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Phylogeny of *Nannizzia incurvata, N. gypsea, N. fulva* and *N. otae* by restriction enzyme analysis of mitochondrial DNA

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Abstract

Sequence divergence among *Nannizzia incurvata*, *N. gypsea*, *N. fulva* and *N. otae* was studied genetically using digestion profiles of mitochondrial DNA with restriction enzymes, *Hae* III, *Hha* I, *Hin*d III and *Xba* I. Only *N. fulva* was subdivided into two types on restriction profiles. Phylogenetic relationships suggest that 1) *N. gypsea* is more closely related to *N. fulva* than *N. incurvata*, 2) the phylogenetic distance between *N. otae* and the other three species is larger than the distances between the other three species.

Introduction

Microsporum gypseum is a species complex with two perfect states, *Nannizzia gypsea* and *N. incurvata*. *M. fulvum* which has been considered synonymous with *M. gypseum*, is an imperfect state of *N. fulva* [1]. These three *Nannizzia* species closely resemble each other morphologically and are distinguished by mating tests.

Recently, mitochondrial DNA (mtDNA) restriction profiles have been used in the comparative study of closely related fungi [3, 4].

In a previous paper [6], analysis of mtDNA made possible the classification of *Sporothrix schenckii* into 11 types based on restriction profiles and also proved to be a sensitive taxonomical means to differentiate *S. schenckii* from related fungi at the molecular level.

In this report, attempts were made to analyze genetically the relationship between these three *Nannizzia* species and *N. otae*, and to differentiate them by using mtDNA restriction profiles.

Materials and methods

Strains used in this study are *N. fulva* (6 strains), *N. gypsea* (13 strains), *N. incurvata* (9 strains), and *N. otae* (7 strains) (Table 1).

Extraction of mtDNA was performed as described previously [6]. Each strain was grown in liquid Sabouraud medium at room temperature for 7 days with constant shaking.

Cells were washed twice with distilled water. The washed cells were incubated in 100 ml of sorbitol buffer (0.9 M sorbitol, 10 mM EDTA, 10 mM Tris-HCl, pH 7.5) with 0.1 ml of 2-mer-

Table 1. Species and strains.

Species	Strain no.	Source	Mating type (mtDNA type)
N. fulva	SM0209	IMI99944	+ (type 1)
	KMU3103	CBS167.64	+ (type 1)
	KMU3106	CBS783.73	+ (type 2)
	SM0210	IMI99943	− (type 1)
	KMU3104	CBS168.64	− (type 1)
	KMU3107	CBS784.73	− (type 2)
N. gypsea	SM8145	From dog	+
	SM8147	From dog	+
	SM8376	Clinical isolate	+
	SM8379	Clinical isolate	+
	SM8398	Clinical isolate	+
	SM8462	Clinical isolate	+
	SM8514	Clinical isolate	+
	SM8522	From dog	+
	SM8523	From dog	+
	SM8377	Clinical isolate	−
	SM8400	Clinical isolate	−
	SM8524	From dog	−
	SM8525	From cat	−
N. incurvata	SM8078	Clinical isolate	+
	SM8080	Clinical isolate	+
	SM8088	Clinical isolate	+
	SM8119	Clinical isolate	+
	SM8198	Clinical isolate	+
	SM8244	Clinical isolate	+
	SM7504	Clinical isolate	−
	SM8243	Clinical isolate	−
	SM8413	Clinical isolate	−
N. otae	SM0215	IMI259269	+
	SM0214	IMI259949	−
	KMU2790	Clinical isolate	−
	KMU2793	Clinical isolate	−
	KMU2803	Clinical isolate	−
	KMU2804	From cat	−
	KMU2848	Clinical isolate	−

CBS: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands
IMI (= CMI): The Commonwealth Mycologycal Institute, Kew, United Kingdom
KMU: Kanazawa Medical University, Ishikawa, Japan
SM: Shiga University of Medical Sience, Otsu, Japan

captoethanol and 30 mg of zymolyase-100T at 37 °C for one hour. After centrifugation at 8000 g, the treated cells were disrupted by sonication in 20 ml of sorbitol buffer for 30 sec under cooling. Cell debris and undisrupted cells were removed by centrifugation at 1600 g for 10 minutes. From the resultant supernatant, mitochondria were obtained by centrifugation at 20000 g for 10 minutes. mtDNA was obtained from the mitochondria by phenol extraction and was digested with restriction enzymes, *Hae* III, *Hha* I, *Hin*d III and *Xba* I, respectively. The digested mtDNA was electrophoresed on 0.8% agarose gels, and the mtDNA fragments in the gels were stained with ethidium bromide, and photographed under 302 nm ultravioletlight.

