

Short Communication

Evolutionary relationships among *Aspergillus oryzae* and related species based on the sequences of 18S rRNA genes and internal transcribed spacers

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The mold *Aspergillus oryzae* is widely used in Japan as a koji mold for the fermentation of sake, miso, and soy sauce and belongs to the genus *Aspergillus* section *Flavi* (Gams et al., 1985), which includes *A. flavus*, *A. sojae*, *A. tamarii*, *A. parasiticus*, and *A. nomius*. *Aspergillus sojae* is also used as a koji mold for soy sauce fermentation. On the other hand, *A. flavus*, *A. parasiticus*, and *A. nomius* are known to commonly infect cereal grains and peanuts; it is also known that many of their isolates produce aflatoxins, the carcinogenic secondary metabolites. Many taxonomical studies on these species have been carried out (Gams et al., 1985; Klich and Pitt, 1988; Murakami, 1971; Raper and Fennell, 1965).

Kurtzman et al. (1986) investigated the DNA relatedness among *A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. sojae*. They showed that all four species had high (69–100%) nuclear DNA homology and similar genomic size. On the basis of these genotypic characters, they proposed that the four taxa represented a single species (Kurtzman et al., 1986). Chemotaxonomic approaches have also been used by Kuraishi et al. (1990) and Yamatoya et al. (1990). The latter attempted to evaluate the conflicting concepts of speciation in *Aspergillus* section *Flavi* by using combinations of chemotaxonomic criteria such as electrophoretic

comparison of enzymes, ubiquinone systems, DNA base composition, and DNA relatedness. They concluded that *A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. sojae* could be accommodated in two species (cf. Samson, 1992, 1994; Sugiyama, 1990).

Chang et al. (1991) have used partial sequence comparisons of 18S rRNA, comprising 558 nucleotides, to determine the evolutionary affinities among 11 species of *Aspergillus* and associated teleomorphs; they have revealed that the sequences of 18S rRNA of *A. oryzae* and *A. flavus* in all sequenced regions (558 nucleotides) are identical. In our previous paper (Nikkuni et al., 1996b), we sequenced the 18S rRNA genes (rDNAs) of *A. oryzae*, *A. awamori*, and their closely related species (total of 7 species) to get information on phylogenetic relationships among these species. The sequence of *A. oryzae* was identical with those of *A. flavus*, *A. sojae*, and *A. parasiticus* in the sequenced region (1,733 nucleotides), but it differed from that of *A. tamarii* (Nikkuni et al., 1996b).

In this study, we sequenced the 18S rDNAs of another 19 strains, including another 16 type species [a total of 18 type species, including those in our previous paper (Nikkuni et al., 1996b)] of the 18 sections (Gams et al., 1985) of *Aspergillus* to learn the phylogenetic position of the section *Flavi* in *Aspergillus*. Furthermore, it is known that rDNA internal transcribed spacers (ITS) are highly divergent in *Fusarium sambucinum* (O'Donnell, 1992), and their sequencing provides good reliability in the detection of close phy-

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Table 1. List of the *Aspergillus* species examined and their accession numbers^a of nucleotide sequence data.

