Genotype Analyses of Human Commensal Scalp Fungi, Malassezia globosa, and Malassezia restricta on the Scalps of Patients with Dandruff and Healthy Subjects

Genotype analyses of human commensal scalp fungi, *Malassezia globosa*, and *Malassezia restricta* on the scalps of patients with dandruff and healthy subjects

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Abstract

Dandruff and seborrheic dermatitis are common afflictions of the human scalp caused by commensal scalp fungi belonging to the genus *Malassezia*. *Malassezia globosa* and *Malassezia restricta* are the predominant species found on the scalp. The intergenic spacer regions of these species' rRNA genes contain short sequence repeats (SSR): (GT)n and (CT)n in *M. globosa* and (CT)n and (AT)n in *M. restricta*. In the present study, we compared the genotypes (SSR) of *M. globosa* and *M. restricta* colonizing the scalps of patients with dandruff and healthy individuals. For *M. globosa*, the genotype $(GT)_{10}$:$(CT)_8$ (40.3%, 25/62) was predominant followed by $(GT)_9$:$(CT)_8$ (14.5%, 9/62) and $(GT)_{11}$:$(CT)_8$ (14.5%, 9/62) in patients with dandruff, whereas the genotypes in healthy subjects were diverse. For *M. restricta*, the genotype $(CT)_6$:$(AT)_6$ (59.7%, 37/62) was predominant followed by $(CT)_6$:$(AT)_8$ (24.2%, 15/62) in patients with dandruff, while four genotypes, $(CT)_6$:$(AT)_6$ (10.5%, 6/57), $(CT)_6$:$(AT)_7$ (22.8%, 13/57), $(CT)_6$:$(AT)_8$ (17.5%, 10/57), and $(CT)_6$:$(AT)_{10}$ (21.1%, 12/57), accounted for 71.9% of all combinations in healthy subjects. The results of this study suggested that *M. globosa* genotype $(GT)_{10}$:$(CT)_8$ and *M. restricta* genotype $(CT)_6$:$(AT)_6$ are strongly involved in the development of dandruff.

Keywords: Dandruff, *Malassezia globosa*, *Malassezia restricta*, Genotype, IGS, rRNA gene

Introduction

Dandruff and seborrheic dermatitis are common afflictions of the human scalp and are considered to be the same basic condition differing only in magnitude. Dandruff and seborrheic dermatitis are categorized into four sequential pathophysiological phases [1]: 1) human commensal scalp fungi (*Malassezia* species) interact with the epidermis; 2) inflammation develops with clinical signs and symptoms, including erythema and itching; 3) proliferation and differentiation in the epidermis are disrupted; and 4) the barrier function of the skin is disrupted. Thus, *Malassezia* species play a significant role in the development of dandruff and seborrheic dermatitis. As *Malassezia* species are lipophilic fungi, they require fatty acids for their growth. Sebum on the human scalp acts as a nutrient source for *Malassezia* growth. Lipases secreted by *Malassezia* species hydrolyze sebum into triglycerides, which are further hydrolyzed into saturated and unsaturated fatty acids. Although the former is used as a nutrient source for other cutaneous microorganisms, the latter (e.g., oleic acid) induces inflammation of the skin [2, 3].

The human body is covered with a great variety of microorganisms, including bacteria and fungi [4, 5]. *Malassezia* species are predominant members of the cutaneous fungal microbiome at all sites of the body and account for over 40% of the total fungal microbiome [6]. Of the 14 species identified to date, the most clinically significant species are *Malassezia globosa* and *Malassezia restricta* [7, 8]. These microorganisms

are associated with the development or exacerbation of *Malassezia*-related skin diseases including dandruff, seborrheic dermatitis, pityriasis dermatitis, and atopic dermatitis [9-11]. Both *M. globosa* and *M. restricta* are detected in patients' skin regardless of disease type, whereas other species are detected in <40% of cases. However, the distribution ratio of these two species differs between skin diseases [12-18]. In seborrheic dermatitis, *M. restricta* is predominant over *M. globosa*, while *M. globosa* is predominant over *M. restricta* in pityriasis versicolor.

