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Short Report

Phylogenetic Analysis of *Histoplasma capsulatum* Based on Partial Sequence of the D1/D2 Region of the 28S rRNA Gene

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Abstract

In order to confirm the phylogenetic relationships of *Histoplasma capsulatum*, the partial sequences of large subunit (28S) ribosomal gene (D1/D2 region) of 49 isolates were studied. The similarity values of the 49 isolates were more than 99.0% across 617 base pairs, however, the 49 isolates were divided into 9 groups. These 9 groups were independent of 3 varieties, var. *capsulatum*, var. *farciminosum* and var. *duboisii*. These results showed that analysis of the nucleotide sequence of the 28S rRNA gene was very effective for identification of *H. capsulatum* and that three varieties of *H. capsulatum* should be reclassified according to the phylogenetic relationship established from analysis of the D1/D2 region sequences.

Key words: *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, *H. capsulatum* var. *farciminosum*, histoplasmosis, D1/D2 region of 28S rRNA gene

Introduction

Histoplasma capsulatum, the causative agent of histoplasmosis, is a thermo-dependent dimorphic fungus that adopts the mycelial form at room temperature and transforms from the mycelial to yeast form in host tissues or at 35-37°C on certain culture media. The criteria for distinguishing varieties are geographical distribution, host and the size of the parasitic yeast form cells[1, 2]. Partial sequences of the D1/D2 region of the 28S rRNA gene have been used to generate phylogenetic databases for basidiomycetes[6] and ascomycetous yeasts[7]. Partial sequences of the D1/D2 region of the 28S rRNA gene have been determined for only 6 isolates of *Histoplasma capsulatum*. In order to confirm the phylogenetic relationships of *Histoplasma capsulatum*, we examined partial sequences of the D1/D2 region of the 28S rRNA gene of 49 isolates that deposited to the IFM.

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Materials and methods

Isolates: The 49 isolates represented 3 varieties of *H. capsulatum* (28 *H. capsulatum* var. *capsulatum*, 13 *H. capsulatum* var. *duboisii*, and 8 *H. capsulatum* var. *farciminosum*) (Table 1).

DNA sequencing: DNA was extracted with a DEXPAT® Kit (TaKaRa, Ohtsu, Japan) with a modification of the manufacturer's protocol. Approximately 100 μl of fungal mass cultured at 25°C for 2 months on potato dextrose agar (Difco, Franklin Lakes, NJ, USA) slants were transferred to a sterilized microtube (1.5 ml), homogenized with 0.5 ml of DEXPAT® solution and homogenized by a plastic pestle. The mixture was incubated at 100°C for 10 min and centrifuged at 12,000 rpm (13,201 *g*) for 10 min. The supernatant was used as the DNA sample.

DNA extract (2.5 μl), Ready-To-Go™ PCR Beads (Amersham Pharmacia Tokyo, Japan), 20 μl of distilled water and 2.5 μl of 10 pM of each primer were mixed. The primers NL-1[7], NL-2 Hc ver2 (5'-TCT TAT AGC CGG GGG TGC AAT GCG GCC-3'), NL-3[11] and NL-4[7] were used. The primer NL-2 Hc ver2 corresponds to