Species ^b	Strain	Origin	Accession number ^a	
			18S rDNA	ITS
<i>E. herbariorum</i>	JCM 1575	CBS 516.65 ^N	AB008402	
<i>A. restrictus</i>	JCM 1727	CBS 541.65 ^T	AB008407	
<i>A. fumigatus</i>	JCM 1738	CBS 133.61 ^R	AB008401	
<i>A. cervinus</i>	ATCC 16915	WB 5025 ^N	AB008397	
<i>H. ornatus</i>	JCM 2354	NRRL 2256 ^T	AB008406	
<i>A. clavatus</i>	JCM 1718	CBS 513.65 ^L	AB008398	
<i>A. nidulans</i>	ATCC 10074	NRRL 187 ^R	AB008403	
<i>A. versicolor</i>	ATCC 16853	WB 227	AB008411	
<i>A. ustus</i>	ATCC 1041	NRRL 275 ^R	AB008410	
<i>A. terreus</i>	ATCC 1012	NRRL 255 ^T	AB008409	
<i>A. flavipes</i>	ATCC 24487	NRRL 302 ^T	AB008400	
<i>A. ochraceus</i>	JCM 1958	CBS 108.08 ^R	AB008405	
<i>A. wentii</i>	JCM 2724	ATCC 10552 ^T	AB008412	
<i>A. oryzae</i>	ATCC 1011	CBS 102.07 ^R	D63698 ^c	AB008417
<i>A. oryzae</i>	IFO 4181			
<i>A. oryzae</i>	RKG 2	Nepal ^d		
<i>A. flavus</i>	NFRI 1212	NRRL 11612	D63696 ^c	AB008415
<i>A. flavus</i>	ATCC 16883	WB 1957 ^N		AB008416
<i>A. flavus</i>	ATCC 10124	NRRL 484		AB008414
<i>A. flavus</i>	NFRI 1096	Thailand		
<i>A. sojae</i>	IFO 4386		D63700 ^c	AB008419
<i>A. parasiticus</i>	NFRI 1153	NRRL 2999	D63699 ^c	AB008418
<i>A. parasiticus</i>	ATCC 16869	WB 465		
<i>A. toxicarius</i>	IFO 31250	NJK 4044 ^{T,e}		AB008421
<i>A. tamarii</i>	JCM 2259		D63701 ^c	AB008420
<i>A. nomius</i>	NFRI 1214	NRRL 13137	AB008404	
<i>A. niger</i>	IFO 6341	ATCC 6275	D63697 ^c	
<i>A. awamori</i>	IFO 4033		D63695 ^c	
<i>A. candidus</i>	JCM 1867	CBS 567.65 ^N	AB008396	
<i>C. cremea</i>	IFO 32021	WB 5081 ^T	AB008399	
<i>A. sparsus</i>	JCM 2357	CBS 139.61 ^T	AB008408	
<i>A. avenaceus</i>	IFO 7539	NRRL 517 ^T	AB008395	
<i>A. zonatus</i>	IFO 8817	NRRL 5079 ^T	AB008413	

^a The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data base with these accession numbers.

^b Abbreviations for generic names: *E*, *Eurotium*; *A*, *Aspergillus*; *H*, *Hemicarpaceles*; *C*, *Chaetosartorya*.

^c Sequence data were from the previous paper (Nikkuni et al., 1996b).

^d Nikkuni et al. (1996a).

^e Strain derived from the type according to Murakami et al. (1983).

^{T, N, L, R} According to Samson and Gams (1985): T, strain derived from the type; N, strain from the neotype; L, strain from the lectotype; R, representative strain.

logenetic distance (Messner et al., 1995). Therefore we also sequenced the ITS regions for a phylogenetic analysis of closely related species of the section *Flavi*.

Each strain of *Aspergillus* species (see Table 1) was cultured in 100 ml YM broth (Difco, Detroit, MI, USA) or YM broth containing 30% glucose in a 500-ml Erlenmeyer flask at 150 rpm and 30°C for 1 to 7 days. Mycelia were harvested with a filter paper (Toyo No. 2) washed with deionized water, immersed into liquid nitrogen, and lyophilized. Genomic DNA was isolated from the lyophilized mycelia according to the methods of Raeder and Broda (1985), described previously (Nikkuni et al., 1996b).

The 18S rDNA was selectively amplified by PCR by

using the synthesized oligodeoxynucleotide primers described previously (Nikkuni et al., 1996b), although a thermocycler (Gene Amp PCR System 2400, Perkin Elmer, Foster City, CA, USA) was used. The ITS regions including the 5.8S rDNA were selectively amplified by PCR using the primer pair of ITS4 and ITS5 (White et al., 1990) and the *Taq* polymerase (Ampli *Taq* Gold, Perkin Elmer). After 94°C for 1 min, 40 cycles of the program 94°C/22 s, 50°C/10 s, and 72°C/25 s were performed in the thermocycler (Gene Amp PCR System 2400, Perkin Elmer). The amplified DNA was purified with Centricon-100 column (Amicon, Beverly, MA, U.S.A.).

The nucleotide sequences of the PCR products

were determined in both directions by the dideoxynucleotide chain termination method (Sanger et al., 1977) by using *Taq* polymerase (Perkin Elmer) and the dye primer (–21M13, Perkin Elmer) with a DNA sequencer (model 377, Perkin Elmer).