The fungal rRNA gene consists of four subunits (18S, 5.8S, 26S, and 5S) with spacer regions (two internal transcribed spacers [ITSs] and two intergenic spacers [IGSs]) located between the subunits (**Fig. 1**) [7]. The 26S and ITS regions are widely used for species taxonomy and/or identification. The IGS regions show remarkable intraspecific diversity compared to the other subunits and spacer region; therefore, IGS analysis can be used for strain typing or molecular epidemiology [19-23]. In addition, we found that the IGS regions of *M. globosa* and *M. restricta* have short sequence repeats (SSR): (GT)n and (CT)n for *M. globosa*, and (CT)n and (AT)n for *M. restricta* (**Figs. 2 , 3**) [20, 21].

In the present study, we analyzed the genotypes of both *M. globosa* and *M. restricta* colonizing the scalps of patients with dandruff and healthy individuals, and we found that specific genotypic strains selectively colonized the patients' scalps.

Materials and Methods

Subjects

Samples were obtained from 62 Japanese patients with dandruff and 57 healthy subjects control subjects (**Table S1**). Subjects treated with antimicrobial agents in the previous 4 weeks were excluded. This study protocol was approved by the Institutional Review Board of our institution, and informed consent was obtained from each individual prior to enrollment.

Collection of scale samples and *Malassezia* DNA extraction

Scale samples were obtained from the scalp by swabbing with Falcon™ polyester fiber-tipped applicators (Becton, Dickinson, and Co., Sparks, MD). Briefly, a 3- × 3-cm area of the scalp was swabbed 15 times back and forth on the x-axis and 15 times back and forth on the y-axis.

Swabs were placed in 1.5-mL Eppendorf tubes with 1 mL of lysis solution (100 mM Tris-HCl pH 8.0, 30 mM EDTA, and 0.5% SDS) and incubated for 15 min at 100°C. The lysis solution was then transferred to a new tube and combined with 2 volumes of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol). The solution was then vortexed and centrifuged at 14000 rpm. The aqueous phase was transferred to a new

tube, combined with chloroform-isoamyl alcohol (24:1, vol/vol), and centrifuged at 14000 rpm. DNA was precipitated with 2.5 volumes of ethanol in the presence of 3 M sodium acetate and Ethachinmate™ (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The DNA pellet was resuspended in 30 μL of TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and stored at –20°C until use.

Quantitative analysis of *Malassezia* DNA on the scalp by real-time PCR

The level of colonization by *Malassezia* was quantified by real-time PCR with TaqMan probes according to the method of Sugita et al. [18]. DNA from *M. globosa*, *M. restricta*, and all *Malassezia* species was analyzed using the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA).

Determination of *M. globosa* and *M. restricta* genotypes

The IGS 1 regions of *M. globosa* and *M. restricta* were amplified by nested PCR using species-specific primers. For analysis of the *M. globosa* genotype, the PCR conditions were as follows: denaturation at 94°C for 1 min followed by 30 cycles of 30 s at 94°C, 30 s at 54°C, and 30 s at 72°C, with a final extension at 72°C for 10 min. The primers used were gb-F1 (GCTTTCGAGTGCATACCACACT) and gb-R1 (GGAAATAGGATGAGAGAAACA). For nested PCR, 1 μL of the first amplification product was added to a new reaction tube and PCR was performed as follows:

denaturation at 94°C for 1 min followed by 30 cycles of 30 s at 94°C, 30 s at 54°C, and 30 s at 72°C, with a final extension at 72°C for 10 min. The primers used were gb-F2 (TGCATACCACACTCGAGCGCTT) and gb-R2 (ATGTGGTAGTACGACATAGAGA). For analysis of the *M. restricta* genotype, the PCR conditions were the same as those used for *M. globosa*. The primer sequences were restF1 (CGACCTAGTCGACTACATCCTA), restR1 (GTGTATGTTCGGAGATACAAGC), restF2 (CTAGTCGACTACATCCTACTG), and restR2 (GGAGATACAAGCCTCCATTCG). All products were directly sequenced and analyzed for the number of SSR: (GT)n and (CT)n for *M. globosa* and (CT)n and (AT)n for *M. restricta*.

Results

Level of colonization on the scalp by *Malassezia* species

DNA from all *Malassezia* species and from *M. globosa* and *M. restricta* were quantified by real-time PCR. The level of colonization by all *Malassezia* species in patients with dandruff was approximately three times greater than that in healthy subjects (**Fig. 4**). The amounts of DNA from the two major species in the patients were also greater than those in the healthy subjects. In dandruff patients, *M. restricta* and *M. globosa* accounted for 74 and 6 % of all *Malassezia* species, respectively, and 90 and 5 % of all Malasseia species in healthy subjects (**Fig. 5**). The ratio of *M. restricta* to all *Malassezia*

species in patients with dandruff was slightly lower than that in healthy individuals.

