The ISME Journal (2009) 3, 498–502

www.nature.com/ismej

SHORT COMMUNICATION

Malassezia furfur fingerprints as possible markers for human phylogeography

George Gaitanis[1,2], Aristea Velegraki[1], Evangelos C Alexopoulos[3], Evangelia Kapsanaki-Gotsi[4], Lilia Zisova[5], Yuping Ran[6], Hao Zhang[6], George Arsenis[1], Ioannis D Bassukas[2] and Jan Faergemann[7]

[1]*Mycology Laboratory, Medical School, National and Kapodistrian University of Athens, Athens, Greece;* [2]*Department of Skin and Venereal Diseases, Medical School, University of Ioannina, Ioannina, Greece;* [3]*Department of Public health, Medical School, University of Patras, Patras, Greece;* [4]*Division of Ecology and Systematics, Biology Department, National and Kapodistrian University of Athens, Athens, Greece;* [5]*Department of Dermatology and Sexually Transmitted Diseases, Medical University, Plovdiv, Bulgaria;* [6]*Department of Dermatovenereology, West China Hospital, Sichuan University, Chengdu, PR China and* [7]*Department of Dermatology, Sahlgrenska University Hospital, SE Goteborg, Sweden*

Malassezia furfur **was the first species described within the cosmopolitan yeast genus** ***Malassezia,*** **which now comprises 13 species. Reported isolation rates of these species from healthy and diseased human skin show geographic variations. PCR-fingerprinting with the wild-type phage M13 primer (5′-GAGGGTGGCGGTTCT-3′) was applied to investigate phylogeographic associations of** ***M. furfur*** **strains isolated from Scandinavians residing permanently in Greece, in comparison to clinical isolates from Greek, Bulgarian and Chinese native residents. Seven** ***M. furfur*** **strains from Scandinavians were compared with the Neotype strain (CBS1878), CBS global collection strains (*n* = 10) and clinical isolates from Greece (*n* = 4), Bulgaria (*n* = 15) and China (*n* = 6). Scandinavian, Greek and Bulgarian** ***M. furfur*** **strains mostly formed distinct group clusters, providing initial evidence for an association with the host's geographical origin and with the underlying skin condition. These initial data address the hypothesis that** ***M. furfur*** **could be a eukaryotic candidate eligible for phylogeographic studies.**

The ISME Journal (2009) **3,** 498–502; doi:10.1038/ismej.2008.112; published online 20 November 2008
Subject Category: microbial population and community ecology
Keywords: eukaryote; fingerprinting; phylogeography; *Malassezia*

The genus *Malassezia* includes 10 anthropophilic and obligatory lipophilic species (*M. globosa, M. restricta, M. slooffiae, M. obtusa, M. furfur, M. sympodialis, M. japonica, M. yamatoensis, M. dermatis, M. nana*) and 3 zoophilic species (*M. pachydermatis, M. caprae, M. equina*) (Cabañes *et al.*, 2007; Ashbee, 2007). It is considered a member of the class Exobasidiomycetes that mainly includes plant pathogens (Begerow *et al.*, 2006). *Malassezia* yeasts show global distribution on human skin and display adaptable, occasionally species-specific biological properties related to their human host. (Xu *et al.*, 2007). Moreover, worldwide epidemiological studies sampling healthy volunteers and *Malassezia* associated dermatoses, such as pityriasis versicolor, atopic dermatitis and seborrheic dermatitis, report isolation of mixed species from healthy and diseased skin, as well as clear geographic variations in *Malassezia* species isolation rates (Ashbee, 2007).

In this study PCR-fingerprinting, a widely available method offering direct multi-centre assessment of primary epidemiological data, was used to investigate potential phylogeographic associations of *M. furfur* strains isolated from Scandinavians permanently residing in Greece, as well as clinical isolates from Greece, Bulgaria and China. *M. furfur* strains formed distinct group clusters, associated with the host's geographical origin and with the underlying skin condition.

