

Detection and Mutation Screening of *malassezia sympodialis* Sequences Coding for the Mal s 1 Allergen Implicated in Atopic Dermatitis

To the Editor:

Malassezia yeasts are implicated in atopic dermatitis pathogenesis through production of IgE antibodies that recognize proteins of *Malassezia sympodialis* extract in atopic patients (Johansson and Karlström, 1991). In these patients, skin prick and patch tests are positive to recombinant *Malassezia* allergens related to T cells with T helper 2-like cytokine profile (Johansson *et al*, 2002). Schmidt *et al* (1997) sequenced and expressed the 1176 bp cDNA encoding for the 37 kDa *M. sympodialis* ATCC 42132¹ major allergenic protein Mal s 1.² The genus *Malassezia*, which originally included *M. furfur*, *M. pachydermatis*, and *M. sympodialis*, has only recently been expanded to include *M. globosa*, *M. obtusa*, *M. slooffiae*, and *M. restricta* (Guého *et al*, 1996). Therefore, molecular screening methods for *Malassezia* allergen coding sequences have not yet been developed.

A polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP)–based system for detecting and screening *Mal s 1* sequences coding for peptides rendering the allergenic protein functional is reported herein for the first time. The method has been assessed using 36 strains comprising: 6 *M. furfur* (CBS 6001, CBS 7019, IP 1363–82, 3 clinical strains CS); 14 *M. sympodialis* (ATCC 42132, CBS 7222, GM 301, GM 310, 10 CS); 2 *M. obtusa* (CBS 7876 and GM 211); 4 *M. restricta* (CBS 7877, 3 CS); three *M. slooffiae* (CBS 7956, 2 CS); 3 *M. pachydermatis* (CBS 1879, ED 206, dog otitis); and 4 *M. globosa* (CBS 7966, 3 CS). The icosameric primers, forward Mal s 1a (5'-AACTTGACGCCGAGGACAC-3') and reverse Mal s 1b (5'-ATGGCGTAGGG- GGCGCGAGC-3') (Interactiva Biotechnologie GmbH, Ulm, Germany) were designed from the *Mal s 1* cDNA (GenBank accession no. X96486) to amplify sequences coding for peptides 2, 3, 5, 7, and 9 of the functional protein. Primer interactions were controlled by BLAST (GenBank, www.ncbi.nih.gov/ebank/index.html).

Genomic DNA was prepared from pure cultures as described previously (Gaitanis *et al*, 2002). The 100 µL optimized PCR reaction mixture contained 5 µL PCR buffer (500 mM KCl, 100 mM Tris–HCl, 15 mM MgCl₂, 50 µg gelatin), 0.2 M 2-pyrrolidone (Sigma-Aldrich, St Louis, Missouri), to exclude amplification of atypical products (Chakrabati and Schutt, 2001), 100 pmol of each primer, 20 mM of each deoxyribonucleoside triphosphate (Clontech, Palo Alto, California), 1.2 U of *Taq* polymerase (Promega, Madison, Wisconsin), and 400 ng template DNA. The PCR reactions were performed in a computer-controlled robocycler (Gradient 40, Stratagene, La Jolla, California) with heated lid, without mineral oil, at an initial denaturation cycle (5 min at 94°C), followed by 38 cycles of 1 min at 94°C, 1 min at 61°C (for achieving

high stringency conditions), and 2 min at 72°C, and a final extension step of 5 min at 72°C.

Only *M. sympodialis* template DNA generated the expected 740 bp amplicon visualized in ethidium bromide-stained 2% agarose gels run for 1 h at 60 V. As these sequences refer to the translated region of the protein, it can be acknowledged that absence of a specific PCR product in all other strains of the *Malassezia* species tested denotes lack of the functional protein. This is in accordance with current observations showing that *M. globosa* and *M. restricta*, despite possessing numerous IgE-binding components, lack *M. sympodialis* IgE-binding allergens (Zargari *et al*, 2003). IgE sensitization in atopic patients was lower when the *M. furfur* CBS 1878 extract was used, compared with that of *M. sympodialis* ATCC 42132 or with commercial extracts (m70 ImmunoCAP, Pharmacia, Diagnostic AB, Uppsala, Sweden) (Mayser and Gross, 2000). Moreover, the involvement of *M. sympodialis* allergens in atopic dermatitis aetiopathology was recently demonstrated, because condition severity was associated with *M. sympodialis* reactivity when ImmunoCAP and recombinant Mal s allergen mix were compared (Johansson *et al*, 2003). Thus, the issue of *Malassezia*-induced hypersensitivity in atopic patients could have been previously overlooked.