Table 1. *Histoplasma capsulatum* isolates

Variety/	IFM no.	Strain name or number in other culture collection	Source	Country	Accession no.	Groups
capsulatum						
	5396	MTU 16001, TIMM 0713	Unknown	USA	AB176464	III
	5397	MTU 16002, TIMM 0714	Human/sputum	USA	AB176465	III
	5398	MTU 16003, TIMM 0715	Human/sputum	USA	AB176466	III
	5399	MTU 16010, TIMM 0722	Human/skin	USA	AB176467	III
	5400	MTU 16011, TIMM 0723	Unknown	USA	AB176468	III
	5401	MTU 16013, TIMM 0725	Human/lung	USA	AB176469	III
	5405	MTU 16018, IP 110575, TIMM 0730	Unknown	USA	AB176470	VI
	5407	MTU 16021, IP 637, TIMM 0733	Unknown	USA	AB176471	III
	5408	MTU 16031, B-580	Unknown	USA	AB176472	III
	5410	MTU 16033, B-17624	Unknown	USA	AB176473	IX
	5411	MTU 16034, B-2984	Unknown	USA	AB176474	III
	5412	MTU 16041, 80-4-3a	Unknown	USA	AB176475	III
	40752	12	Unknown	Unknown	AB176476	I
	41329	CDC 105	Human	USA	AB176477	IX
	41330	Ohino NHL 2966-1	Human	USA	AB176478	V
	41331	Ohino NHL 2966-2	Human	USA	AB176479	V
	46003	KUM 967, FMJ 502	Human	USA	AB176480	III
	46159	G 217B	Human	USA-Brazil*	AB176481	III
	47750	IBB	Human/lung	Japan-China*	AB176482	IV
	49200	CB-99-8	Human/skin	Brazil	AB176483	VI
	49721	Verdemi	Human/oral mucosa	Argentina	AB176484	VII
	50248	Benitez	Human/skin	Argentina	AB176485	VII
	50249	Fernandez	Human/oral mucosa	Argentina	AB176486	VII
	50250	Reggiani	Human/lymph node	Argentina	AB176487	VII
	50251	Gomez	Human/skin	Argentina	AB176488	VII
	50955	Myanmar	Human/lymph node	Japan-Myanmar*	AB176489	VII
	50958	NIH 02777	Human/skin	Thailand	AB176490	VII
	50959	NIH 02778	Human/blood	Thailand	AB176491	VII
duboisii						
	5406	MTU 16020, TIMM 0732, IP 634	Unknown	USA	AB176492	III
	5415	MTU 16022, IP 263	Unknown	Africa	AB176493	II
	5416	MTU 16023, IP 527, TIMM 0737	Unknown	Africa	AB176494	II
	5417	MTU 16024, TIMM 0738, IP 638	Unknown	Africa	AB176495	II
	41319	ATCC 22636, CBS 137.72(+)	Soil	USA	AB176496	III
	41320	ATCC 22635, CBS 136.72(−)	Soil	USA	AB176497	III
	41332	CDC B-650	Unknown	Africa	AB176498	II
	50954	Uganda	Human/CSF	Japan-Uganda*	AB176499	III
	52673	E2(2)	Human	Nigeria	AB176500	II
	52674	E7G	Human	Nigeria	AB176501	II
	52675	EB11	Human	Nigeria	AB176502	II
	52676	EB9	Human	Nigeria	AB176503	II
	52677	EGINT	Human	Nigeria	AB176504	II
farciminosum						
	5418	MTU 16042, 80-64-1a	Unknown	USA	AB176505	VII
	41333	SM 1024	Horse	USA	AB176506	VIII
	41335	CDC B-22	Horse	USA	AB176507	VIII
	41612	76103	Human/skin	Japan	AB176508	VII
	49109	Tsuchiura-1	Human/liver	Japan-Thailand*	AB176509	VII
	49110	Tsuchiura-2	Human/skin	Japan-Thailand*	AB176510	VII
	52876	SM 1025	Horse	USA	AB176511	II
	52877	SM 1026	Horse	USA	AB176512	VIII

Abbreviations for culture collections: ATCC, American Type Culture Collection, USA; CBS, Centraalbeaurau voor Schimmelcultures, The Netherlands; CDC, Centers for Disease Controls and Prevention, USA; IP, Institute Pasteur, France; KUM, Department of Dermatology, School of Medicine, Kanazawa University. Japan; MTU, Department of Bacteriology, Faculty of Medicine, University of Tokyo, Japan; NHL, National Institute of Hygienic Sciences, Japan; NIH, National Institute of Health Thai, Thailand; SM, Department of Dermatology, Shiga University of Medical Science, Japan; TIMM, Research Center for Medical Mycology, Teikyo University., Japan.; IFM, Institute of Food Microbiology, Chiba University; which was the former name of the Research Center for Pathogenic Fungi and Microbial Toxicoses.