To detect the similarities and differences between these four species, sequence divergence within the mtDNA from the four species was

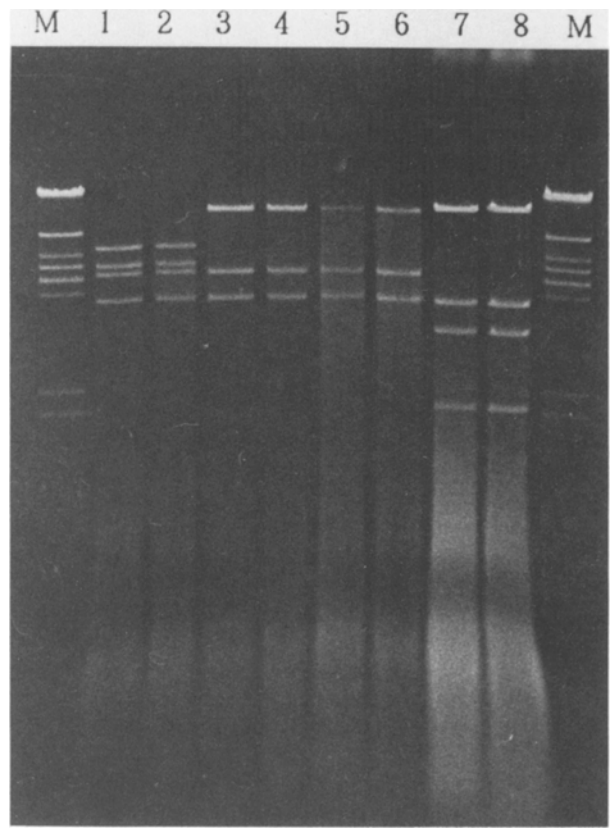

Fig. 1. Restriction profiles of mtDNA digested with *Hae* III. Lane M: size marker, mixture of *Hin*d III digest and *Eco* RI digest of λ DNA. Lane 1: *N. incurvata* (+), Lane 2: *N. incurvata* (−), Lane 3: *N. gypsea* (+), Lane 4: *N. gypsea* (−), Lane 5: *N. fulva* (+), Lane 6: *N. fulva* (−), Lane 7: *N. otae* (+), Lane 8: *N. otae* (−).

roughly estimated by using the formula [20] of Nei and Li [5], and a phylogenetic diagram was made using the method of Fitch and Margoliash [2].

Results and discussion

Digestion of mtDNA from *N. gypsea* and *N. fulva* with *Hae* III showed identical restriction profiles which differed from those of *N. incurvata*. Two bands, 5.6 and 4.4 Kbp, were common to the three species and the 4.4 Kbp band was also shared by *N. otae* (Fig. 1). These lengths of bands were roughly estimated from gels electrophoresed with another size marker, λ DNA digested with *Hind* III and *Eco* RI. The relationship between *N. gypsea* and *N. fulva* was suggested to be the closest among the four species.

Digestion with *Hha* I showed different restriction profiles of the mtDNA from each of the four species. Each species produced two bands. *N. gypsea* and *N. fulva* shared a common band of 16 Kbp, and *N. incurvata*, *N. gypsea* and *N. otae* shared a common band of 6.1 Kbp (Fig. 2), suggesting that *N. gypsea* genetically lies between *N. incurvata* and *N. fulva*.

Digestion with *Hind* III showed different restriction profiles in the four species. *N. incurvata* and *N. gypsea* shared common bands of 5.2, 1.8, 1.3 and 0.95 kbp. *N. incurvata*, *N. gypsea* and *N. fulva* had three common bands of 1.8, 1.3 and