Seven *M. furfur* strains isolated from the forehead (A) or midscapula area (B) of Scandinavian volunteers residing permanently in Greece for 0.5–47 years (median 16) were compared with the Neotype (CBS 1878) (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands), global strains ($n = 10$) and 25 clinical isolates from Greece ($n = 4$), Bulgaria ($n = 15$) and China ($n = 6$; Table 1). For isolation and identification to species level, *Malassezia* strains

Correspondence: A Velegraki, Mycology Laboratory, Medical School, National and Kapodistrian University of Athens, M Asias 75–77, Goudi, Athens 11527, Greece.
E-mail: aveleg@med.uoa.gr
Received 17 July 2008; revised 2 October 2008; accepted 8 October 2008; published online 20 November 2008

Table 1 Epidemiological data of the Scandinavian, Bulgarian, Chinese, the Neotype (ATCC 14521 (CBS1878)) and global *M. furfur* strains included in the study

Code no.	*Gender*	*Age (years)*	*Stay in Greece*	*Site of isolation*	*Region of origin*	*Disease*
GS1B	Female	64	16	Back	Greece resident Scandinavian	HS
GS2A	Male	53	1	Face	Greece resident Scandinavian	HS
GS2B	Male	53	1	Back	Greece resident Scandinavian	HS
GS4A	Female	59	32	Face	Greece resident Scandinavian	HS
GS9A	Female	50	16	Face	Greece resident Scandinavian	HS
GS9B	Female	50	16	Back	Greece resident Scandinavian	HS
GS19A	Female	52	15	Face	Greece resident Scandinavian	SD
B1	Female	1	NA	Scalp	Bulgaria	DF
B2	Female	6	NA	Scalp	Bulgaria	DF
B3	Male	25	NA	Back	Bulgaria	PV
B5	Male	29	NA	Back	Bulgaria	PV
B10	Female	14	NA	Back	Bulgaria	PV
B13	Male	42	NA	Face	Bulgaria	SD
B14	Male	33	NA	Face	Bulgaria	SD
B15	Male	23	NA	Presternal Area	Bulgaria	SD
B19	Female	50	NA	Face	Bulgaria	SD
B21	Male	45	NA	Face	Bulgaria	SD
B22	Male	39	NA	Scalp	Bulgaria	SD
B23	Male	19	NA	Face	Bulgaria	SD
B28	Male	2	NA	Face	Bulgaria	SD
B29	Female	5	NA	Face	Bulgaria	SD
B412	Female	6	NA	Scalp	Bulgaria	SD
CH98	Male	30	NA	Ear	China	Otitis
CH100	Female	34	NA	Chest	China	PV
CH102	Female	27	NA	Back	China	PV
CH106	Male	33	NA	Chest	China	Folliculitis
CH107	Female	6	NA	Nail	China	Nail
CH114	Male	0.5	NA	Head	China	PV
CBS1878			NA	Human		DF
CBS5333			NA	Human	Canada	
CBS6001			NA	Human	India	PV
CBS7019			NA	Human	Finland	PV
CBS7983			NA	Human	France	Blood isolate
CBS7984			NA	Elephant	France	
CBS7985			NA	Ostrich	France	
CBS8736			NA	Woman	Canada	PV
CBS8737			NA	Human	Canada	Toe nail
CBS9580			NA	Human	Romania	
CBS9584			Greek	Human	Greece	DF
CBS9585			Greek	Human	Greece	SD
CBS9596			Greek	Human	Greece	DF
EM7036			Greek	Human	Greece	Blood isolate

Abbreviations: B, Bulgarian *M. furfur* strains; CH, Chinese; DF, Dandruff; GS, Scandinavians residing in Greece; SD, seborrheic dermatitis; PV, pityriasis versicolor; HS, healthy subjects; CBS, Centraalbureau voor Schimmelcultures; NA, not applicable.