The sequences of 1,733 nucleotides of entire genes, except for about 40 nucleotides at the 5' ends and about 30 at the 3' ends, of 26 species of *Aspergillus*, including our previous paper (Nikkuni et al., 1996b), were determined. However, both sequences of *A. sparsus* and *A. candidus* consisted of 1,732 nucleotides, and the sequence of *A. ochraceus* was 1,734 nucleotides in the same sequenced regions. As a result of alignment, the sequences of 1,731 nucleotides of 26 species of *Aspergillus* were used for the present phylogenetic analysis.

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data base with the accession numbers shown in Table 1.

The evolutionary distance (*Knuc*) between sequences was calculated by Kimura's formula (Kimura, 1980) by using a computer program, Biorearch Sinca (Fujitsu, Tokyo, Japan). A phylogenetic tree was prepared by the neighbor-joining (NJ) method (Saitou and Nei, 1987) from the *Knuc* data by using the same computer program, Biorearch Sinca (Fujitsu). Bootstrap confidence values were calculated from 1,000 replications.

The *Knuc* values of 25 species of *Aspergillus*, including those of our previous paper (Nikkuni et al., 1996b), to *A. oryzae* were 0 (*A. flavus*, *A. sojae*, *A. parasiticus*), 0.00058 (*A. tamarii*), 0.00173 (*A. nomius*), 0.00580 (*A. fumigatus*, *Chaetosartorya creamea*), 0.00638 (*A. niger*, *A. awamori*, *Eurotium herbariorum*), 0.00639 (*A. terreus*), 0.00696 (*A. restrictus*), 0.00755 (*A. clavatus*), 0.00756 (*A. flavipes*, *Hemicarpenateles ornatus*), 0.00813 (*A. wentii*), 0.00872 (*A. ochraceus*), 0.00931 (*A. sparsus*), 0.00988 (*A. versicolor*), 0.00989 (*A. avenaceus*, *A. cervinus*), 0.01106 (*A. ustus*), 0.1165 (*A. nidulans*), 0.1225 (*A. candidus*), and 0.01460 (*A. zonatus*). The result shows that *A. oryzae* is closely related to the other species of the section *Flavi*. The sequences of *A. tamarii* and *A. nomius* differed from the *A. oryzae* sequence at a single nucleotide and three nucleotide positions. The results obtained also indicate that *A. avenaceus* and *A. zonatus*, both classified into the *A. flavus* group by Raper and Fennell (1965), are not so closely related to *A. oryzae*.

Figure 1 shows the phylogenetic tree constructed by the NJ method (Saitou and Nei, 1987). The *Aspergillus* species examined could be divided into four main clusters: 1, 2, 3, and 4. Cluster 1 was composed of *A. niger*, *A. awamori*, *A. candidus*, *A. cervinus* and

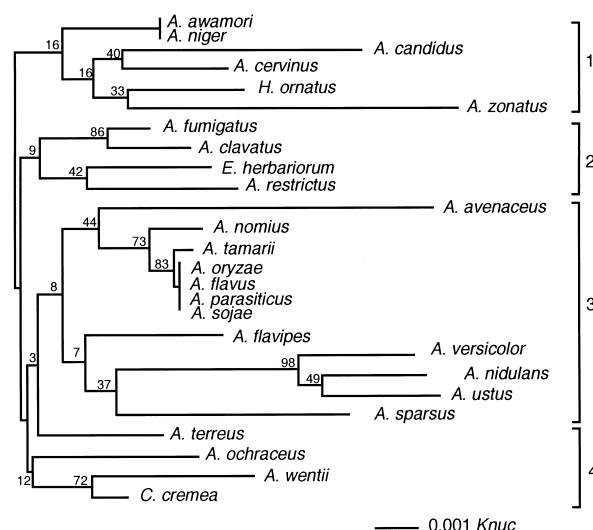


Fig. 1. Neighbor-joining tree for 26 *Aspergillus* species with or strictly lacking the teleomorph based on continuous 1731-nucleotide of 18S rDNA sequences.

Bootstrap confidence values were calculated from 1,000 cycles.

