

Note

Comparison of Groupings among Members of the Genus *Aspergillus* Based on Phylogeny and Production of Bioactive Compounds

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Three hundred sixty-six *Aspergillus* strains preserved at the National Institute of Technology and Evaluation (NITE) were compared as to phylogenetic relationships (11 species-clusters) based on the DNA sequences of the D1/D2 domains of LSU rRNA and ITS regions, including the 5.8S rRNA and biological activities of their secondary metabolites. The results showed relatively well correlation between the phylogenetic distribution and the production of bioactive compounds, especially, antimicrobial activities.

Key words: *Aspergillus*; phylogeny; biological activity

The filamentous fungus *Aspergillus* spp. includes approximately 250 species,¹⁾ commercially and clinically important microorganisms. The genus *Aspergillus* has played a central role in the production of traditional fermented foods such as soy sauce, soybean paste, and rice wine throughout the last thousand years.^{2,3)} Recently, more than 1,200 biological metabolites, including derivatives have been discovered in metabolites of *Aspergillus* strains (according to “The Chapman and Hall/CRC Combined Chemical Dictionary”). *Aspergillus* species such as *A. versicolor*,⁴⁾ *A. ozyae*,^{5,6)} and *A. tamarii*,⁷⁾ are among the well-known producers of useful bioactive compounds.

On the other hand, many fungal toxins called mycotoxins are also produced by species of *Aspergillus*. For example, aflatoxins, which are capable of causing disease and death in humans and other animals, are produced by *A. flavus* and *A. parasiticus*,^{8,9)} but only four species of *Aspergillus*, *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger*, are commonly associated with invasive infections in humans.^{10,11)} There have been reports suggesting that the causative agents of aspergillosis are species specific, but production of natural

products has not yet been effectively related to phylogeny.¹²⁾ The aim of this study was to examine *Aspergillus* strains as to their phylogenetic relationships and the production of bioactive compounds. Phylogenetic analysis was based on DNA sequences of the D1/D2 domain of LSU rRNA and an internal transcribed spacer region including 5.8S rRNA (ITS-D1/D2).

Three hundred sixty-six *Aspergillus* strains (69 species) preserved at the Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE) of Japan, were used. Total DNA was extracted with a DNeasy Plant Kit (Qiagen, Tokyo). PCR amplification employed a pair of universal primers, ITS5¹³⁾ and NL-4.¹⁴⁾ Direct sequencing was performed on an Applied Biosystems 3130xl DNA Analyzer and with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Japan, Tokyo). For cycle sequencing reactions, primers ITS5, ITS4, ITS3, NL-1, and NL-4 were used.^{13,14)} All sequences are accessible at the web site of NITE (<http://www.nbrc.nite.go.jp>). Phylogenetic analysis was conducted by the neighbor-joining method with PAUP* version 4.0b10.¹⁵⁾ Four sequences of Onygenales species were added in analysis as outgroups, *Spiromastix warcupii* (AY527407 and AY176723), *Coccidioides immitis* (AB232890), *Paracoccidioides brasiliensis* (AF038360), and *Ajellomyces capsulatus* (AF038354). Support for individual nodes was assessed by bootstrap analysis, resampling the datasets 1,000 times.

All of the *Aspergillus* strains used were cultured at 28 °C for 14 d. Two solid culture media, composed of 3 g of oatmeal (Quaker Oats Company) and 10 ml of V8 Mix Juice (Campbell Soup Company) (medium A), and 3 g of brown rice, 1 g of yeast extract, and 9 ml of supplement solution (1 mg/ml of sodium tartarate and 1 mg/ml of KH₂PO₄) (medium B), were used as the

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Abbreviations: NITE, National Institute of Technology and Evaluation; NBRC, NITE Biological Resource Center; DPPH, 1,1-diphenyl-2-picrylhydrazyl

production medium. The mycelium was extracted with 80% acetone. Acetone extracts were prepared for measurement of biological activities, and the samples were stored at -40°C until use.