Analysis of *Malassezia* genotypes

PCR products that included the GT and CT repeats of the *M. globosa* IGS were approximately 300 bp in length (**Fig. 2**) while those that included the CT and AT repeats of the *M. restricta* IGS were approximately 500 bp in length (**Fig. 3**).

For the *M. globosa* IGS genotypes, 6-19 repeats were detected for GT while 6-15 repeats were detected for CT in the DNA of the patients with dandruff and healthy individuals. A total of 31 combinations were noted. The genotype $(GT)_{10}$:$(CT)_8$ (40.3%, 25/62) was predominant followed by $(GT)_9$:$(CT)_8$ (14.5%, 9/62) and $(GT)_{11}$:$(CT)_8$ (14.5%, 9/62) in patients with dandruff, whereas the genotypes in the healthy subjects were diverse. The genotypes $(GT)_{10}$:$(CT)_8$, $(GT)_{11}$:$(CT)_8$, and $(GT)_{17}$:$(CT)_{13}$ accounted for 14.0% (8/57), 14.0% (8/57), and 10.5% (6/57), respectively, of the healthy subjects (**Fig. 6**).

For the *M. restricta* IGS genotypes, 3-9 repeats were detected for CT while 3-15 repeats were detected for AT in the DNA of the patients with dandruff and healthy individuals. A total of 15 combinations were found. The genotype $(CT)_6$:$(AT)_6$ (59.7%, 37/62) was predominant followed by $(CT)_6$:$(AT)_8$ (24.2%, 15/62) in patients with dandruff, while the genotypes $(CT)_6$:$(AT)_6$ (10.5%, 6/57), $(CT)_6$:$(AT)_7$ (22.8%, 13/57), $(CT)_6$:$(AT)_8$ (17.5%, 10/57), and $(CT)_6$:$(AT)_{10}$ (21.1%, 12/57) accounted for 71.9% of all

combinations (**Fig. 7**).

Both *M. globosa* and *M. restricta* were detected in all cases. A total of 68 combinations of *M. globosa* and *M. restricta* genotypes were obtained from 119 cases (**Table S2**). Three combinations of each genotype [30.6% (*M. globosa* $[GT]_{10}$:$[CT]_8$; *M. restricta* $[CT]_6$:$[AT]_6$), 12.9% (*M. globosa* $[GT]_9$:$[CT]_8$; *M. restricta* $[CT]_6$:$[AT]_6$), and 9.7% (*M. globosa* $[GT]_{11}$:$[CT]_8$; *M. restricta* $[CT]_6$:$[AT]_8$)] predominated in the dandruff patients, while no specific genotype combination was found among the 68 combinations in healthy subjects.

Discussion

In the present study, we found that specific genotypic strains of *M. globosa* and *M. restricta* selectively colonized the scalps of patients with dandruff. *Malassezia* species are responsible for the induction of dandruff on the scalp. In fact, scalp symptoms of dandruff are improved by treatment with antifungal agents or pyrithione zinc shampoo [24-26]. Gao et al. [6] determined the levels of *Malassezia* colonization on the forehead, forearm, behind the ears, inner elbows, foreleg, and axillae of healthy human subjects by qPCR, and they found that *Malassezia* species were predominant in the skin fungal microbiome. In the cheek and scalp areas, the fungal microbiome consisted mainly of *M. restricta* followed by *M. globosa* [27, 28]. In the present study, *M. restricta* accounted for 98.7 ± 7.2% of the total fungal microbiome on the healthy scalp. This was also the

predominant microorganism over *M. globosa* on the patients' scalps. The level of *Malassezia* colonization on the scalps of patients should be greater than that on the healthy scalp as patients with dandruff secrete larger amounts of sebum than healthy subjects. In fact, the scalps of the patients showed three-fold greater levels of *Malassezia* colonization compared with the scalps of the healthy individuals.

Malassezia globosa possesses 15 lipase genes in its genome [29]. This number is higher than that in the non-lipophilic yeasts *Saccharomyces cerevisiae* and *Cryptococcus neoformans*. The presence of multiple genes for secreted lipases suggests that this species utilizes fatty acids from external sources as nutrients. Lee et al. [30] confirmed the expression of several lipase genes from *M. globosa* and *M. restricta* on the scalps of patients, and one of the *M. restricta* lipase genes (MRE-0242) was strongly expressed on the patients' scalps. Therefore, high levels of lipase production by *Malassezia* species may contribute to the clinical severity of dandruff. The high level of lipase expression may be a characteristic of this genus or a response to the chemical composition of a patient's sebum.