were grown on modified Dixon's agar at 32 °C (Gaitanis *et al*., 2006a, b). *M. furfur* strains were identified by their inability to grow on Sabouraud-dextrose agar without lipid supplementation, the positive assimilation of Tweens 20, 40, 60, 80 and Cremophor El (Guého *et al*, 1996; Mayser *et al*, 1997). Conventional identification was confirmed by PCR–RFLP analysis of the internal transcribed spacer 2 region (Gaitanis *et al* 2006a, b). DNA was extracted by a modified hexadecyltrimethylammonium bromide method (Velegraki *et al*, 1999). Additional automated DNA extractions (Maxwell 16, Promega, Madison, WI, USA) were carried out from single, 2–3 mm diameter *M. furfur* colonies from Dixon's agar, using a modification (not shown) of the Maxwell 16 Cell DNA Purification protocol (Promega), yielding 20 ng DNA (A260/A280: 1.03). Automated *M. furfur* extraction was engaged to evaluate its standardization aptitude and throughput in upcoming medium-scale intercontinental population studies, its basic downstream applications, such as direct use in PCR, restriction enzyme digestion and agarose gel electrophoresis, and downstream real-time PCR research applications.

For PCR fingerprinting, the minisatellite-specific core sequence of the wild-type phage M13 (5′-GAGGGTGGCGGTTCT-3′) (Meyer and Mitchell, 1995) was used. The 25 μl of the PCR master mix contained 2 μl $MgCl_2$ (25 mM), 2.5 μl $MgCl_2$-free storage buffer B (Promega), 1.5U Taq Polymerase (Promega), 0.015 mM of each dNTP (HT Biotechnology Ltd, Cambridge, UK), 50 pmol of the M13 primer and 200–250 ng of template DNA. The PCR reaction was carried out in a Techgene Techne