To identify the biological activities of the metabolites produced by the *Aspergillus* strains, we employed the following biological systems: antimicrobial, antioxidative, and hemolytic activities. The antimicrobial activity of the metabolites was estimated by the paper disk method on Mueller-Hinton agar medium (Eiken Chemical, Tokyo) for bacteria, and Sabouraud's dextrose agar (Becton Dickinson, Franklin Lakes, NJ) for fungi. Antioxidative activity was estimated by the DPPH (1,1-diphenyl-2-picrylhydrazyl)-scavenging method, as described previously.¹⁶⁾ As for hemolytic activity, rabbit erythrocytes were obtained from Nippon Bio-Test Laboratories (Tokyo) and used within 7 d, as described previously.¹⁷⁾

The results of the phylogenetic analysis of the *Aspergillus* species preserved at NITE based on the ITS-D1/D2 region are shown in Fig. 1. *Aspergillus* strains were assigned to 11 species-clusters, based mainly on clades supported by high bootstrap values, excluding the cluster G and J. Although the strains in

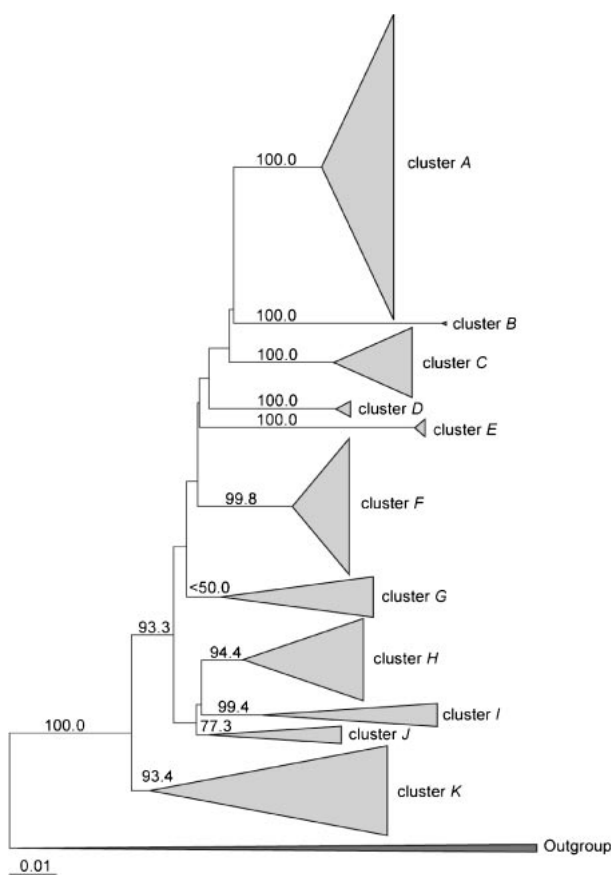


Fig. 1. Neighbor Joining Tree of NBRC *Aspergillus* Strains Based on the ITS-D1/D2 Region.

Values at the node are bootstrap values calculated from 1,000 replications. Members clustering in different groups are listed in Table 1. Bar, 0.01 Knu.

the cluster G and J were supported by low bootstrap, the strains in the cluster G (*A. carneus*, *A. janus*, and *A. terreus*) and the cluster J (*A. itaconicus* and *A. wentii*) have been accepted into *Aspergillus* section *Cremeri* and *Aspergillus* sections *Terrei* and *Flavipedes* respectively.¹⁸⁾