We studied the relationship between the genotypes of microorganisms and their virulence. The rRNA gene is responsible for protein synthesis and is not directly associated with virulence. However, the rRNA genotypes of pathogenic fungi are correlated with their virulence factors or source of origin. Secreted aspartic protease (SAP) is a virulence factor of the pathogenic yeast *Candida albicans*, and three

genotypes of the large subunit are correlated with SAP production ability [31]. Another example is observed in *Trichosporon asahii*, which causes both deep-seated opportunistic infections in immunocompromised hosts and summer-type hypersensitivity pneumonitis as type III or IV allergies in healthy subjects. Of the several *T. asahii* IGS genotypes, genotype I strains are infectious, while type III strains are involved in allergic reactions [32].

With regard to *Malassezia* genotypes, we found that strains of specific genotypes selectively colonized the skin of patients with atopic dermatitis [33]. That is, the genotype of *M. globosa* was correlated with *M. globosa*-specific IgE antibody levels in the sera of patients with atopic dermatitis. Patients were divided into two groups according to their specific IgE antibody level: ≥ 30 IU/ml and < 30 IU/mL. In the < 30 IU/mL group, 16 genotypes were almost equivalently distributed from 2.1 to 12.5% for each genotype, while in the > 30 IU/mL group, 92.3% of patients showed the genotype $(GT)_{10}$:$(CT)_8$. In this study, the genotype $(GT)_{10}$:$(CT)_8$ was also predominant in patients with dandruff (40.3%). Therefore, *M. globosa* genotype $(GT)_{10}$:$(CT)_8$ seems to be common in both dandruff and atopic dermatitis. Currently, the relationship between the *M. restricta* genotype and the species-specific IgE antibody level is under investigation. Our previous study also indicated that the IGS sequences of *M. restricta* colonizing the skin of seborrehic dermatitis patients consisted phylogenetically of two groups: one that included patients with seborrehic dermatitis and another that included both patients with

seborrheic dermatitis and healthy subjects [21]. Although it is unclear whether the specific genotypic strains of *M. globosa* and *M. restricta* induce or exacerbate dandruff, or whether they selectively colonize the scalps of dandruff patients, these strains were predominantly found on the scalps of patients when compared to healthy individuals. This raises the question why specific genotypic strains of both microorganisms are predominant on the scalps of patients with dandruff. This may be due to differences in the chemical composition of scalp sebum, water content, and/or pH of the skin surface of patients with dandruff and healthy subjects. Therapeutic agents given to dandruff patients may also affect selective skin microbial colonization. However, none of the patients in the present study received any antibacterial agents.

In conclusion, we found that specific genotypic strains of *M. globosa* and *M. restricta* predominated in patients with dandruff. The virulence of these microorganisms against the scalp is still unknown. However, the genotype of *Malassezia* species should be investigated when determining the relationships between *Malassezia* species and virulence against the scalp.

Conflict of interest

No conflicts of interest exist for any of the authors due to financial, commercial, or other affiliations.

Legends to Figures

Fig. 1

Primary structure of the fungal rRNA gene

The fungal rRNA gene consists of four subunits (18S, 5.8S, 26S, and 5S) and two spacer regions (ITS and IGS). Approximately 100 copies are present in the genome.

ITS, internal transcribed spacer; IGS, intergenic spacer.

Fig. 2

(GT)n and (CT)n repeats in the IGS region of *M. globosa*

Two representative examples are shown.

Fig. 3

(CT)n and (AT)n repeats in the IGS region of *M. restricta*

Two representative examples are shown.

Fig. 4

The level of *Malassezia* colonization as determined by qPCR

The levels of all *Malassezia* species, *M. restricta*, and *M. globosa* were determined.

PT, patients; HS, healthy subjects.

Fig. 5

M. restricta and *M. globosa* colonization

The ratios of the levels of *M. restricta* and *M. globosa* colonization relative to all *Malassezia* species are shown.

Blue, *M. restricta*; red, *M. globosa*; green; other *Malassezia* species

Fig. 6

Distribution of *M. globosa* genotypes colonizing the scalps of patients with dandruff and healthy subjects

Blue, patients with dandruff; red, healthy subjects.

n:n = the numbers of (GT)n and (CT)n.

