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Phylogenetic analysis of newly described *Neosartorya* species

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Abstract

Isolates representing newly described *Neosartorya* species, and isolates with abnormal morphologies from *Aspergillus* section *Fumigati* were examined by phylogenetic analysis of sequences of part of their β-tubulin gene. Phylogenetic analyses supported the earlier suggestions that heterothallism is a derived character, and that sexuality was lost several times during the evolution of *Aspergillus* section *Fumigati*. The heterothallic *N. fennelliae* and *N. udagawae* strains were found to be closely related to the homothallic *Neosartorya* sp. NRRL 4179 and *N. aureola*, respectively. *Aspergillus* sp. FRR 1266, which was earlier described as a variant of *A. fumigatus*, was found to be closely related to *A. viridinutans*. Another abnormal asexual isolate was found to be closely related to *A. fumigatus* and *N. fischeri*. Phylogenetic relationships among newly described *Neosartorya* species and other taxa were successfully established based on phylogenetic analysis of β-tubulin sequences.

Introduction

The genus *Neosartorya* (family Trichocomaceae) was established by Malloch & Cain (1972) to accommodate teleomorphs of species belonging to the '*Aspergillus fischeri* series' of the *A. fumigatus* species group (Raper & Fennell 1965; *Aspergillus* section *Fumigati* according to Gams et al. 1985). This section now involves the anamorphs of 17 sexual *Neosartorya* species, and 5 asexual *Aspergilli* (Horie et al. 1992, 1995a, b; Udagawa et al. 1993; Samson 1994; Yaguchi et al. 1994). Several taxonomic methods have been applied to clarify the evolutionary relationships among species of *Aspergillus* section *Fumigati*. Recently, carbon source utilization spectra and isoenzyme patterns of 16 species of this section have been examined (Varga et al. 1997), while Geiser et al. (1998) analyzed the phylogenetic relationships among 11 sexual *Neosartorya* species, and 5 asexual Aspergilli by sequencing parts of their β-tubulin and hydrophobin genes. However, several homothallic and one heterothallic species described recently by Japanese authors (Horie et al. 1992, 1995a, b; Udagawa et al. 1993; Yaguchi et al. 1994) have not been included in these analyses. Some of these species were suggested to be related to other *Neosartorya* species based mainly on their similar ascospore morphologies (e.g. *N. tatenoi* was suggested to be related to *N. fischeri* and *N. hiratsukae* based on their similar ascospore surface ornamentations; Horie et al. 1992). Our aim was to examine the relationship of the newly described *Neosartorya* species together with some abnormal asexual isolates to other species in *Aspergillus* section *Fumigati*. We wished to clarify the taxonomic position of the third heterothallic *Neosartorya* species, *N. udagawae*, and to elucidate whether the similar ascospore ornamentations observed between some species indicate their close taxonomic relationship.

Materials and methods

Strains

The strains examined are listed in Table 1. Strains were maintained on malt extract agar slants. The

Table 1. The *Aspergillus* and *Neosartorya* strains examined in this study (for the other species included, see Geiser et al. 1998)

Species	Strain number, origin and reference	GenBank accession numbers
Aspergillus sp.	FRR 1266 (soil, Australia; Rinyu et al. 1995)	AF132223
Aspergillus sp.	JV3 (cocoa beans, unknown origin)	AF132222
N. botucatensis^T	CBM-FA 0672 (soil, Brazil; Horie et al. 1995a)	AF132225
N. multiplicata^T	CBM-FA 0710 (soil, Taiwan; Yaguchi et al. 1994)	AF132228
N. paulistensis^T	CBM-FA 0690 (soil, Brazil; Horie et al. 1995a)	AF132231
N. primulina^T	CBM-FA 0685 (tea, Japan; Udagawa et al. 1993)	AF132229
N. tatenoi^T	CBM-FA 0022 (soil, Brazil; Horie et al. 1992)	AF132227
N. udagawae^T	CBM-FA 0702 (soil, Brazil; Horie et al. 1995b) (mating type A)	AF132226
N. udagawae^T	CBM-FA 0703 (soil, Brazil; Horie et al. 1995b) (mating type a)	AF132230
Neosartorya sp.	NRRL 4179 (soil, Australia; Peterson 1992)	AF132224

Abbreviations: CBM, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan; FRR, CSIRO Food Research Culture Collection, North Ryde, New South Wales, Australia; NRRL, Agricultural Research Service Culture Collection, Peoria, Illinois, USA.

strains are available upon request from the culture collections listed in the footnote of Table 1.