*; isolated in Japan from patients of foreign nationality.

CSF, cerebrospinal fluid.

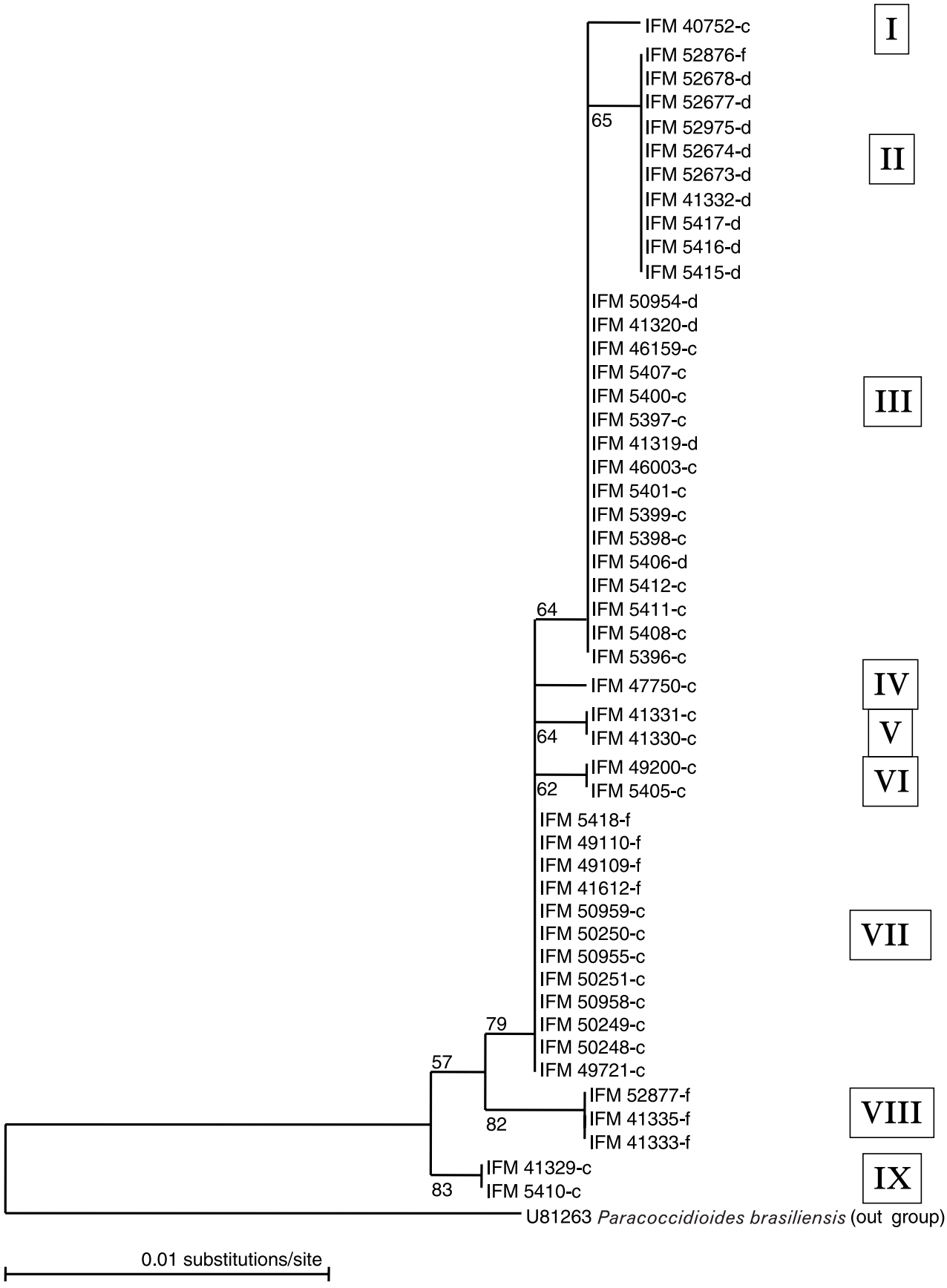

Fig. 1. Neighbor joining (NJ) tree for D1/D2 domains of three varieties of *Histoplasma capsulatum*. Bootstrap values derived from 10,000 replicates are shown as percentages.
The scale bar represents a difference corresponding to 0.01 (1.0%). "-c" indicates *H. capsulatum* var. *capsulatum*, "-d" incdicates *H. capsulatum* var. *duboisii*, and "-f" indicates *H. capsulatum* var. *farciminosum*

nt 1104 to 1130 of the D1/D2 region of the 28S rRNA gene of *Ajellomyces capsulatus* (Accession no. AF038354). The reaction mixture was subjected to 1 cycle of denaturation at 95°C for 4 min, 30 cycles of amplification at 94°C for 1 min, 55 or 58°C for 1 min, and 72°C for 2 min, and a final extension cycle at 72°C for 10 min with a PCR Thermal Cycler MP (TaKaRa). PCR products were separated by electrophoresis on 1.0% agarose gels in 1x TBE buffer (0.04 M Tris-boric acid, 0.001 M EDTA [pH 8.0]) and visualized by ethidium bromide staining. PCR products were purified with a PCR purification kit (QIAquick®, Qiagen, Tokyo, Japan), labeled with BigDye® terminator Ver. 1.1 (Applied Biosystems, Foster City, CA, USA) per the manufacturer's protocol and with primers of NL-1, NL-2[11], NL-2 Hc ver2, NL-3, and NL-4. The labeled samples were directly sequenced by ABI PRISM® 3100 sequencer (Applied Biosystems). DNA sequences were aligned with GENETEX-MAC genetic information processing software (Software Development Co., Ltd., Tokyo, Japan). Sequences were aligned with CLUSTAL W (version 1.6)[12]. A phylogenetic tree was constructed by the neighbor-joining (NJ) method. The sequence was listed accession number U81263 under Genbank, which is a sequence of a ribosomal RNA gene from *Paracoccidioides brasiliensis*, was used as an outgroup sequence for phylogenetic analysis. The nucleotide sequences for all strains examined were registered in the DNA Data Bank of Japan (DDBJ) under the accession numbers shown in Table 1.