Fig. 7

Distribution of *M. restricta* genotypes colonizing the scalps of patients with dandruff and healthy subjects

Blue, patients with dandruff; red, healthy subjects.

n:n = the numbers of (CT)n and (AT)n.

Supporting Information

Table S1

Subjects included in this study

Table S2

Genotypes of *M. globosa* and *M. restricta* found in this study

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Fig. 1

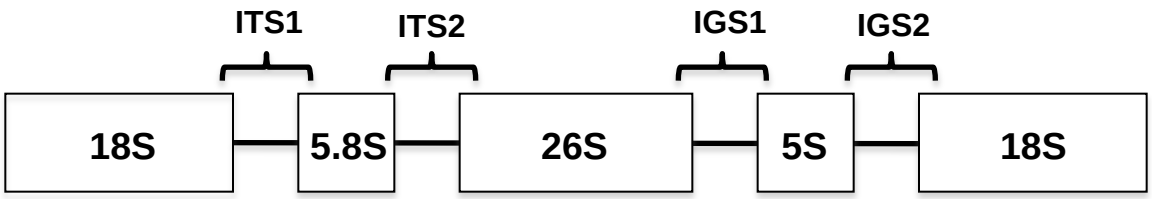

Fig. 2

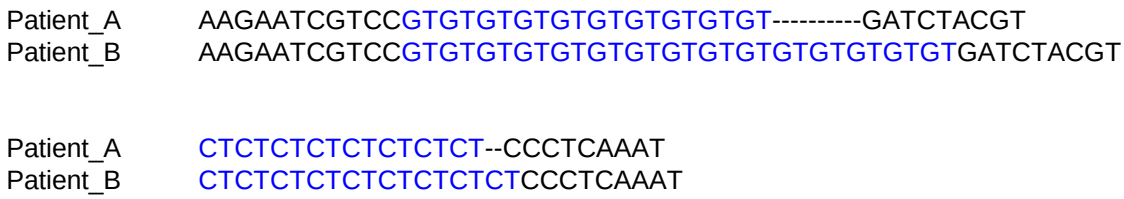
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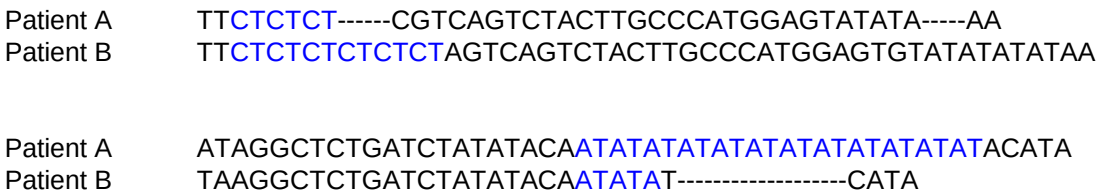
Fig. 3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Patient B TTCTCTCTCTCTCTAGTCAGTCTACTTGCCCATGGAGTGTATATATATAA
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Patient B TAAGGCTCTGATCTATATACAATATAT----------CATA