H. ornatus. Cluster 2 included *A. fumigatus*, *A. clavatus*, *E. herbariorum*, and *A. restrictus*. Cluster 3 included *A. oryzae*, *A. flavus*, *A. sojae*, *A. parasiticus*, *A. tamarii*, *A. flavipes*, *A. versicolor*, *A. nidulans*, *A. ustus*, and *A. sparsus*. Cluster 4 was composed of *A. terreus*, *A. ochraceus*, *A. wentii*, and *C. creamea*. *Aspergillus zonatus* and *A. avenaceus*, both classified into the *A. flavus* group by Raper and Fennell (1965), were included in cluster 1 and cluster 3, respectively. On the other hand, all the species of section *Flavi* examined were included in cluster 3.

Our results show that a comparison of 18S rDNA sequence can provide a means for analyzing phylogenetic relationships of *Aspergillus*, whose morphological classification was established by Raper and Fennell (1965) and reviewed, nomenclaturally reconstructed, by Gams et al. (1985).

Since the sequencing of ITS provides good reliability in the detection of close phylogenetic distance (Messner et al., 1995), we sequenced the ITS regions and 5.8S rDNA of 6 species: 12 strains of the section *Flavi*, namely, three of *A. oryzae* (ATCC 1011, IFO 4181, and RKG-2), four of *A. flavus* (ATCC 16883, ATCC 10124, NFRI 1096, and NFRI 1212), two of *A. parasiticus* (ATCC 16869 and NFRI 1153), and one each of *A. sojae* IFO 4386, *A. toxicarius* IFO 31250, and *A. tamarii* JCM 2259. The ITS regions and 5.8S rDNA of these 12 strains were reproducibly amplified by using the primer pairs of ITS4 and ITS5 (White et al., 1990).

The sequences of 157 nucleotides of the 5.8S rDNA of 12 strains from 6 species of the section *Flavi* were identical. On the other hand, the sequences of the in-

ternal transcribed spacers ITS1 and ITS2 of the 12 strains examined were grouped into three ITS types, designated A, B, and C, whose complete sequences are shown in Fig. 2. An identical nucleotide is indicated by a hyphen, and a gap in a sequence is marked with an asterisk (*). The sequences of 181 nucleotides of ITS1 and 169 nucleotides of ITS2 of *A. oryzae* ATCC 1011 were identical with those of the other two strains of *A. oryzae* (A ITS type). Although no differences were found in the 181-nucleotide sequence of ITS1 among the three strains of *A. oryzae* and the four strains of *A. flavus* (ATCC 16883, ATCC 10124, NFRI 1096, and NFRI 1212), these seven strains were further divided into three groups, designated as A1, A2a, and A2b because of the sequences of ITS2 (positions 497 to 500 and position 384 in Fig. 2). A1 consisted of three strains of *A. oryzae* and *A. flavus* ATCC 16883. A2a consisted of two strains of *A. flavus* (ATCC 10124 and NFRI 1096), and A2b was *A. flavus* NFRI 1212, whose nucleotide sequence of ITS2 differed from A2a type at a single nucleotide (position 384 in Fig. 2, where "C" in A2b was replaced with "G"). On the other hand, the sequences of ITS1 and ITS2 of *A. sojae* were identical with those of the two strains of *A. parasiticus*, and *A. toxicarius* (B ITS type). The sequences of ITS1 and ITS2 of *A. tamarii* (C ITS type) differed from those of

A. oryzae, *A. flavus*, or *A. parasiticus*.

The phylogenetic tree of these strains was constructed by the NJ method (Saitou and Nei, 1987) from the *Knuc* values (Kimura, 1980) based on their nucleotide sequences of ITS1 and ITS2 after alignment. These six species, 12 strains of *Aspergillus* section *Flavi*, could be divided into three groups (Fig. 3). The first group was composed of *A. oryzae* and *A. flavus* (A ITS type in Fig. 2). The second included *A. sojae*, *A. parasiticus* and *A. toxicarius* (B ITS type in Fig. 2), and the third consisted of *A. tamarii* (C ITS type in Fig. 2). Yamatoya et al. (1990) revealed the dendrogram based on the similarity values of the electrophoretic mobilities of enzymes from 41 isolates in

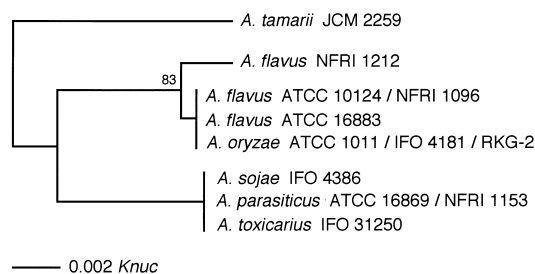


Fig. 3. Neighbor-joining tree for 12 strains of *A. oryzae* and five related species based on ITS1 and ITS2 sequences.

