Indian J Med Res 139, March 2014, pp 431-437

Association of *Malassezia* species with dandruff

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Received November 9, 2012

***Background & objectives: Malassezia* species implicated with dandruff vary at different geographical locations. The present study was conducted to determine the spectrum and distribution of *Malassezia* species in dandruff patients and healthy individuals.**

***Methods*: Patients with dandruff from northern (Chandigarh) and southern (Manipal, Karnataka) parts of India (50 each) and healthy individuals (20) were included in the study. Dandruff severity was graded as mild, moderate and severe. *Malassezia* spp. isolated were quantified and identified by phenotypic characters and molecular methods including PCR-RFLP and DNA sequencing.**

***Results*: Number of *Malassezia* spp. retrieved was significantly higher ($P<0.001$) in dandruff cases (84%) as compared to healthy individuals (30%). Isolation of *Malassezia* spp. was significantly higher ($P<0.01$) in patients from southern India. In moderately severe cases *M. restricta* was single most predominant (37.8%) isolate from patients of northern part of India and *M. furfur* (46.4%) from patients of southern part of India. *Malassezia* density was significantly associated with the severity of dandruff ($P<0.001$).**

***Interpretation & conclusions*: Our results on a limited number of individuals show that *Malassezia* spp. associated with dandruff varies in different regions of the country and the density of yeasts increases with severity of disease.**

Key words Dandruff - epidemiology - *Malassezia* - molecular identification

Dandruff is a common persistent, relapsing inflammatory condition affecting the areas rich in sebaceous glands[1]. The condition is characterized by the flaky white to yellowish scales seen on the scalp and less frequently on the nasolabial folds, behind the ears, eyebrows, and intertriginous areas. The prevalence of dandruff in population varies between 30-95 per cent[2]. Besides the discomfort, this disorder is also socially embarrassing and affects the self esteem of the patient[3]. *Malassezia* species play a role in pathogenesis of this condition along with stress, fatigue, weather extremes, oily nature of skin, use of shampoos, immunosuppressed status (AIDS), and neurological disorders. *M. restricta* and *M. globosa* are generally considered to be the causative agents of dandruff[4]; the species implicated vary depending on geographical location of the host. *M. furfur, M. sympodialis, M. obtusa*, and *M. slooffiae* are the other species associated with this condition[5].

Though cases of dandruff are widely prevalent in India[3], the prevalence of this disease, associated factors including *Malassezia* species are not studied in Indian population. The present study was conducted to determine the spectrum of *Malassezia* spp. causing dandruff and existing as commensal on the scalp of healthy individuals at two geographical locations (northern and southern parts of India). In addition, features associated with dandruff were also studied.

Material & Methods

This study was conducted in the departments of Medical Microbiology and Dermatology, Venerology & Leprosy, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh in collaboration of department of Microbiology, Kasturba Medical College, Manipal, Karnataka, India.

One hundred patients (50 each from northern and southern part of India) and 20 healthy individuals (10 each from northern and southern part of India) without features of dandruff (controls) were enrolled in this study. The study was conducted during September 2011 to August 2012. Based on the presence or absence of visible flakes over the scalp, the study population was categorized as dandruff patients or healthy individuals, respectively. The dandruff patients were defined as individuals having inflammation of the scalp in the form of red, scaly, itchy and flaking rash[2]. The study protocol was approved by the Institute's Ethics Committee of PGIMER, Chandigarh. The samples were collected after obtaining written informed consent of the patients. Study area was elected on the basis of convenience to perform field study. The patients from northern part of India belonged to two villages *viz*., Dhuhar (total population ~2500) and Nial (total population ~2400) of Punjab State, whereas patients from southern part of India belonged to two villages *viz*., Karki (total population ~2800) and Manki (total population 3000) of coastal Karnataka. The distance between the two study areas is approximately 2000 km. The climate of chosen area in southern part of India is hot with high humidity almost all through the year (temperature ~22-35ºC, humidity ~65-90%), northern part of India is dry and hot (temperature ~35-45ºC, humidity ~40-45%) in summer and cold (temperature ~5-15 ºC, humidity ~70-80%) in winter. Sampling was carried out at convenience by random screening for the dandruff cases in the selected villages. Both children and adults (3-50 yr) were included in the study. On the day of sampling, the participants were advised not to wash their scalp. Patients and controls applying topical antifungals and/or steroid to the scalp preceding a month of sampling were not enrolled. The scalp hair were categorized as either oily or dry on visual observation. Hair fall of the patients was recorded as mild, moderate and excess on the basis of self assessment of the patients. In addition, use of cleansing agents (bath soap or shampoo), applying/ not applying oil to the scalp, type of oil applied and its frequency were recorded. Dandruff severity was graded as mild, moderate and severe on the basis of size, colour and surface of the flakes or scales. The severity score was defined as mild (score up to 24), moderate (25-60) and severe (>60) dandruff[6,7].