Results and Discussion

Sequences generated as part of this study were entered into GenBank with serial accession numbers from AB176464 to AB176512 (Table 1). As shown in Fig. 1, the similarity values of the 49 isolates were 99.0% across the D1/D2 region of the 28S rRNA gene. These 49 isolates were then divided into 9 groups. None of the sequences were matched with the GenBank data such as AF038353, AF038354, AF071950, AF071951, AF071952, and AY235020 but was independent from the related sequences of *Onygenales*; *Ajellomyces dermatitidis* (AF038358), *Paracoccidioides brasiliensis* (U81263), and *Coccidioides immitis* (AY17613), and the similarity values were less than 96%. These results indicate that partial sequences of the D1/D2 region of the 28S rRNA gene are useful for species level identification of *H. capsulatum*.

Three of the groups: II, III and VII, consisted of 2 or 3 varieties that were confirmed by their site of isolation, host, cell size of parasitic form, internal transcribed spacer (ITS) regions of rRNA genes, and authorization by culture collections (Fig. 1). Some previous phylogenetic relationships of *H. capsulatum* isolates from a variety of regions established on the basis of DNA sequences of ITS regions of rRNA genes and partial sequences of genus encoding antigen precursor, fatty acid desaturase, alpha tubulin, ADP-ribosylation factor, H antigen precursor, and delta-9 fatty acid desaturase were questionable[3-5]. The isolates belonging to the genotypes consisting of different varieties had originated from distant countries. The results that the 9 groups were independent of the 3 varieties, reinforce these suspicions.

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