DNA isolation

For DNA extractions, the strains were grown in a liquid minimal medium (Pontecorvo et al. 1953) at 30 °C for 48 hours on a rotary shaker. Nucleic acids were isolated as described by Leach et al. (1986).

DNA amplification and sequencing

Part of the β-tubulin gene of the strains was amplified using primers benA1 and benA2 (Geiser et al. 1998). The 100 l reaction mixtures contained 200 M of each dNTP, 1 M of each primer, 2.5 U *Taq* DNA polymerase (Promega), and about 100 ng genomic DNA in an amplification buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $mgCl_2$, 0.1% Triton X-100). The reaction mixture was overlaid with a drop of mineral oil. The MJ Research programmable thermal controller (MJ Research Inc., USA) was programmed for an initial denaturation step (94 °C, 5 min), 35 steps consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min, followed by a final extension step at 72 °C for 5 min. The PCR products were precipitated with ethanol, and electrophoresed in agarose gels according to standard protocols (Sambrook et al. 1989). DNA fragments were purified from the excised agarose blocks using Genelute spin columns (Supelco). Direct sequencing of the fragments was performed on an ABI 373A DNA sequencer (Applied Biosystems Inc.) using dye deoxy terminator reaction chemistry. Sequences were determined from both strands using benA1 and benA2 as primers.

Data analysis

Sequences were aligned manually, and analyzed using the outgroup rooting method with *A. clavatus* designated as outgroup taxon. Gaps were treated as a fifth nucleotide state. Phylogenetic analyses were conducted by programs in the PHYLIP software package version 3.57c (Felsenstein 1995). The DNAPARS program was used to carry out parsimony analysis. Indices of support (bootstrap values) for internal branches were generated by 1000 replications of the bootstrap procedure using programs SEQBOOT, DNAPARS and CONSENSE of the PHYLIP package (Felsenstein 1985, 1995). Distance matrices were generated by the DNADIST program, based on the two-parameter model (Kimura 1980). Topology and branch length of the phylogenetic trees were evaluated by the least-square method of Fitch & Margoliash (1967) from the distance matrices, using FITCH of the PHYLIP package. The DNA sequences of part of the β-tubulin gene of the examined strains can be obtained from GenBank under the accession numbers listed in Table 1.

Results and discussion

Several taxonomic studies have been carried out recently on *A. fumigatus*, and other species belonging to *Aspergillus* section *Fumigati*. Among other approaches, secondary metabolite profiles and micromorphology of the strains were examined (Frisvad & Samson 1990; Samson et al. 1990), DNA reassociation kinetics of some of the species were recorded (Peterson 1992), restriction fragment length polymorphisms were applied (Girardin et al. 1995), and isoenzyme and amplified DNA patterns of some species were compared (Rinyu et al. 1995). This study supplements the work of Varga et al. (1997) and Geiser et al. (1998) who examined the carbon source utilization, isoenzyme patterns and sequences of the β-tubulin and hydrophobin genes of most species belonging to *Aspergillus* section *Fumigati*. We wished to clarify the evolutionary relationships of 6 newly described *Neosartorya* species, an unusual *N. glabra* strain, and two asexual isolates with slightly abnormal morphologies to other species in *Aspergillus* section *Fumigati*.

The benA1 and benA2 primers amplified an about 450 bp fragment in each strain. Parsimony analysis resulted in 47 equally parsimonious trees of 564 steps. Bootstrap analysis of the data set was carried out using 1000 replicas. The resulting majority-rule consensus tree is shown in Figure 1. A phylogenetic tree based on genetic distances between the examined strains is displayed in Figure 2. An *A. clavatus* strain (H522; Geiser et al. 1998) was used as outgroup in these experiments.

Besides the evolutionary relationships described by Geiser et al. (1998), the following conclusions could be drawn from the trees (Figures 1 and 2). *Neosartorya udagawae* was found to belong to a different clade than the other two heterothallic species, *N. spathulata* and *N. fennelliae*. Geiser et al. (1998) observed that "forcing *N. spathulata* and *N. fennelliae* to form a single clade did not result in a significantly less likely tree", consequently, evolution of heterothallism may represent a single event within *Aspergillus* section *Fumigati*. However, the high bootstrap values obtained in the cases of *N. fennelliae* and *N. udagawae*, and 10 informative nucleotide site changes support the hypothesis that *N. udagawae* in not closely related to the other two heterothallic *Neosartorya* species (refusal of the hypothesis is supported only by two site changes). The large genetic distances observed between *N. udagawae* and *N. fennelliae* (0.092), and