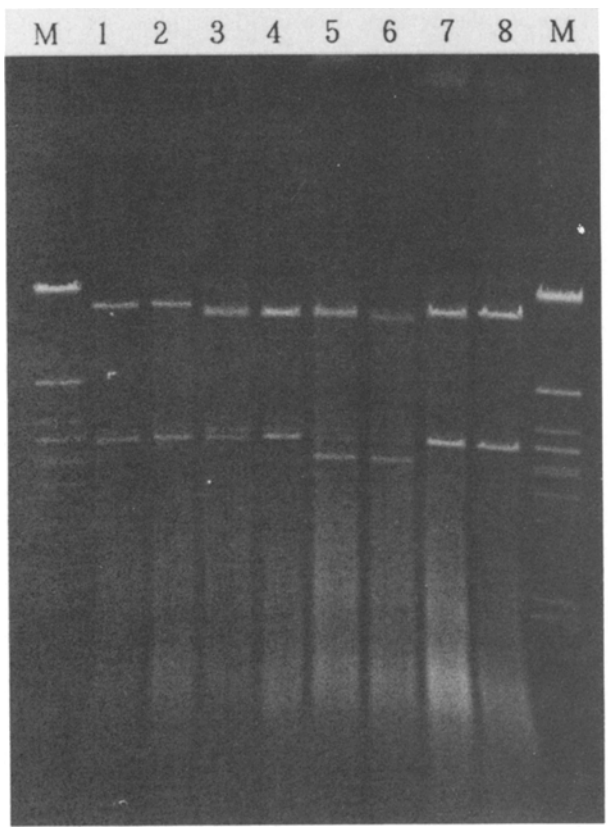

Fig. 2. Restriction profiles of mtDNA digested with *Hha* I. Lane M: size marker. Lane 1: *N. incurvata* (+), Lane 2: *N. incurvata* (−), Lane 3: *N. gypsea* (+), Lane 4: *N. gypsea* (−), Lane 5: *N. fulva* (+), Lane 6: *N. fulva* (−), Lane 7: *N. otae* (+), Lane 8: *N. otae* (−).

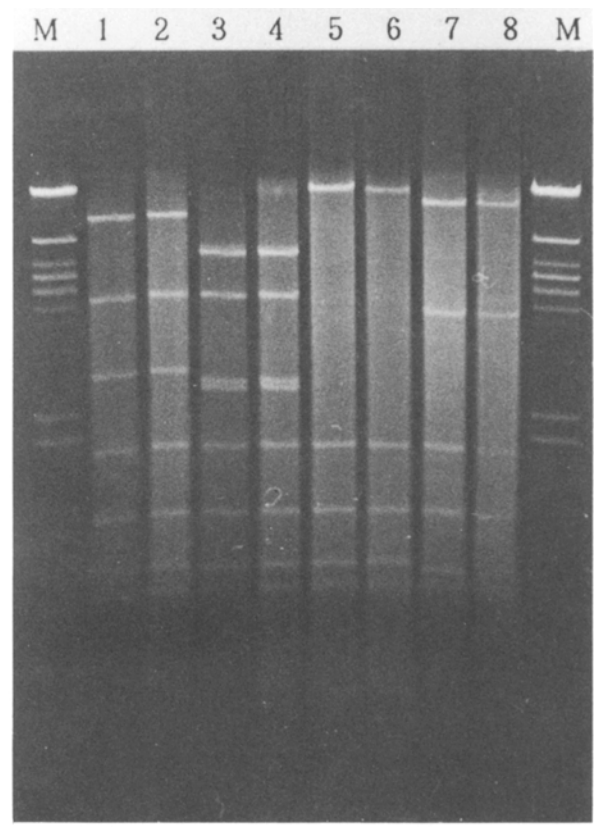

Fig. 3. Restriction profiles of mtDNA digested with *Hind* III. Lane M: size marker. Lane 1: *N. incurvata* (+), Lane 2: *N. incurvata* (−), Lane 3: *N. gypsea* (+), Lane 4: *N. gypsea* (−), Lane 5: *N. fulva* (+), Lane 6: *N. fulva* (−), Lane 7: *N. otae* (+), Lane 8: *N. otae* (−).