Samples were collected from relatively severely affected areas of the patients. Flakes or scales were collected by partitioning the hair with a sterile comb and scrapping approximately one inch area using blunt scalpel. In healthy individuals, the scrapings were collected from vertex and temporal regions. The samples were inoculated over the surface of modified Dixon's agar slants[8], transferred to the laboratory on the same day and incubated at 30^0C in a humid chamber up to four weeks. Colonies appearing like *Malassezia* were counted depending on the growth rate. *Malassezia* species isolated from southern region (Kasturba Medical College, Manipal) were transferred to PGIMER, Chandigarh for identification. At PGIMER all the isolates were identified on the basis of macroscopic and microscopic features (Fig. I), biochemical, and physiological tests[8,9].

The identity of the isolates were further confirmed by PCR-RFLP (restriction fragment length polymorphisms) of ITS2 (internal transcribed spacer 2) region rDNA gene and DNA sequencing of 26s region of rDNA gene[10,11]. Standard strains of *M. furfur* (Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India, MTCC-1374), *M. restricta* (The Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, CBS-7877), *M. obtusa* (CBS–7876), *M. globosa* (CBS-7966), *M. pachydermatis* (CBS–1879), *M. slooffiae* (CBS-7956), *M. sympodialis* (CBS-7222), *M. japonica* (CBS–9348), *M. yamatoensis* (CBS-9725) were used as controls. Genomic DNA isolation was performed by microwave irradiation technique[12]. Briefly, a colony of *Malassezia* yeast was transferred to a PCR tube and warmed three times for one minute each in a microwave. The supernatant was used as template for PCR analysis. For PCR- RFLP, amplification was done using the

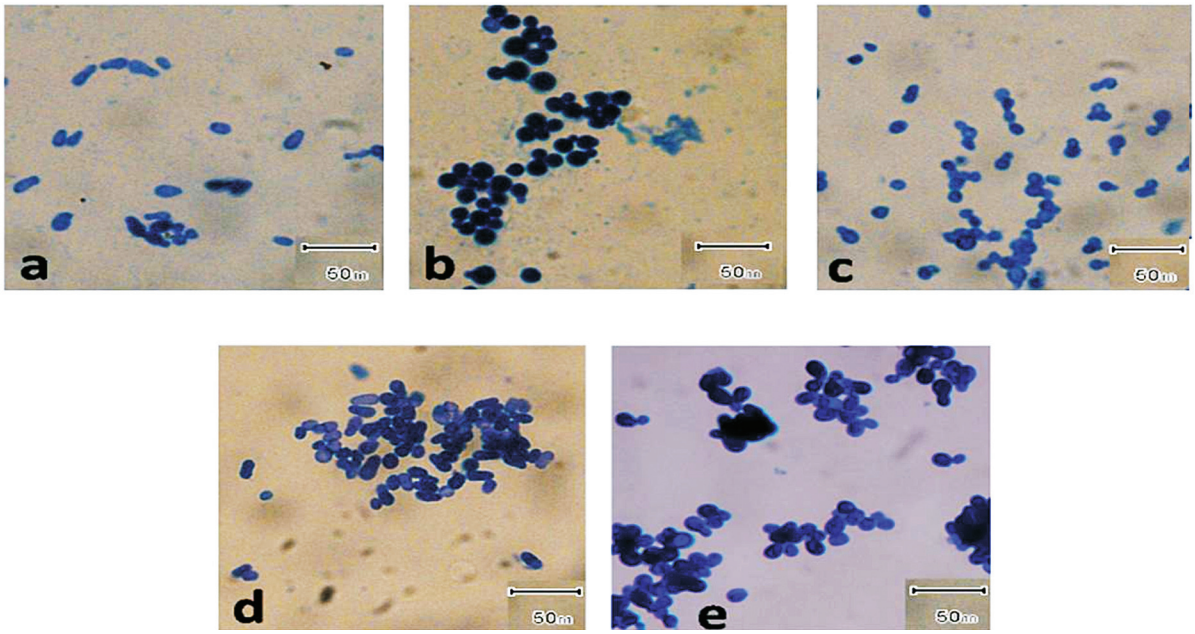

Fig. 1. Photomicrographs of different *Malassezia* species stained by methylene blue. **(a)** *M. furfur*; **(b)** *M. globosa*; **(c)** *M. restricta*; **(d)** *M. slooffiae*; and **(e)** *M. sympodialis*. Magnification 1000X.