Fig. 4

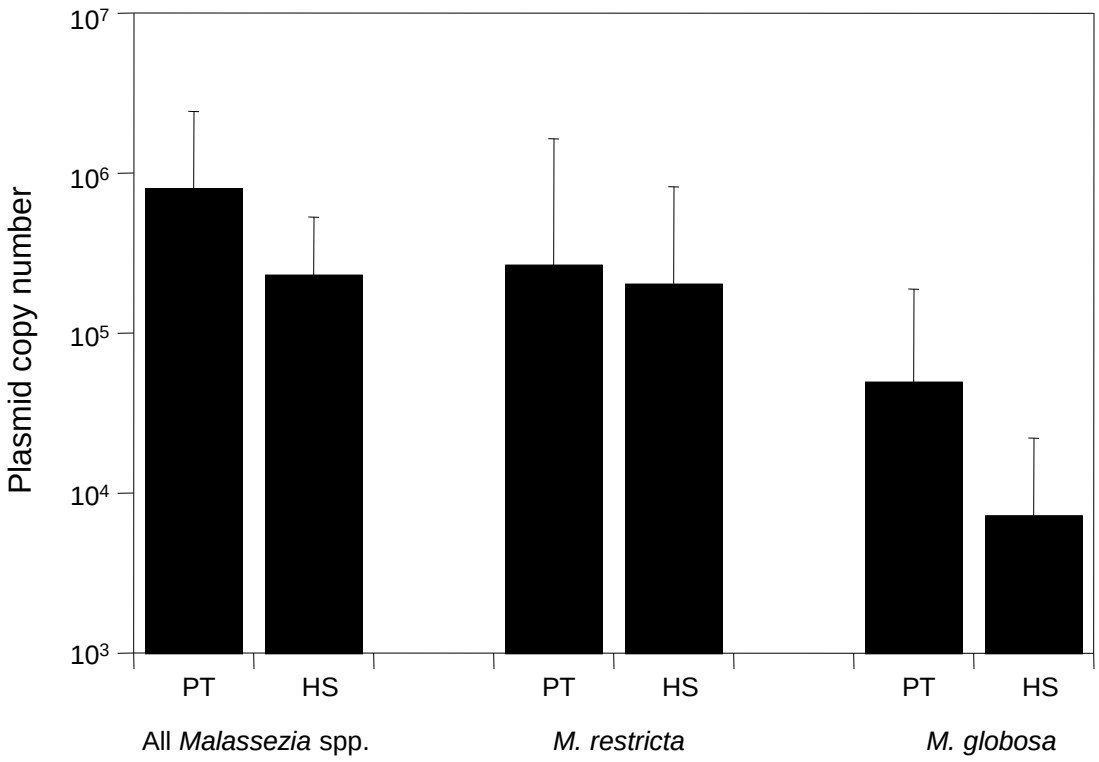
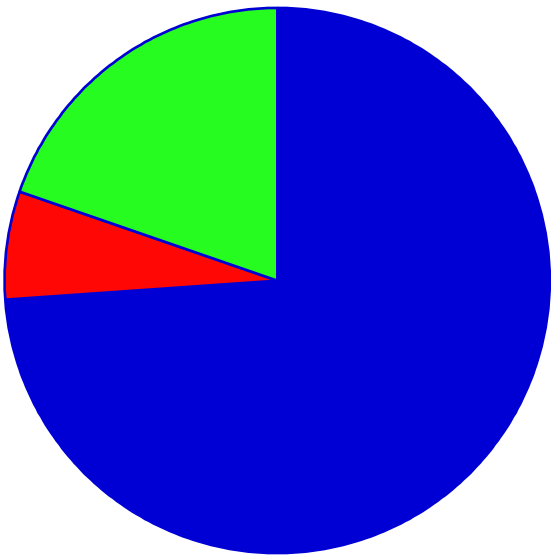
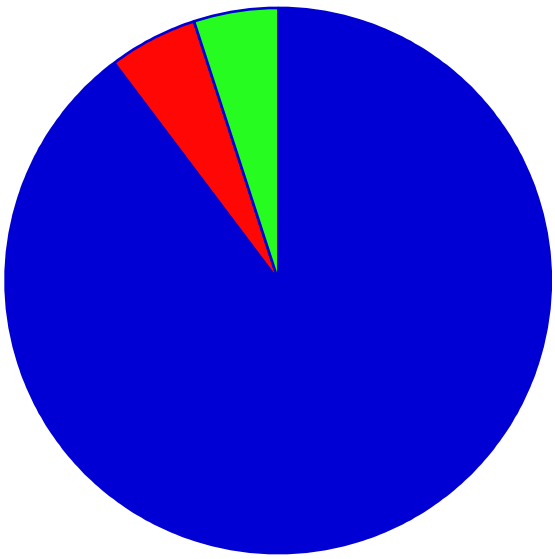

Plasmid copy number
10^7
10^6
10^5
10^4
10^3
PT
HS
PT
HS
PT
HS
All Malassezia spp.
M. restricta
M. globosa