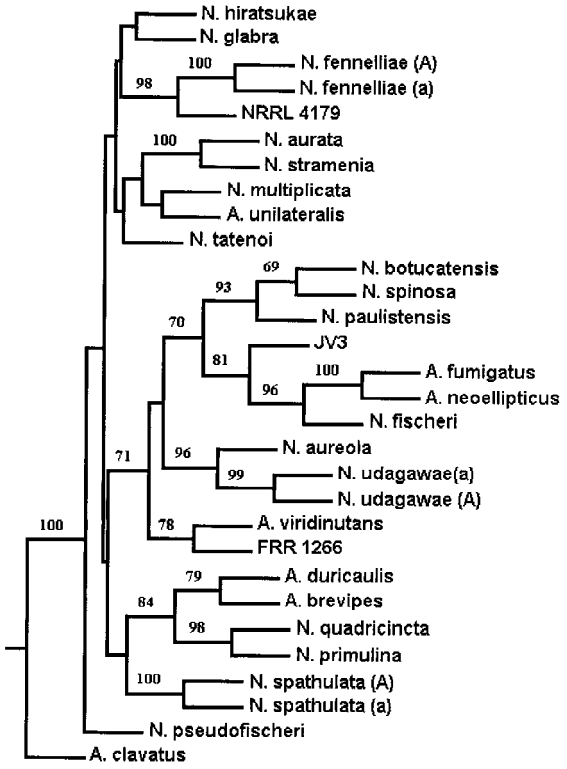

Figure 1. Majority-rule consensus tree based on phylogenetic analysis of sequences of the β-tubulin gene of species in *Aspergillus* section *Fumigati*. Numbers above branches indicate bootstrap values; only values >50% are shown.

between *N. udagawae* and *N. spathulata* (0.095) as compared to those observed, e.g., between *N. udagawae* and *N. aureola* (0.036) or between *N. fennelliae* and *Neosartorya* sp. NRRL 4179 (0.041) also indicate that these species are not closely related (data not shown). The observation that *N. udagawae* is unrelated to *N. fennelliae* and *N. spathulata* on the dendrogram further supports the opinion of Geiser et al. (1998), who concluded that heterothallism is a derived character in the *Neosartorya* genus.

Asexual species were scattered through the dendrogram supporting the conclusions of Geiser et al. (1998) that sexuality was lost several times throughout the evolution of *Aspergillus* section *Fumigati*. Besides the four independent losses of the *Neosartorya* sexual state suggested by Geiser et al. (1998), an additional evolutionary event could have been necessary to explain the position of the asexual isolate JV3 on the cladogram (Figure 1).

Close relationship was observed between *A. fumigatus*, *A. fumigatus* var. *ellipticus*, *N. fischeri* and an asexual *Aspergillus* strain (JV3) isolated from mouldy

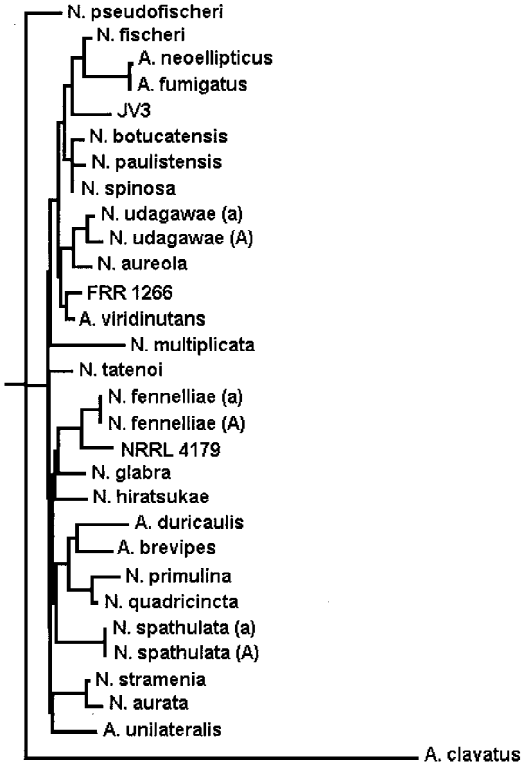

Figure 2. Phylogenetic tree based on genetic distances of the sequence data between the strains examined. *A. clavatus* was used as an outgroup. Horizontal branch lengths are drawn to scale and represent the expected number of base substitutions.