primers ITS3 (5' GCATCGATGAAGAACGCAGC 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (Sigma, Banglore). Enzymatic digestion was carried out using three restriction enzymes *viz*. Alu1, Ban1 and MspA1 (New England Biolabs, Ipswich, USA). The amplicons and enzyme mixture was incubated at 37^0 C for three hours. For each digestion reaction 12.5 µl of PCR product, 1.5 µl buffers and 1µl of corresponding enzyme were added. For each species, three separate reaction mixtures were made using three different restriction enzymes. The restriction patterns of clinical isolates were compared with band pattern of the standard strains of *Malassezia* (Table I and Fig. 2). Sequencing of the 26S rDNA was done by amplifying this region with universal primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5'-GGTCCGTGTTTCAAGACGG)[11]. Purification of amplified gene product was performed by gel extraction kit (QIAquick, Quiagen, Bangalore). The elute was used as the purified gene product for sequencing PCR.

Table I. Amplified product size (bp) and expected length of restriction fragments (bp) obtained after digesting with different restriction enzymes

Malassezia spp	Amplicon size (bp)	AluI	BanI	MspAI
M. furfur	548	308, 240	391, 157	527, 21
M. obtuse	545	NRS	397, 168	NRS
M. pachydermatis	530	414, 116	NRS	501, 29
M. japonica	526	393, 133	182, 199, 145	497, 29
M. slooffiae	504	390, 114	NRS	477, 27
M. globosa	470	221, 16, 240	NRS	447, 30
M. restricta	450	NRS	187, 273	433, 27
M. sympodialis	425	NRS	NRS	287, 109, 29

NRS, non restriction site
M. yamotoensis did not yield any digested fragments by any of the enzymes used