To test whether Mal s 1 amplified sequences reflect a homogeneous *M. sympodialis* population structure in the cohort tested, all PCR products were screened for mutations by SSCP analysis. For maximum resolution of single-stranded DNA bands the 740 bp product was digested (Gaitanis *et al*, 2002) with *AluI* (Fig 1). For SSCP, 12 µL of the 283 bp, 266 bp, and 179 bp *AluI* fragments recovered from 3% low melting point agarose gels (FMC, Bio-Products, Rockland, Maine) and purified (MBI, Fermentas, Lithouania) were added to 27 µL loading buffer [95% formamide, 20 mM ethylenediamine tetraacetic acid, 0.05% bromophenol blue, and 0.05% xylene cyanol (all from Sigma, St Louis, Missouri)], heated to 95°C for 10 min and quenched on ice for denaturation. Loading of the large fragments on 10% nondenaturing polyacrylamide gel 37.5:1 and of the 179 bp on 29:1 acrylamide/bisacrylamide (Sigma) was followed by electrophoresis at 350 V overnight at 8°C. The gel was visualized after silver nitrate (Sigma) staining (Gathof *et al*, 1995) revealing no mutations in the tested sequences (Fig 2). Mal s 1 a/b PCR product sequencing (QIAGEN, European Oligo Production Facility, Cologne, Germany) and alignment (BLAST, GenBank) of ATCC 42132, CBS 7222, GM 310, and one *M. sympodialis* CS, confirmed both the SSCP results and *Mal s 1* clonality in our strain population. Testing a larger sample size can disclose if this is a geographically confined or a general phenomenon.

The proposed PCR–SSCP system detected homogeneous *Mal s 1* allergen sequences solely in *M. sympodialis* strains. This system can be applied more broadly by deriving primers from *M. furfur* (*sensu lato*) *Mal f 2*, *3*, and *4* genes (Yasueda *et al*, 1998; Onishi *et al*, 1999) to assay reliably whether their corresponding allergen coding sequences are also homogeneously distributed in *Malassezia* populations. As ImmunoCAP and atopic dermatitis studies have been based on *M. sympodialis* ATCC 42132 (Johansson and

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¹Originally identified as *M. furfur* (*sensu lato*), and renamed on October 24, 2001.

²Formerly Mal f 1, and renamed on October 24, 2001.

Figure 1. Restriction map (NEBcutter V 1.0, tools.neb.com/NEBcutter/index.php3) of the *Mal s 1 a/b* PCR product and sequences of *M. sympodialis* ATCC 42132 PCR product (GenBank accession no. AY283740). (A) Selection of *Mal s 1a/b* *Alu I*-restriction fragments for SSCP was based on the restriction map generated from the published *M. sympodialis* ATCC 42132 sequence (accession No. X96486). (B) Comparison and alignment of the 740 bp PCR amplified nucleotide sequences (GenBank accession Nos AY283740–AY283743) with the published cDNA sequence, demonstrated 100% identity and the absence of introns in the amplified gene region.



A

5'CACAATCTATGACCGTACTCGCCAGGTGTTCTACCAATCGAATCTGTACAAAGGCCGCA TTGAGGTCTACAACCCAAAGACCCAGTCACACTTTAATGTGGTCATTGACGGTGCCCTCGTC CAATGGCGATGGTGAGCAGCAGATGTCGGGACTGTCTCTCTGACCCACGACAACTCGAA GCGTTTGTTCGCTGTTATGAAGAATGCGAAGTCGTTCAACTTTGCCGACCAGTCGAGCCAT GGTGCAAGCTCGTTCCACAGCTTCAACCTCCCTCTCTCGGAGAACAGCAAGCCTGTCTGGT CTGTAAACTTCGAGAAGGTCCAGGACGAGTTTGAAAAGAAGGCTGGCAAGCGCCCTTCG GTGTGGTGCACTCGGCTCAGGACCGTGATGGCAACTCTTACGTGGCCCTTTGCACTGGGTAT GCCGGCCATTGCCCGTGTGAGTGCTGATGGAAAGACGGTGAGCACCTTTGCCCTGGGAATC GGGCAATGGAGGTCAGCGTCCCGGGTACTCAGGTATCACGTTCCGACCCTCACTCGAACAA GCTGATCGCGTTTGGTGGCCCTCGCGCCCTGACTGCCTTCGATGTGAGCAAGCCTTATGCA TGGCCGAGCCTGTAAAGATTAACGGCGACTTTGGCACGCTTTCGGGTACCGAGAAGATT GTTACGGTCCCGGTAGGCAATGAGAGTGTG 3'

B

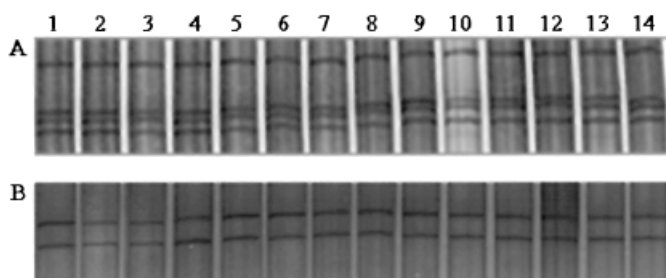


Figure 2. Identical *Alu I* *Mal s 1 a/b* SSCP profiles of the 14 *M. sympodialis* type, reference, and clinical strains. (A) SSCP profiles of the 283 bp and 266 bp restriction fragments. (B) SSCP profiles of the 179 bp restriction fragments. The 179 bp fragment codes for functional peptides 2, 3, 5, and the 266 bp fragment for peptides 7 and 9.

Karlström, 1991; Zargari *et al*, 1995; Johansson *et al*, 2003), *sensu stricto*, identification of *Malassezia* species and evaluation of *Mal s 1* population level polymorphisms, coupled with direct detection of *Mal s 1* sequences in skin scales (Gaitanis *et al*, 2002; Gemmer *et al*, 2002) are warranted.

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