Wicklow (1983a) offers examples where strains initially identified as *A. flavus* have been reclassifed as *A. oryzae* because of changes in culture characteristics during laboratory subculture. In the present study, cultures were selected for DNA fingerprinting if they were associated with Oriental food fermentations. Our limited results show that the genotypic diversity of atoxigenic strains of *A. oryzae* vs. *A. sojae* mirrors what has been found in wild populations of *A. flavus* and *A. parasiticus*. Atoxigenic strains of *A. parasiticus* are rarely isolated from nature (Cole et al. 1994; Horn et al. 1996). As one example, while aflatoxin production was reported for all strains of *A. parasiticus* isolated from an Illinois corn field, atoxigenic strains of *A. flavus* were common (McAlpin et al. 1998). Loss of aflatoxin production in *A. parasiticus* may be less frequent because of strong selection in a soil habit, regardless of latitude, and because it possesses duplicated chromosome regions with multiple copies of aflatoxin biosyntheis genes, including the aflR gene regulating aflatoxin B_1 synthesis (Liang et al. 1996). Wang and Fang (1986) describe chu as originating in northern China where it was prepared in midsummer using cracked wheat. The wheat was first soaked and steamed, cooled, and then gathered into a pile 6 cm deep and covered with leaves. Within 7 days the wheat became covered with a yellow mass (*Aspergillus* mycelia and spores), which indicated that the grain had been converted to chu.

Atoxigenic strains of *A. flavus* are recorded at a much greater frequency in northern latitudes than in near-subtropical to tropical regions (Manabe et al. 1976; Shearer

et al. 1992; Wicklow et al. 1998). It should follow that many of the naturally occurring wild strains of *A. flavus* that colonized piles of steamed wheat in northern China, converting them into chu, were already atoxigenic before becoming further domesticated. None of the DNA fingerprints of domesticated yellow-green aspergilli examined in this study matched any of the DNA fingerprints in our limited database for "wild" strains of *A. flavus* (316 unique fingerprints) or *A. parasiticus* (33 unique fingerprints) isolated from crop fields in Georgia, Illinois, or Iowa (McAlpin and Mannarelli 1995; Wicklow et al. 1998; McAlpin et al. 1998, 2002; Wicklow et al., unpublished data). There was no reason to believe that we would find such a match in fingerprints, but the availability of the database invited such a comparison.

The ancient and modern history of the distribution of mold inoculum for chu or koji throughout the Orient will never be fully understood . Chu or koji mold isolates that produce identical DNA fingerprints probably originated from the same ancient clonal populations. Extensive periods of genetic isolation and selection in different fermentation environments can lead to a divergence in colony, microscopic, physiological, and chemical phenotypic characteristics. Rather than to suggest the repeated domestication of the same "wild" clonal populations, it would seem more likely that in both ancient and modern times a successful chu or koji starter would have been spread throughout East Asia.

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