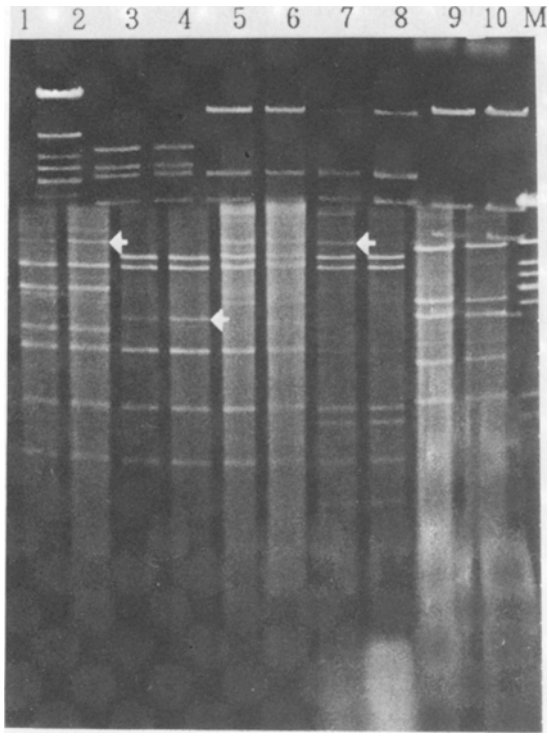

Fig. 4. Restriction profiles of mtDNA digested with *Xba* I. Lane M: size marker. Lane 1: *N. incurvata* (+), Lane 2: *N. incurvata* (−), Lane 3: *N. gypsea* (+), Lane 4: *N. gypsea* (−), Lane 5: *N. fulva* (+ · type 1), Lane 6: *N. fulva* (− · type 1), Lane 7: *N. fulva* (+ · type 2), Lane 8: *N. fulva* (− · type 2), Lane 9: *N. otae* (+), Lane 10: *N. otae* (−). Arrows: Fragments by incomplete digestion.

0.95 Kbp, and four species showed two common bands of 1.8 and 1.3 Kbp. On restriction profiles by *Hind* III, *N. incurvata* and *N. gypsea* were suggested to be the most closely related among the four species.

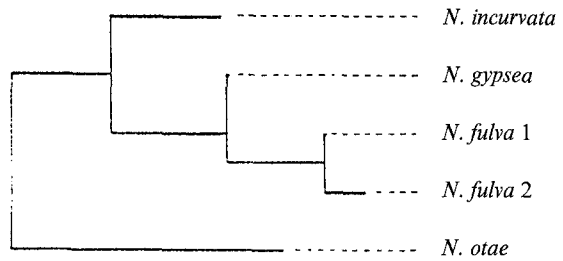

Fig. 5. A constructed phylogenetic tree. The distance between two species is proportional to the sum of the horizontal lengths of the branches. The vertical lengths of the branches are arbitrary. Any two branches are free to rotate over the axis leading to them. Lengths of the last branches toward *N. gypsea* and toward *N. fulva* 1 are nearly zero.

Digestion of mtDNA from *N. fulva* with *Xba* I showed two different restriction profiles and this species was divided into two types, types 1 and 2. The profile of type 1 was identical to that of *N. gypsea*. Type 2 of *N. fulva* and *N. gypsea* showed four common bands of 7.7, 7.0, 2.3 and 1.7 Kbp. *N. incurvata* and type 2 of *N. fulva* showed three common bands of 7.0, 2.3 and 1.7 Kbp. *N. incurvata* and *N. gypsea* showed four common bands of 7.0, 3.5, 2.3 and 1.7 Kbp. Four species including *N. otae* showed a common band of 2.3 Kbp. From these results, *N. gypsea* and *N. fulva* were suggested to be the most closely related among the four species. The size of mtDNA from these four species ranged from approximately 23 to 25 Kbp.

Calculated values of sequence divergence are presented in the lower left half of Table 2. A phylogenetic tree constructed from these sequence divergence is shown in Fig. 5. And reconstructed

Table 2. Sequence divergence and reconstructed distance.

	N. incurvata	*N. gypsea*	*N. fulva* 1	*N. fulva* 2	*N. otae*
N. incurvata		0.0373	0.0557	0.0632	0.0820
N. gypsea	0.0380		0.0162	0.0236	0.0821
N. fulva 1	0.0560	0.0160		0.0080	0.1005
N. fulva 2	0.0610	0.0240	0.0080		0.1080
N. otae	0.0820	0.0790	0.1060	0.1080	

Sequence divergences (numbers of nucleotide substitutions per site) are presented in the lower left half, and reconstructed distances (leg lengths in Fig. 5) are presented in the upper right half.

distances found by summing the leg lengths in Fig. 5 are presented in the upper right half of the Table 2. The percent 'standard deviation' for this tree is 2.63

This phylogenetic diagram revealed that 1) *N. gypsea* is more closely related to *N. fulva* than *N. incurvata*, 2) the phylogenetic difference between *N. otae* and the three species is larger than the distances between three species.

The results indicate that phenotypic classification is not completely consistent with genotypic classification among *N. incurvata*, *N. gypsea* and *N. fulva*. The restriction profiles of mtDNA, however, proved to be species specific and therefore provided a sufficient taxonomic tool to differentiate and identify these species, if mating tests are sterile. On the other hand, mtDNA polymorphism was not observed at a high rate in this study, suggesting that at present mtDNA analysis may not be useful in the molecular epidemiology of these species.

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