Fig. 5



Patients



Healthy subjects

Fig. 6

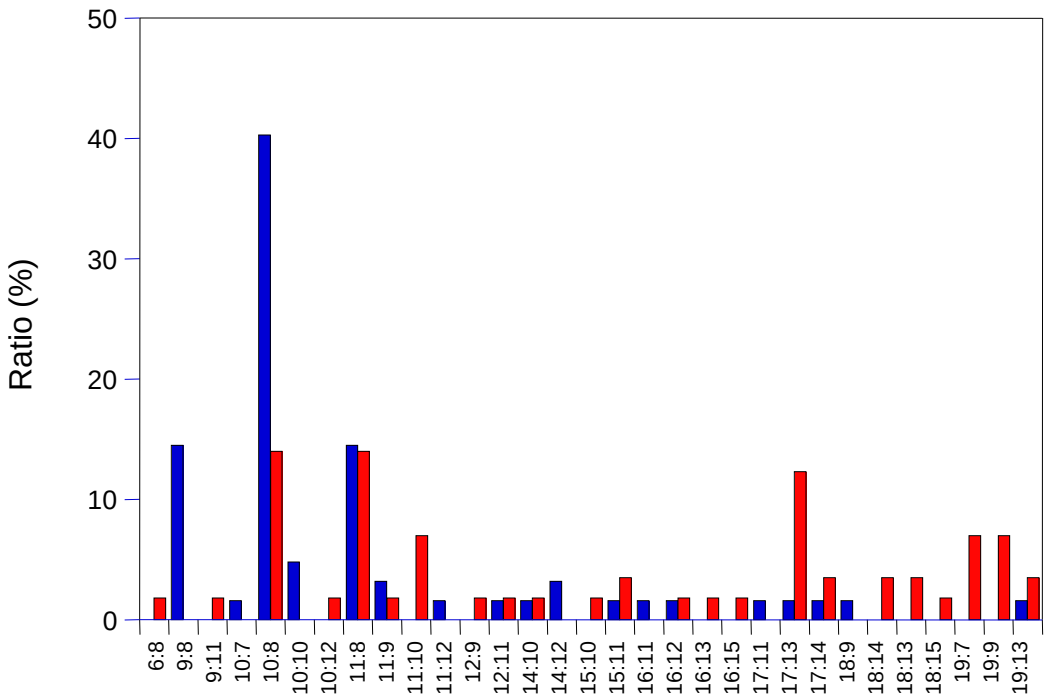

Ratio (%)
50
40
30
20
10
0
6:8
9:8
9:11
10:7
10:8
10:10
10:12
11:8
11:9
11:10
11:12
12:9
12:11
14:10
14:12
15:10
15:11
16:11
16:12
16:13
16:15
17:11
17:13
17:14
18:9
18:14
18:13
18:15
19:7
19:9
19:13

Fig. 7

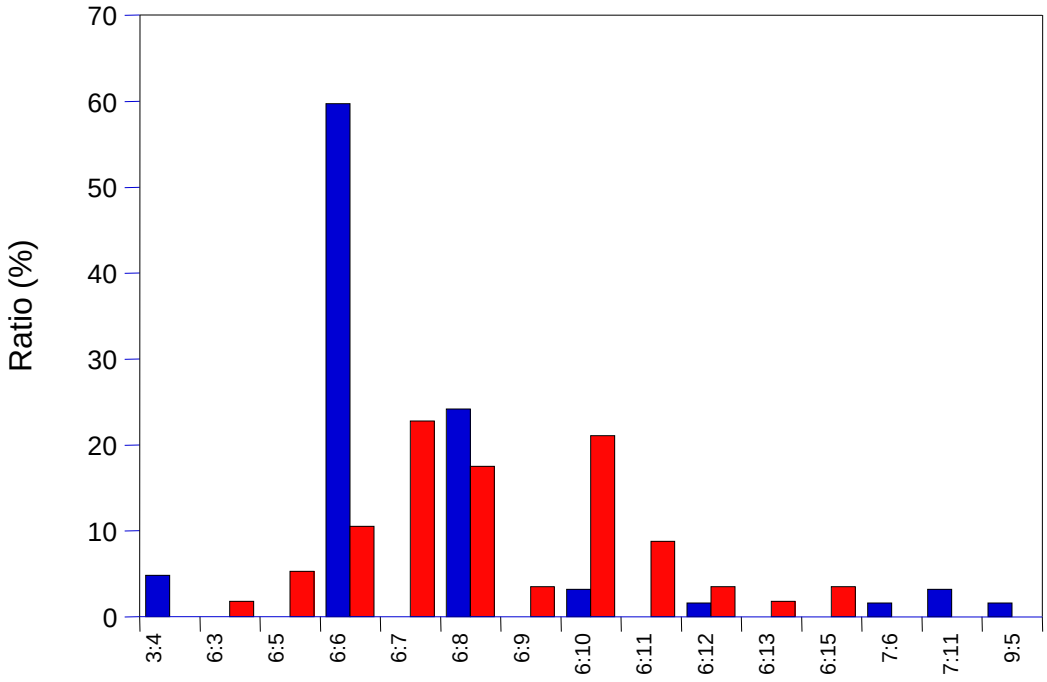

Ratio (%)
70
60
50
40
30
20
10
0
3:4
6:3
6:5
6:6
6:7
6:8
6:9
6:10
6:11
6:12
6:13
6:15
7:6
7:11
9:5