cocoa beans (Figure 1). Close relationship of these strains was also supported by carbon source utilization data (Varga et al. 1997; data not shown). Isolate JV3 exhibited different mtDNA restriction patterns from the other *A. fumigatus* strains examined (Rinyu et al. 1995; J. Varga, unpublished observations), and this was the only isolate among the examined strains which produced ochratoxin A (J. Varga, unpublished observations). Some *A. fumigatus* isolates have been shown earlier to be able to produce ochratoxins (Abarca et al. 1997). Other *A. fumigatus* isolates should also be examined to clarify whether this strain fits into the rather broad species concept of *A. fumigatus*. Another asexual isolate, FRR 1266 was found to be closely related to *A. viridinutans*, although the vesicles of this isolate are in an upright position, in contrast with the nodding conidial heads characteristic to *A. viridinutans* isolates (Raper & Fennell 1965). Isolate FRR 1266 was earlier found to be only distantly related to *A. fumigatus* based on isoenzyme analysis, mtDNA restriction profiles, PCR-based methods (Rinyu et al. 1995) and sequence analysis of an alkaline protease gene (Katz et al. 1998). More *A. viridinutans* isolates should be examined to clarify whether FRR 1266 is a member of this species, in which case the presence of nodding heads is not a reliable taxonomic character in *Aspergillus* section *Fumigati*. Among the examined newly described *Neosartorya* species, *N. botucatensis* and *N. paulistensis* were found to be closely related to *N. spinosa*. The species descriptions of these taxa also mention the possibility of such relationship, based on the surface morphology of the ascospores of these species; all three species produce hyaline to pale yellowish brown ascospores with well-separated equatorial crests, and convex surfaces bearing spine-like projections (Kozakiewicz 1989; Horie et al. 1995a). *Neosartorya primulina* was found to be closely related to *N. quadricincta*, which observation is supported by the similar colony morphologies of the two species (Udagawa et al. 1993), and their identical *Hae*III digested mitochondrial DNA profiles (J. Varga, unpublished observations), although the ascospore morphologies of *N. primulina* and *N. quadricincta* are quite distinct. Udagawa et al. (1993) suggested that *N. primulina* could be related to *N. hiratsukae* based on their restricted growth on Czapek agar medium, and their similarly colored ascomata, but this hypothesis was not supported by sequence data. Based on our results, the several narrow crests observed on the ascospores of *N. primulina* could be homologous with the four equatorial crests of the ascospores of *N. quadricincta* (Kozakiewicz 1989; Udagawa et al. 1993). Mating type strains of the heterothallic *N. udagawae* species were found to be related to the homothallic *N. aureola* species. Both species produce ascomata with hyaline to pale yellowish brown peridia and ascospores, and the surface ornamentation of the ascospores are also similar (Horie et al. 1995b). *Neosartorya* sp. NRRL 4179, which was earlier assigned to the *N. glabra* species (Raper & Fennell 1965; Kozakiewicz 1989), was found to be closely related to the heterothallic *N. fennelliae* species. Peterson (1992) observed the same relationship based on DNA reassociation kinetics of these strains. Carbon source utilization data also supported that isolate NRRL 4179 is closely related to *N. fennelliae* (data not shown). Raper & Fennell (1965) mentioned that this isolate produces ascospores with narrower equatorial crests than other *N. glabra* isolates. The surface ornamentation of the ascospores of strain NRRL 4179 is also reminiscent of those of *N. fennelliae* (Samson et al. 1990; Peterson 1992). Relationship of *N. tatenoi* and *N. multiplicata* to any of the other species examined was not strongly supported as indicated by the low bootstrap values observed

(Figure 1). Although the surface ornamentation of the ascospores of *N. tatenoi* is similar to those of *N. fischeri* (Horie et al. 1992), only distant relationship was observed between these taxa. *Neosartorya multiplicata* is the only *Neosartorya* species which produces more or less globose ascospores without equatorial crests (Yaguchi et al. 1994). This species was described as closely related to *N. primulina* based on their similar ascospore morphology (Yaguchi et al. 1994). This suggestion is not supported by sequence data. *N. pseudofischeri* was found to be the most basal taxon in *Aspergillus* section *Fumigati* based on both parsimony and distance methods (Figures 1 and 2), in accordance with the results of Geiser et al. (1998).

In conclusion, phylogenetic analysis of protein coding sequences like the β-tubulin gene was found to be a very useful tool for examining evolutionary relationships within *Aspergillus* section *Fumigati*. Such an approach is planned to be used for clarifying the phylogenetic relationships in other economically important Aspergilli like species belonging to *Aspergillus* sections *Circumdati* and *Flavi*.

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