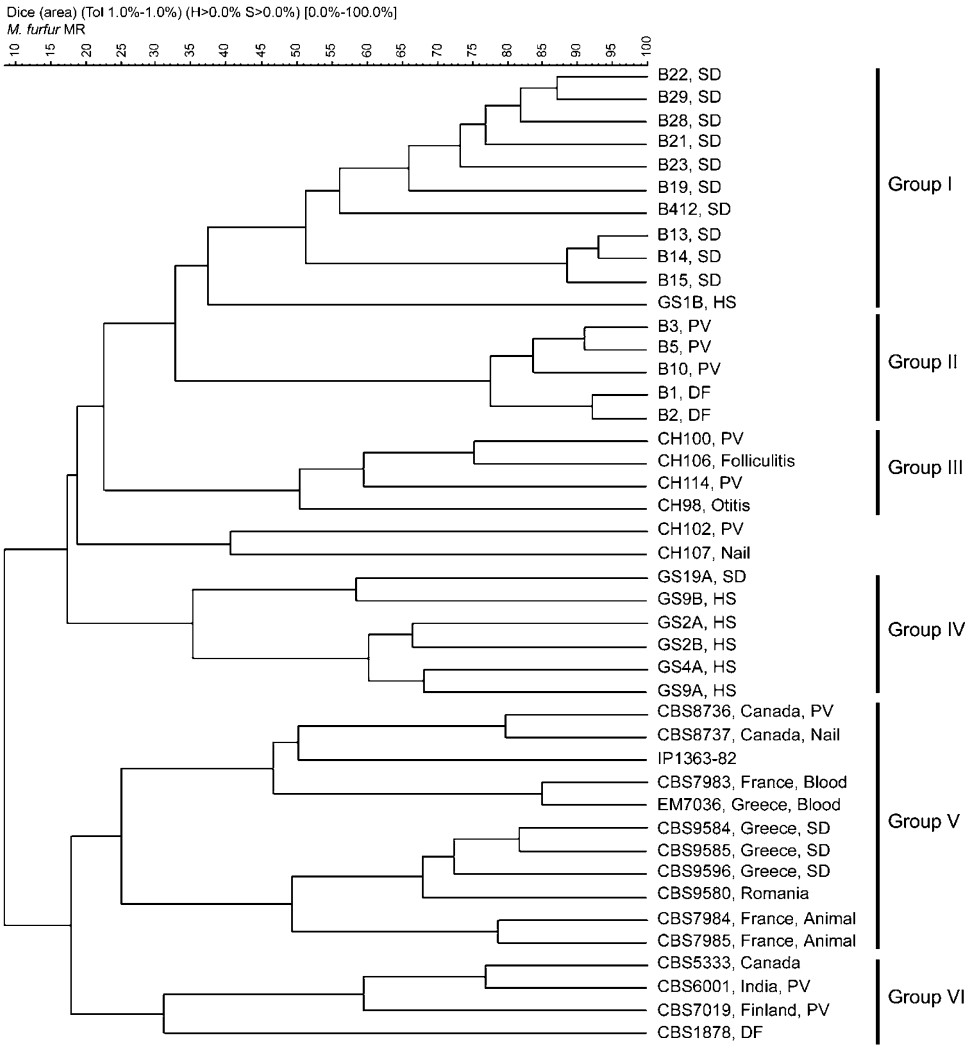

Figure 1 Rooted dendogram constructed using unweighted pair-group method with arithmetic averages (UPMGA) analysis of *M. furfur* microsatellite PCR fingerprints as generated by Bionumerics software. B: Bulgarian *M. furfur* strains; CH: Chinese; GS: Scandinavians residing in Greece; SD: seborrheic dermatitis; PV: pityriasis versicolor; HS: healthy subjects; CBS: Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands. In case there was a simultaneous isolation of two *M. furfur* strains from the forehead and midscapula area they were differentiated as A and B, respectively.

thermal cycler (Stone Staffordshire, UK) and consisted of 1 initial denaturation cycle (5 min; 94 °C) and 45 amplification cycles (1 min; 94 °C, 1 min; 42 °C and 2 min; 72 °C). PCR products were electrophoresed in 4–6% 29:1 acrylimide/bis-acrylimide stacking gels for 3.5 h at 180 V, followed by ethidium bromide staining and UV visualization (Herolab, E.A.S.Y., Weisloch, Germany). The M13 PCR-fingerprinting has been exhaustively validated previously in the basidiomycetous yeast *Cryptococcus neoformans* (Meyer and Mitchell, 1995). In this study, the stability of M13 fingerprints for all *M. furfur* strains was confirmed in five independent experiments within a 3-year period.

The dendrogram was constructed by the Bionumerics software Version 4 (Bio-Maths, Bilthoven, Belgium; Figure 1) using the Dice Coefficient of similarity and cluster analysis with the unweighted pair-group method with arithmetic averages (UPGMA) using 1.00% position tolerance and no optimization, to obtain the greatest variation in similarity. A cutoff point of 24% similarity was used discriminating six major groups.

Overall, *M. furfur* fingerprints showed a trend to associate with the host's geographic origin and a correlation with the underlying skin condition (Figure 1). Six out of seven Scandinavian *M. furfur* isolates formed Group IV with 35% homology forming a distinct cluster from Groups V and VI (20% homology) that included all Greek and the global selection of strains (Figure 1). The single *M. furfur* Scandinavian (GS1A) isolate that was grouped together with the Bulgarian isolates in Group I (37% similarity; Figure 1) could represent a transient member (Mayser *et al.*, 2001) of the *Malassezia* skin flora.

Generally, data on skin *Malassezia* population dynamics are scant (Paulino *et al*., 2006) and not directly comparable. Yet, indicative of the same phylogeographic trend is that a *M. furfur* seborrheic dermatitis isolate from a Dutch male residing for 5 years in China showed identical complete internal transcribed spacer sequences with *M. furfur* CBS7982, from a European healthy individual (Ran *et al*., 2008).

Bulgarian strains clustered in Groups I and II with clear disease associations. Specifically, seborrheic dermatitis isolates ($n=10$) clustered in Group I with 51% similarity and pityriasis versicolor ($n=3$) and dandruff (DF) ($n=2$) isolates in Group II (similarity 77%). Although the number of isolates analyzed is small, the observed disease-specific *M. furfur* clustering concurs with previously recorded disease-specific *Malassezia* species subtypes (Paulino *et al*, 2006; Tajima *et al*, 2008) based on IGS, internal transcribed spacer and 18S sequencing. Interestingly, the two systemic *M. furfur* isolates from France and Greece (Table 1) clustered together showing their common European and disease origin (Gupta *et al*, 2004). Of the Chinese isolates, four out of six formed a single Group III with 22% homology to Group I, whereas two out of six grouped separately. More strains from this large geographic region should be analyzed to reveal consequential human phylogeographic segregation. Variable *M. furfur* subtypes (Figure 1: strains GS4A/GS4B and GS9A/GS9B) from different anatomical regions showed different M13 fingerprints. As multiple *Malassezia* species are isolated from individuals (Gaitanis *et al*., 2006b; Tajima *et al*, 2008), an analogous intra-species polymorphism is expected.