Table S1. Subjects involved

Subjects	Gender	Number of subject	Age (year)	
			Mean ± SD	Range
Patients with dandruff	Malae	38	36.4 ± 10.6	20 - 73
	Female	24	30.4 ± 10.2	8 - 75
Healthy subejct	Malae	36	27.7 ± 8.3	20 - 49
	Female	21	25.5 ± 6.0	21 - 38

SD, standard deviation

Table S2 Genotypes of *M. globosa* and *M. restricta* found in this study

	M. globosa		*M. restricta*		Number of cases		Percentage (%)	
	(GT)n	(CT)n	(CT)n	(AT)n	Patients	Healthy subjects	Patients	Healthy subjects
1	6	6	6	5	0	1	0.0	1.8
2	9	8	3	4	1	0	1.6	0.0
3	9	8	6	6	8	0	12.9	0.0
4	9	11	6	10	0	1	0.0	1.8
5	10	7	7	11	1	0	1.6	0.0
6	10	8	3	4	1	0	1.6	0.0
7	10	8	6	6	19	1	30.6	1.8
8	10	8	6	7	0	1	0.0	1.8
9	10	8	6	8	4	1	6.5	1.8
10	10	8	6	9	0	2	0.0	3.5
11	10	8	6	10	1	0	1.6	0.0
12	10	8	6	11	0	2	0.0	3.5
13	10	8	6	15	0	1	0.0	1.8
14	10	10	6	6	1	0	1.6	0.0
15	10	10	7	6	1	0	1.6	0.0
16	10	10	7	11	1	0	1.6	0.0
17	10	12	6	6	0	1	0.0	1.8
18	11	8	6	6	3	0	4.8	0.0
19	11	8	6	7	0	3	0.0	5.3
20	11	8	6	8	6	1	9.7	1.8
21	11	8	6	10	0	3	0.0	5.3
22	11	8	6	11	0	1	0.0	1.8
23	11	9	6	6	2	0	3.2	0.0
24	11	9	6	11	0	1	0.0	1.8
25	11	10	6	7	0	2	0.0	3.5
26	11	10	6	6	0	1	0.0	1.8
27	11	10	6	13	0	1	0.0	1.8
28	11	12	6	6	1	0	1.6	0.0
29	12	9	6	8	0	1	0.0	1.8
30	12	11	6	6	0	1	0.0	1.8
31	12	11	6	8	1	0	1.6	0.0
32	14	10	6	3	0	1	0.0	1.8
33	14	10	6	8	1	0	1.6	0.0
34	14	12	6	6	1	0	1.6	0.0
35	14	12	6	8	1	0	1.6	0.0
36	15	10	6	8	0	1	0.0	1.8
37	15	11	6	7	0	1	0.0	1.8
38	15	11	6	8	1	0	1.6	0.0
39	15	11	6	11	0	1	0.0	1.8
40	16	11	9	5	1	0	1.6	0.0
41	16	12	6	7	0	1	0.0	1.8
42	16	12	6	10	1	0	1.6	0.0
43	16	13	6	8	0	1	0.0	1.8
44	16	15	6	10	0	1	0.0	1.8
45	17	11	3	4	1	0	1.6	0.0

46	17	13	6	7	0	2	0.0	3.5
47	17	13	6	8	0	1	0.0	1.8
48	17	13	6	10	0	1	0.0	1.8
49	17	13	6	5	0	1	0.0	1.8
50	17	13	6	6	1	0	1.6	0.0
51	17	13	6	10	0	1	0.0	1.8
52	17	14	6	8	1	0	1.6	0.0
53	17	14	6	12	0	1	0.0	1.8
54	17	14	6	15	0	1	0.0	1.8
55	18	9	6	6	1	0	1.6	0.0
56	18	13	6	10	0	2	0.0	3.5
57	18	14	6	5	0	1	0.0	1.8
58	18	14	6	12	0	1	0.0	1.8
59	18	15	6	7	0	1	0.0	1.8
60	18	15	6	10	0	1	0.0	1.8
61	19	9	6	12	1	0	1.6	0.0
62	19	7	6	6	0	1	0.0	1.8
63	19	7	6	8	0	2	0.0	3.5
64	19	7	6	10	0	1	0.0	1.8
65	19	9	6	6	0	1	0.0	1.8
66	19	9	6	10	0	1	0.0	1.8
67	19	13	6	7	0	2	0.0	3.5
68	19	13	6	8	0	2	0.0	3.5