Molecular typing of *Aspergillus* section *Flavi* by mitochondrial cytochrome *b* gene analysis

Koji YOKOYAMA*, Li WANG, Haruo TAKAHASI*, Nobuaki KASE*, Makoto MIYAJI and Kazuko NISHIMURA

An important and problematic section within *Aspergillus* is sect. *Flavi*. Because of their industrial, agronomic and medical importance, the accurate identification and classification is necessary. Traditional methods are based on the morphological characters, but the high degree of morphological similarities make identification difficult, therefore the taxonomy is clouded and controversial [1-4].

In the past, various methods had been used to the identification and classification of the sect. *Flavi*. But the results of these studies were varied and could not give a clear taxonomic conclusions. Recently, the several molecular attempts have been carried out as tools for studying the phylogenetic relationships, as well as for classifying and identifying the sect. *Flavi*. For example, based on RAPD analysis, only *A. parasiticus* and *A. sojae* were differentiated from each other [5]. The sequence divergence of aflR genes (hybridization patterns) showed that *A. oryzae* and *A. sojae* were similar to those of *A. parasiticus* [6]. Based on PCR-SSCP (single-strand conformation polymorphism) analysis, the strains were divided into four group, *A. flavus*, *A. oryzae*, *A. parasiticus/A. sojae*, *A. tamarii* and *A. nomius* [4]. The analysis of the PCR-RFLP patterns showed except for *A. tamarii*, there were very similar among the other five species. Based on the analysis of the enzyme electrophoretic patterns, *A. flavus*, *A. parasiticus* and *A. tamarii* showed distinct patterns [2, 7], but *A. oryzae* was very similar to those of *A. flavus*, and *A. sojae* to those of *A. parasiticus*. While Klich and Mullaney (1987) reported that *A. oryzae* and *A. flavus* could be differentiated based on the Sma I digestion patterns using total DNA [8]. Using electrophoretic comparison of enzymes and ubiquinone systems, Yamatoya et al. (1990) found that isolates of *A. flavus*, *A. parasiticus*, *A. oryzae* and *A. sojae* could be accommodated in two species: *A. flavus* and *A. parasiticus* [2].

The substitution rate in mammalian mt DNA was five to ten times higher than that in chromosomal gene [9]. Therefore, it can giving a magnified view of genetic differences among species and allow determination of the relationships among closely related species [10]. Recently, mt DNA analysis by restriction fragment-length polymorphisms (RFLP) has become a useful methods for taxonomy of the fungi [11, 12, 13]. The mt cytochrome *b* gene was often
chosen as a phylogenetic probe in birds, mammals and fish [14-19].

To demonstrate the sequences of mt cytochrome b gene can be used for phylogenetic classification and will prove especially valuable identification for fungi, we had firstly reported this gene were very useful and powerful tool in fungi [20]. We had studied major five species of pathogenic *Aspergillus* [20] and other fungi, for example, *Zygomycetes*, genus *Aspergillus*, *Penicillium*, *Neosartorya*, and so on (unpublished). These sequences and phylogenetic tree have confirmed it.

Here we provide an independent assessment of evolutionary relationships among sect. *Flavi*, based on nucleotide sequences from mt cytochrome b gene. And using the sequences as a tool for classification and identification.

**Materials and Methods**

**Fungal strains.**

31 strains of *Aspergillus* sect. *Flavi* representing five species used in this study were obtained from several preserved facilities.

**Primers**

mt DNA fragments containing sequences of the cytochrome b gene were amplified by PCR using designed primers Elm (5’-TGAGGTGCTACAGTTATTAC-3’) and rEME2 (5’-AAAATAGCATAGAAAGGTAA-3’) or E2 (5’-GGTAGATAGMTCTTAAWATAGC-3’). To designed these primers, we compared the published amino acid sequences of mt cytochrome b of potato, corn, *A. nidulans*, *N. crassa*, *S. cerevisiae* and *Rhodospirillum rubrum*, and searched for highly conserved regions. [20]. Elm was used as forward primer and E2 or rEME2 as reverse primers.