The undemanding sampling procedure and the satisfactory *M. furfur* isolation rate of 5–23.3% (Sweden, Sandström-Falk *et al*., 2005; Iran, Tarazooie *et al*., 2004) may appoint *M. furfur* a candidate eukaryotic organism for studying human phylogeography. Moreover, compared with other *Malassezia* species, *M. furfur* survives longer in storage, thus supporting retrospective studies, strain exchange and inter-laboratory investigations. Also, *M. furfur* is designated as a Biosafety Level 2 agent, this not impending international handling and distribution (Devlin, 2006).

Generally, PCR-fingerprinting holds an inborn methodological disadvantage, because the randomly amplified DNA fragments are used in a deterministic way to identify similarity or dissimilarity of isolates. By contrast, the minisatellite-derived oligonucleotide M13 is capable of achieving specific amplification of inter-repeat sequences by target minisatellite sequences dispersed throughout the fungal genome. Despite the somewhat inscrutable role of minisatellites in genomic plasticity on an evolutionary time scale, M13 PCR-fingerprinting used as a single-typing method in yeasts has been proven, upon stringent PCR reaction parameter standardization, highly reproducible. Since it is long shown to successfully detect inter- and intra-species variation (Meyer and Mitchell, 1995), it represents a cost-effective initial screening method engaging *M.furfur* in human phylogeography studies.

To our knowledge, this is the first time that a eukaryote serves as a potential marker for human phylogeography. Human viruses (HIV, dengue virus, rabbies virus) (Holmes, 2004) and bacteria (*Helicobacter pylori*) (Falush *et al*., 2003) have been used previously for this purpose. By contrast, other human fungal pathogens of global distribution, such as *Trichophyton rubrum,* though shown clonally reproduced in two geographically distinct populations, (Gräser *et al*., 2007), maintain genetic homogeneity (Summerbell *et al*., 1999) rendering it unsuitable for this purpose.

The data presented herein have to be confirmed with inclusion of additional *M. furfur* isolates from more geographically defined population cohorts using robust typing methods, such as multilocus sequencing, to expand knowledge on *Malassezia* species ecology and pathobiology and further assess its candidacy for phylogeograpic studies.

Acknowledgements

This study was encouraged by the International Society for Human and Animal Mycology (ISHAM) *Malassezia* Working Group and funded by the Greek State Scholarships Foundation (IKY) and the University of Athens Grant 'Kapodistrias' K.A. 70/4/5905 and K.A 70/3/6915. We thank the Scandinavian community of Athens, the NOKIA Hellas personnel and Helena Boundouvi for organizing the sampling sessions, G Tzanakaki and K Kessanopoulos, National Reference Laboratory for Meningococcal Disease, School of Public Health, Athens, Greece, for use of the Bionumerics Software and S Kritikou for technical assistance.

Conflict of interest

The authors declare no conflict of interest.

References

Ashbee HR. (2007). Update on the genus *Malassezia*. *Med Mycol* **45**: 287–303.

Begerow D, Stoll M, Baurer R. (2006). A phylogenetic hypothesis of *Ustilagomycotina* based on multiple gene analyses and morphological data. *Mycologia* **98**: 906–916.

Cabañes FJ, Theelen B, Castellá, Boekhout T. (2007). Two new lipid-dependent *Malassezia* species from domestic animals. *FEMS Yeast Res* **7**: 1064–1076.

Devlin RK. (2006). Invasive fungal infections caused by *Candida* and *Malassezia* species in the neonatal intensive care unit. *Adv Neonatal Care* **6**: 68–77.

Falush D, Wirth T, Linz B, Pritchard J, Stephens M, Kidd M *et al.* (2003). Traces of human migrations in *Helicobacter pylori* populations. *Science* **299**: 1582–1585.

Gaitanis G, Robert V, Velegraki A. (2006a). Verifiable single nucleotide polymorphisms of the internal transcribed spacer 2 region for the identification of 11 *Malassezia* species. *J Derm Sc* **43**: 214–217.

