Journal of Dermatological Science (2006) **43**, 214—217

Journal of Dermatological SCIENCE

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LETTER TO THE EDITOR

Verifiable single nucleotide polymorphisms of the internal transcribed spacer 2 region for the identification of 11 *Malassezia* species

To the Editor,

KEYWORDS
Malassezia;
Identification;
ITS;
SNP;
PCR-RFLP

Malassezia yeasts are the globally distributed agents of pityriasis versicolor and are implicated in the pathogenesis of seborrheic and atopic dermatitis [1]. The taxonomic status of the genus has relatively recently expanded to include seven species [2]. Moreover, in the last 4 years *Malassezia dermatis* [3], *M. japonica* [4], *M. nana* [5] and *M. yamatoensis* [6] have been appended to the genus and their species status as well as their contribution to skin disease is under investigation [1].

The current conventional identification schemes of the lipophilic *Malassezia* species [2,7] has inbuilt difficulties as the results are not always unequivocal due to the variable Tween assimilation profiles of *M. slooffiae* and *M. nana* strains [5]. The identification of the new species was based on comparison of the %G + C content and on sequence analysis of the Large Subunit rDNA and Intergenic Spacer regions [2—6].

Our aims were to upgrade the ITS 3/4 PCR-RFLP system developed previously [8] and assess its capacity for reliable identification of 11 Type, reference and clinical *Malassezia* species.

DNA was extracted from pure cultures of 120 strains of 11 *Malassezia* species (Table 1) and the Internal Transcribed Spacer 2 (ITS 2) region was amplified by PCR employing the ITS 3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (INTERACTIVA Biotechnologie GmbH, Ulm, Germany) as previously described [8]. The ITS 3/4 PCR products of the novel species *M. dermatis* and *M. nana* were selectively recovered from the agarose gel (QIAEX® II Gel Extraction Kit, QIAGEN, Hilden, Germany), sequenced bi-directionally (Biogenomica, Athens, Greece) and the consensus sequence for each strain was submitted to the GenBank (Acc. nos.: AY390283-5, DQ083032). Selection of the restriction endonucleases was accomplished comparing the expected ITS 3/4 Restriction Fragment Length Polymoprhism (RFLP) profiles through alignment and comparison (Seqman, DNA Star Inc., USA) of the published sequences for *M. furfur* (AF246896, AF522058-9, AY387100-131, AB105150-4), *M. obtusa* (AF521960, AB105155-8, AY387137-8, AY743631), *M. pachydermatis* (AB118937-41, AY387139-42, AY743637, AF522061), *M. japonica* (AB105199), *M. slooffiae* (AY743633, AY387146-56, AF522063), *M. globosa* (AF522060, AY387132-6, AY743630, AJ437693, AY267224), *M. restricta* (AF522062, AJ437694-5, AY267225, AY387143-5, AY743636), *M. yamatoensis* (AB125261-2), *M. nana* (DQ083032), *M. sympodialis* (AB070366, AF522064 and AY387157-195) and *M. dermatis* (AY390283-5, AB070356-60). The relatively small number of the clinically important *M. globosa* and *M. restricta* species tested (15 and 3, respectively out of 120 strains) is due to the difficulty in maintaining them in culture and is depicted into the small number of deposited sequences in the GenBank (9 and 8, respectively out of 123 sequences).

The restriction endonucleases *Alu*I, *Ban*I and *Msp*AI (New England Biolabs, Beverly, MA, USA) were selected for producing distinct RFLP patterns and were used for digestion of the PCR products [8]. Single nucleotide polymorphisms that would alter the predicted restriction profile were recorded and tested for interference with the identification procedure. Electrophoresis of the PCR products and their restriction fragments was performed in 6% 29:1 acrylamide:bis-acrylamide non-denaturating acrylamide gels at 180 V for 3 h and 30 min or, alternatively, in 10 cm long 4% standard agarose gels at 100 V for 4 h. The gels were stained with ethidium

0923-1811/$30.00
doi:10.1016/j.jdermsci.2006.03.013

Table 1 *Malassezia* type, reference and clinical strains of 11 *Malassezia* species tested for actual nucleotide polymorphisms in the ITS 2 amplified region by RFLP analysis

Group	*Malassezia* species (total, *n* = 120)	Strains	ITS 3/4 PCR product (bp)	*Alu*I	*Ban*I	*Msp*AI
I	*M. furfur* (*n* = 35)	CBS1878, CBS7019, CBS9576, CBS9580, CBS9596, GS2A[a], GS2B[a], GS4A[a], GS9A[a], GS9B[a], B1[a], B2[a], B3[a], B5[a], B10[a], B13[a], B14[a], B15[a], B22[a], B412[a], B522[a], CS3[a]	557	306, 251	389, 168	525, 32
		CBS9375, CBS9569, CBS9572, CBS9575, CBS9577, CBS9578, CBS9581, CBS9582, CBS9584, CBS9587, CBS9589, CBS9590, CBS9597			NRS[b]	
I	*M. obtusa* (*n* = 2)	CBS7876, CBS7968	554	NRS[b]	396, 158	NRS
I	*M. japonica* (*n* = 2)	CBS9431, CBS9432	528	394, 134	183, 199, 146	498, 30
I	*M. pachydermatis* (*n* = 10)	CBS1879, CBS1880, CBS1881, CBS1882, CBS1883, CBS1884, CBS1885, CBS6540, CBS7925, CBS9552	529	412, 117	NRS	499, 30
II	*M. slooffiae* (*n* = 4)	CBS7956, CBS7971, CBS7973, CBS8738	505	385, 120	NRS	472, 33
II	*M. globosa* (*n* = 15)	CBS7866, CBS7874, EM5635a[a], EM5635b[a], GS13B[a], GS19A2[a], GS39B[a], GS40B[a], AA2B[a], AA5B[a], XG1A[a], XG1B[a], XG2A[a], XG2B[a], EM6279[a]	477	221, 16, 240	NRS	447, 30
II	*M. restricta* (*n* = 3)	CBS7877, AA6C[a], AA9A[a]	463	NRS	186, 277	432, 31
II	*M. yamatoensis* (*n* = 1)	CBS9725	470	NRS	NRS	NRS
III	*M. sympodialis* (*n* = 44)	CBS7222, CBS9570, CBS9593, CBS9594, CBS9974, CS17[a], GS3B[a], GS8B[a], GS10B[a], GS12B[a], GS15B[a], GS16B[a], GS18A[a], GS18B[a],GS19A1[a], GS19B[a], GS20B[a],GS22B[a], GS24B [a], GS25B[a], GS26B[a], GS29B[a], GS33A[a], GS34B[a], GS37B[a], AA1B[a], AA4B[a], AA13B[a], EM6154[a], EM6265[a], AA3C[a], AA14B[a], AA16B[a], B437[a], B439[a], B5[a], B17[a], B821[a], B822[a], B829[a], B850[a], B880[a], B881[a], XG3[a]	420	NRS	NRS	281, 109, 30
III	*M. dermatis* (*n* = 3)	CBS9145, CBS9169, CBS9170	416	NRS	186, 230	192, 85, 109, 30
III	*M. nana* (*n* = 1)	CBS9557	428	NRS	188, 240	286, 110, 32

The lengths reported (in base pairs) are approximate as small intraspecies variations exist.

[a] Clinical isolates.

[b] NRS: no restriction site.