**Extraction of DNA from cultured strains**

DNA was extracted according to the method described by Wang *et al* [20]. Fungal mycelium was cultivated at 25°C for 3-5 days with shaking in liquid medium PDB (potato dextrose broth), harvested and stored at 8°C. Zymolyase and glass beads were used for the destruction of cells. Mitochondria were then pelleted by centrifugation at 20,000 g for 15 min. PCI (phenol : chloroform : isooamyl alcohol-25 : 24 : 1) and ethanol were used for mt DNA extraction.

**PCR.**

The PCR cycling protocol consisted of the following: each cycle consisted of denaturation for 1 min at 94°C, annealing for 1 min at 50°C and extension for 2 min at 72°C for 30 cycles. A TaKaRa PCR Amplification Kit (Takara Shuzo Co., Ltd., Otsu, Shiga, Japan) was used for amplification with a Sanyo DNA amplifier MIR-D 30. Amplifications were performed in a 100 ul reaction volume, using 1 ml of each primer (approximately 130 ng ml-1). PCR products were subjected to electrophoresis, then visualized and photographed under UV [20].

**Sequencing**

Both the strands of the amplified PCR products were sequenced directly on an ABI prism 377 DNA sequencer using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Division of Perkin-Elmer Japan Co., Ltd.) [20].
Data analysis

DNA sequences and amino acid sequences estimated by using the yeast mt genetic code were aligned and compared using program GENETYX-MAC Genetic Information Processing Software (Software Development Co., Ltd., Tokyo, Japan) and the trees were generated by Unweighted Pair Group Method with Arithmetic mean (UPGMA). The estimation of phylogenetic relationship was done using standard errors for each branching point. Standard errors were calculated by the method of Nei [21].

Detection of Aflatoxins

The culture broth was filtered, and aflatoxin in the filtrate was separated by thinlayer chromatography (TLC) after direct spotting of 10 µl filtrate on precoated Kieselgel plate (Merk, no. 5721, Germany). The plates were developed with asolvent system; chloroform : acetone : n-hexane, 85 : 15 : 20 (v/v). The aflatoxins on the plate were detected and Rf value was compared with standard aflatoxins under UV.

SEM observation

Conidial samples were fixed by OsO4 gas, and corted by goled. Scanning electron microscope (JOL, JSM-6100) was used at an accelerating voltage of 10kv.

Results and Discussion

A total of 402 base pairs (except for the parts of primers) of the mt cytochrome b from Aspergillus sect. Flavi was sequenced. Based on the the nucleotide sequences, all of the strains used in this study were separated into six types D-1, D-2, D-3, D-4, D-5 and D-6, as shown in Figure. We selected representatives from each type, and aligned the sequences. The nucleotide sequences and estimated amino sequences were used to construct the phylogenetic trees with other species of Aspergillus (Figure).

In type D-1 (Figure), A. oryzae var. oryzae (CBS 133.52, ATCC 9362, NRRL 1988) were shown be identical to 3 strains of A. parasiticus (A. sojae). This strain (CBS 133.52, ATCC 9362, NRRL 1988) have been determined to be a A. sojae by Perng-Kuang Chang et al [22]. Our results also confirmed this strain was A. sojae. 3 strains of the strong aflatoxin producer A. parasiticus and 3 strains which labelled as A. flavus were included in type D-2 (Figure). We considered that the type D-2 was A. parasiticus type and the 3 strains of A. flavus belong to A. parasiticus. The critical difference is that A. flavus only produces B aflatoxins or cyclopiazonic acid, or both. A. parasiticus produces both B and G aflatoxins, but never cyclopiazonic acid [3, 23]. However A. flavus IFM 48053 (NRRL 500) did not produce G aflatoxin, A. flavus IFM 5366 did not produce G and B aflatoxins. Concerning the strains of A. parasiticus and A. flavus, Pitt considered that the strains of the two species are very confused in fact. Most researcher who have reported A. flavus have been working with A. parasiticus really [7]. Kozakiewicz also found that the confusion of A. parasiticus and A. flavus using the SEM, and revealed that labbelled as A. flavus which actually belonged to A. parasiticus [24]. Since Sakaguchi and Yamada (1944) described A. sojae as a new species, past half of a century, the discussion concerning it as a synonym of A. parasiticus was continue ( Nehira (1955), Raper & Fennell (1965), Murakami (1971), Christensen (1981) ) [24]. Now A. sojae have been belonged to A. parasiticus in some preserved facilities. Our results strongly suggested that A. sojae and A. parasiticus were not identical, although they
Mycotoxins were most closely related among the others in this study. In the position 310 of the nucleotide sequences, *A. sojae* showed sequence “A” and *A. parasiticus* showed “T”, therefore, they can be distinguished clearly each other. In the position 253, *A. sojae* and *A. parasiticus* can be differentiated from *A. flavus*, *A. oryzae* and *A. tamarii*.