Gaitanis G, Velegraki A, Alexopoulos EC, Chasapi V, Tsigonia A, Katsambas A. (2006b). Distribution of *Malassezia* species in pityriasis versicolor and seborrhoeic dermatitis in Greece. Typing of the major pityriasis versicolor isolate *M. globosa*. *Br J Dermatol* **154**: 854–859.

Gräser Y, Fröhlich J, Presber W, de Hoog S. (2007). Microsatellite markers reveal geographic population differentiation in *Trichophyton rubrum*. *J Med Microbiol* **56**: 1058–1065.

Guého E, Midgley G, Guillot J. (1996). The genus *Malassezia* with description of four new species. *Antonie Van Leeuwenhoek* **69**: 337–355.

Gupta AK, Boekhout T, Theelen B, Summerbell R, Batra R. (2004). Identification and typing of *Malassezia* species by amplified fragment length polymorphism and sequence analyses of the internal transcribed spacer and large-subunit regions of ribosomal DNA. *J Clin Microbiol* **42**: 4253–4260.

Holmes EC. (2004). The phylogeography of human viruses. *Mol Ecol* **13**: 745–756.

Mayser P, Schütz M, Schuppe HC, Jung A, Schill WB. (2001). Frequency and spectrum of *Malassezia* yeasts in the area of the prepuce and glans penis. *BJU Int* **88**: 554–558.

Mayser P, Haze P, Papavassilis C, Pickel M, Gruender K, Guého E. (1997). Differentiation of *Malassezia* species: selectivity of cremophor EL, castor oil and ricinoleic acid for *M furfur*. *Br J Dermatol* **137**: 208–213.

Meyer W, Mitchell T. (1995). Polymerase chain reaction fingerprinting in fungi using single primers specific to minisatellites and simple repetitive DNA sequences: strains variation in *Cryptococcus neoformans*. *Electrophoresis* **16**: 1648–1656.

Paulino LC, Tseng C-H, Strober BE, Blaser M. (2006). Molecular analysis of fungal microbiota in samples from healthy human skin and psoriatic lesions. *J Clin Microbiol* **44**: 2933–2941.

Ran Y, He X, Zhang H, Dai Y, Li L, Bulmer GS. (2008). Seborrheic dermatitis flare in a Dutch male due to commensal *Malassezia* furfur overgrowth. *Med Mycol* **46**: 611–614.

Sandström Falk MH, Tengvall Linder M, Johansson C M-H, Bartosik J, Bäck O, Tarrnhult T *et al.* (2005). The prevalence of *Malassezia* yeasts in patients with atopic dermatis, seborrheic dermatitis and healthy controls. *Acta Derm-Venereol* **85**: 17–23.

Summerbell RC, Haugland RA, Li A, Gupta AK. (1999). rRNA gene internal transcribed spacer 1 and 2 gene sequences of asexual, anthropophilic dermatophytes related to *Trichophyton rubrum*. *J Clin Microbiol* **37**: 4005–4011.

Tajima M, Sugita T, Nishikawa A, Tsuboi R. (2008). Molecular analysis of *Malassezia* microflora in seborrheic dermatitis patients: comparison with other diseases and healthy subjects. *J Invest Dermatol* **128**: 345–351.

Tarazooie B, Kordbacheh P, Zaini F, Zomoridian K, Saadat F, Zeraati H *et al.* (2004). Study of the distribution of *Malassezia* species in patients with pityriasis versicolor and healthy individuals in Tehran, Iran. *BMC Dermatol* **4**: 5.

Velegraki A, Kambouris M, Kostourou A, Chalevelakis G, Legakis NJ. (1999). Rapid extraction of fungal DNA from clinical samples for PCR amplification *Med. Mycol* **37**: 69–73.

Xu J, Saunders CW, Hu P, Grant RA, Boekhout T, Kuramae EE *et al.* (2007). Dandruff-associated *Malassezia* genomes reveal convergent and divergent virulence traits shared with plant and human fungal pathogens. *Proc Natl Acad Sci USA* **104**: 18730–18735.