The representative strains included in the 11 clusters and its bioactivities of the metabolites are shown in Table 1. The rates for positive strains with antioxidant and hemolytic activities were more constant over whole clusters than those with antimicrobial activities. Thus the phylogenetic relationships of the strains were relatively well correlated with the antimicrobial profiles (Table 1). Since *A. oryzae* was used as a safe test filamentous fungus, almost all the strains belonging to the cluster A showed no effect on the test fungal strain. However, four (one strain of *A. pseudotamarii*, one strain of *A. sojae*, and two strains of *A. tamarii*) of the 138 strains showed antifungal activity against the test fungus. *A. pseudotamarii* is closely related *A. tamarii*.¹⁹⁾ In addition, the *A. sojae* and *A. tamarii* have factors in common, such as usage in soy sauce production. Hence antifungal compounds may be common among the stains in the cluster A showing antifungal activity against *A. oryzae*. Three of the strains included in cluster H (*A. fumigatus*, *A. cellulosa*, and *A. clavatus*) showed antimicrobial activities against *K. rhizophila*, *C. albicans*, and *A. oryzae*, but all except one of the 19 strains showed no activity against *E. coli*. The strains in cluster G also showed a similar tendency to have antimicrobial profiles. Moreover, a majority of the strains in the cluster C composed of six species (*A. elegans*, *A. melleus*, *A. ochraceus*, *A. ostianus*, *A. sclerotiorum*, and *A. sulphureus*) containing 32 strains showed antibacterial activity against *E. coli*, but strains with antifungal activities against *A. oryzae* were fewer (one of 13 strains of *A. ochraceus* and four of six strains of *A. sclerotiorum* showed antifungal activity against *A. oryzae*). According to data in the Combined Chemical Dictionary, the strains included in cluster C produce common antibiotics such as aspyrone, asperlactone, and flavacol. Since the strains should be resistant to the common antibiotics, they may have an advantage over other microorganisms. However, cluster analysis of the antioxidative and hemolytic profiles indicated that no relationship between the biological profile and the active strains (Table 1).

The highest rates of positive strains were cluster C, G, G, and J in antibacterial, antifungal, antioxidant, and hemolytic activities respectively. The potential utility of these species of *Aspergillus* is confirmed not only in the fermentation industry but also in that they are used sources of natural bioactive compounds.²⁰⁾ It is clear that this ability is not restricted the genus *Aspergillus*. The patterns of biological activity in other fungal strains preserved at NITE are currently under study. Information on the relationship between taxonomy and bioactive compounds should be useful as to industrial microorganisms.

Table 1. Diversity of the *Aspergillus* Species Based on the DNA Sequences of D1/D2 Domain of LSU rRNA and ITS region including 5.8S rRNA, as defined in Fig. 1.

Cluster	No. of species	Representative species	No. of strains	Numbers of positive strains					
				AK	AE	AC	AA	AO	HL
A	13	<i>A. oryzae</i>	138	42	19	16	4	40	33
B	1	<i>A. avenaceus</i>	2	0	0	0	0	0	0
C	6	<i>A. ochraceus</i>	32	15	12	10	5	6	8
D	1	<i>A. japonicus</i>	8	3	1	1	3	2	0
E	4	<i>A. niger</i>	9	1	0	2	2	1	1
F	9	<i>A. carneus</i>	62	4	1	0	4	8	6
G	4	<i>A. terreus</i>	19	6	0	8	11	8	4
H	11	<i>A. fumigatus</i>	38	20	1	15	17	6	3
I	6	<i>A. restrictus</i>	11	1	0	2	3	3	2
J	2	<i>A. wentii</i>	7	0	0	0	0	2	4
K	13	<i>A. versicolor</i>	40	1	0	5	6	8	11

The biological profiles of 11 clusters are indicated: AK, anti-*K. rhizophila*; AE, anti-*E. coli*; AC, anti-*C. albicans*; AA, anti-*A. oryzae*; AO, antioxidative activity; HL, hemolytic activity. As antimicrobial activity, four microorganisms obtained from NBRC were used as test strains in the susceptibility tests: *Kocuria rhizophila* NBRC 12708 (deposited as *Sarcina lutea*), *Escherichia coli* NBRC 102203, *Candida albicans* NBRC 1385, and *Aspergillus oryzae* NBRC 6215. After incubation at 37 °C for bacteria and 28 °C for fungi for 24 h, the diameters of the inhibition zone were determined, and samples displaying an inhibition zone were considered positive. As antioxidative activity, ascorbic acid (10 µg/ml) was used as a positive control. Samples that showed more than 90% greater radical-scavenging activity than that of the control were recorded as positive. For hemolytic activity, 5 µl of sample solution were added to the wells of round-bottom 96-well microplates (Nunc, Roskilde, Denmark). To each well, 95 µl of PBS and 100 µl of a 4% (v/v) suspension of erythrocytes in PBS were added. After incubation for 2 h at 37 °C, the absorbance (540 nm) of the supernatant as the amount of released hemoglobin was measured with a microplate reader (Molecular Devices, Tokyo). Triton X-100 (1%) was used as a positive control.

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