Bootstrap confidence values were calculated from 1,000 cycles.



Fig. 2. Sequence alignment of the ITS types of 12 strains of *A. oryzae*, *A. flavus*, *A. sojae*, *A. parasiticus*, *A. toxicarius*, and *A. tamarii* amplified with the primer pair ITS4 and ITS5 (White et al., 1990).

The sequence of *A. oryzae* ATCC 1011, A1 was used as the reference sequence. The numbering of the sequence begins with the first nucleotide in the ITS1. An identical nucleotide is indicated by a hyphen, and a gap in a sequence is marked with an asterisk (*). 18S, 5.8S, and 28S are 18S rDNA, 5.8S rDNA, and 28S rDNA, respectively. DDBJ accession numbers for the sequences are AB008414 through AB008421.

Aspergillus section *Flavi*, and they showed that *A. flavus* and other very closely related *Aspergillus* taxa formed one major cluster that could be divided into two subclusters, corresponding to *A. flavus* and *A. parasiticus*. A good correlation was found between the dendrogram based on the sequences of ITS regions obtained in this study and the dendrogram reported by Yamatoya et al. (1990). It may support Sugiyama's suggestion (1990) that *A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. sojae* can be accommodated in two species.

The sequences of ITS regions could distinguish *A. oryzae* from *A. parasiticus*, and *A. flavus* from *A. parasiticus*, but *A. oryzae* could not be distinguished from *A. sojae*, *A. flavus*, and *A. parasiticus* based on the sequence of 18S rDNA. This result shows that the comparison of ITS sequences can be used for analyzing phylogenetic relationships of closely related species. Although the number of strains examined is very limited, the variation of ITS2 sequence was observed among *A. flavus* strains. These were divided into three groups because of the sequences of ITS2, and *A. oryzae* and *A. parasiticus*, whose sequences were identical among strains of the respective. The ITS1 in *Saccharomyces cerevisiae* is known as an essential gene for the production of 18S rRNA (Musters et al., 1990). This may be the reason for the higher variation of ITS2 sequences than of ITS1 sequences.

Kurtzman et al. (1986) investigated DNA relatedness among *A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. sojae* and have shown that all four species have high (69–100%) nuclear DNA homology and similar genomic size; they have proposed from these results that the four taxa represent a single species. On the other hand, various studies on the differentiation of these four species have been reported. Klich and Mullaney (1987) have found that *Sma* I digests of total DNA can be used for a differentiation of *A. flavus* from *A. oryzae*. Moody and Tyler (1990a, b) have reported that the DNA restriction fragment length polymorphisms (RFLP) clearly distinguished *A. flavus*, *A. parasiticus*, and *A. nomius*. The random-amplified polymorph DNA (RAPD) method has been employed by Yuan et al. (1995), and they reported that three decameres, OPA-04, OPB-10, and OPR-1, allowed adequate discrimination between *A. sojae* and *A. parasiticus* in RAPD analysis. A DNA probe was constructed to distinguish among strains of *A. flavus* by DNA fingerprinting techniques (McAlpin and Mannarelli, 1995). Recently, several aflatoxin pathway genes, including *A. parasiticus aflR*, have been cloned from *A. flavus* and *A. parasiticus* (Yu et al., 1995). The *aflR* homologs in members of *Aspergillus* section *Flavi* were investigated by Chang et al. (1995). They revealed that the same zinc finger domain region was

present in all the *A. sojae*, *A. flavus*, and *A. parasiticus* strains examined and in some of the *A. oryzae* strains. These four species can be distinguished by their specific nucleotide positions of the genes (Chang et al., 1995).

Murakami (1971) and Klich and Pitt (1988) have revealed the species-level difference between *A. flavus* and *A. oryzae*, based on physiological and morphological studies. The nucleotide sequence comparisons obtained in this study indicated that the species in the section *Flavi* are very closely related and that a comparison of ITS sequences can be useful for analyzing phylogenetic relationships of these closely related species.

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