The type D-4 including 8 strains of *A. flavus*, 3 strains of *A. flavus* var. columnarius, 1 strain of *A. flavus* var. *flavus* and 3 strains of *A. oryzae*. Concerning the relationships of *A. flavus* and *A. oryzae*, it has long been argued. Some workers [23, 24, 25, 26] considered that *A. oryzae* and *A. sojae* are "domesticated" forms of *A. flavus* and *A. parasiticus*, respectively, since they are morphologically identical but do not produce the corresponding aflatoxins. Based on our data, we considered that *A. flavus* and *A. oryzae* are same organism, *A. oryzae* is domesticated and so that don't produce aflatoxin. Concerning two varieties of *A. flavus*, some researcher attempted to consistently differentiate *A. flavus* var. columnarius from *A. flavus* var. flavus, but were not successful. Columanrity of conidial heads was found to be an inconsistent character [3].

Two strains of *A. tamarii* and 2 strains of *A. oryzae* were shown the identical sequences and were placed in the type D-5 (Figure). This type was considerable different from the other species. All four strains were shown brownish colonies. We considered that the 2 strains of labelled as *A. oryzae* were misidentified and they are *A. tamarii* actually.

*A. flavus* IFM 41933 belonged to type D-6 (Figure). Those strains was isolated from clinical sample and identified as *A. flavus*. This type was very near to type D-5 and as well as have brownish colonies.

We examined 17 strains described as *A. flavus* divided them into types D-2, D-3, D-4 and D-6, and *A. oryzae* also divided into three types D-1, D-4 and D-5. This suggests that the identification of the strains of sect. *Flavi* were very confusion because their morphological similarities.

Nucleotide based-tree (Figure) showed that the species of sect. *Flavi* were very closely relationships each other, seem to the variation of intraspecies in other *Aspergillus* species such as *A. nidulans*, *A. terreus* and *A. niger*. On the other hand, alignment of the estimated amino acid sequences showed that there were no differences in all the strains used in this study (Figure). In previous study, we have shown clearly the accordance of amino acid sequences among species [20]. For example, based on the amino acid sequences, the strains of *A. nidulans* were divided into three groups, while based on amino acid sequences, they could not be divided into any additional groups and showed an identical amino acid sequence (Figure). Therefore, we suggest that the species of sect. *Flavi* represented subspecies or varieties of a single species. But, if necessary, in fermentation industry, give *A. sojae* and *A. oryzae* distinct name are all right, although they belong to the same species genetically. Based on the high degree of DNA complementarity, Kurtzman et al. (1986) found that the similarities between *A. flavus* and *A. oryzae* to be 100%, *A. parasiticus* and *A. sojae* showed 91% relatedness. Complementarity between these two groups, demonstrated in the *A. flavus* - *A. parasiticus* paring was 70%. *A. tamarii* showed 40-55% relatedness with the foregoing species [27]. Therefore, they proposed that these species represented a same species and reduced *A. parasiticus* to the status of a subspecies and *A. oryzae* and *A. sojae* to the status of varieties of *A. flavus*. But Klich and Pitt [28] and
others [8] didn’t agreed this taxonomy and they stated that this is irrelevant in practicality, the toxin producers and fermentation cultures at species level and have distinct name were essential. Cytochrome b sequences support the DNA complementarity data of Kurtzman et al.

Productivity of aflatoxins is not in keeping with DNA type on Aspergillus sect. Flavi. We considered aflatoxin productivity is not used for criteria of taxionomy.

Each DNA type of section Flavi have a characteristic surface of spore (Figure), but it is not uniform in a species. Characteristics of spore surface are various in same strain. This feature dose not use for identification or classification.

In conclusion, our data showed that the mt cytochrome b sequences analysis is important and useful for phylogenetically classifying and accurately identifying the species of sect. Flavi, and in the future may allow construction of a natural taxonomy of Aspergillus and other genera.

References