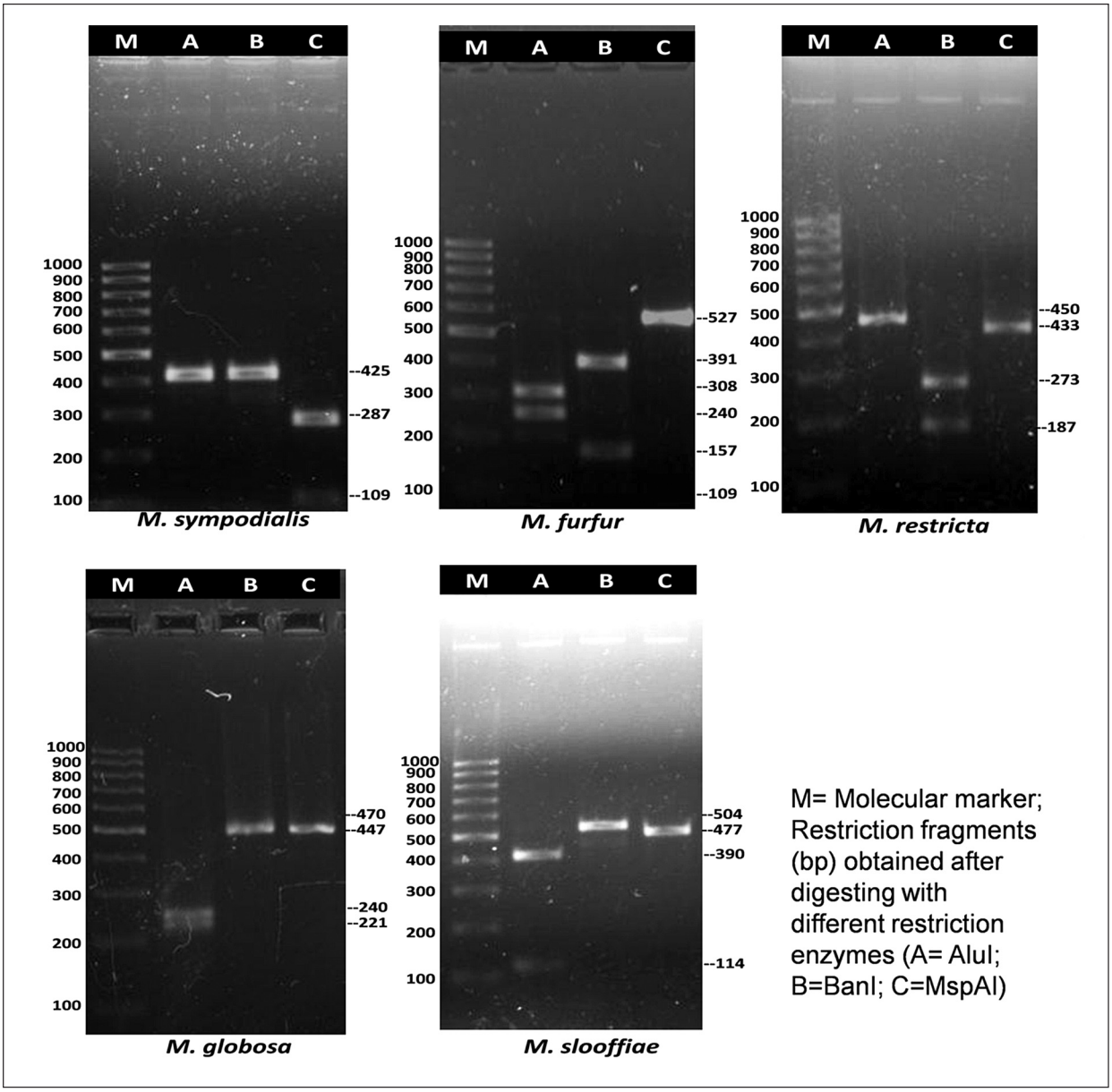

Fig. 2. Agrose gel picture of PCR-RFLP profiles of few *Malassezia* species after digestion with restriction enzymes- AluI, BanI, MspAI.

Sequencing PCR was performed for both the strands using above mentioned primers and Big Dye Terminator Cycle Sequencing Kit, Version 3.1 (Applied Biosystems, USA). All the sequencing reactions were purified and analyzed on ABI 3130 Genetic Analyzer (Applied Biosystems, USA). For each isolate, the consensus sequences were prepared from the sequence obtained by forward and reverse primers using Bionumerics software (version 6.5, Applied Maths, Ghent, Belgium). The consensus sequences were compared with the sequences of the GenBank DNA database (*http://www.ncbi.nlm.nih.gov/Genbank/index.html*) and CBS-KNAW fungal biodiversity centre database (*http://www.cbs.knaw.nl/*).

Statistic analysis: Comparison of *Malassezia* spp. distribution based on severity, hair type and hair fall in different groups (mild, moderate and severe) of dandruff patients was done by Chi-squared test (Software- Epi Info™ 7.1.0.6 and OpenEpi version 2.3, Rolin School of Public Health, Emory University, Antlanta, Georgia, *www.Openepi.com*).

Results

Age of the patients varied between 3 and 50 years. Forty per cent of the patients were in the age range of 10 and 19 years and 34 per cent were between 20 and 29 years. Females were 61 per cent and males 39 per cent. *Malassezia* culture positivity rate was significantly higher in patients from southern India, compared to northern India (47 cases vs. 37 cases; $P<0.01$). *M. furfur* and *M. restricta* were isolated in significantly higher number ($P<0.05$) from patients of southern part of India as compared to patients from northern part of India (Table II). Of the 20 healthy individuals, *Malassezia* species could be isolated from 6 (30%) individuals from southern India, of whom 5 were *M. furfur* and one was *M. slooffiae*. None of the samples collected from the healthy individuals of northern India yielded *Malassezia*. The average number of colonies isolated from mild, moderate and severe dandruff cases from northern part of India was 4.5, 10.6 and 14.4 cfu, respectively, whereas from healthy individuals, mild and moderate dandruff cases of southern India was 6, 24 (samples from 5 patients had confluent growth and the colonies could not be counted) and 24 cfu (samples from 12 patients showed confluent growth), respectively. Isolation of *Malassezia* spp. among dandruff patients and healthy individuals using different cleansing agent and application of oil is given in Table III.

In northern India, *M. globosa* and *M. restricta* were isolated from all the age groups studied. In patients of southern India, *M. furfur*, *M. globosa* and *M. restricta* were almost equally distributed among all the age groups. Although statistically insignificant, in population of northern India, *M. restricta* was associated more in males (48%) and *M. globosa* in females (43%). In patients from southern India, *M. furfur* was predominantly isolated from male patients (60%) and mixture of *M. globosa* and *M. restricta* from females (32%). *M. restricta* was significantly isolated from majority (44.5%) of the patients having oily hair. The details of distribution of *Malassezia* spp. according to geographical location, hair fall and severity are given in Table II.

All the isolates were identified by the band pattern obtained after PCR-RFLP of ITS2 region and 37 of them were subjected to DNA sequencing. Representative sequences of the isolates are deposited in the GenBank database with the accession numbers JN651930 to JN651965 and JN642245.The isolates have been deposited at the National Culture Collection of Pathogenic Fungi, PGIMER, Chandigarh, India.

Discussion

In the present study, two geographically different locations were studied. Geographically, northern and southern regions of India are distinct in terms of environmental conditions. *M. restricta* and *M. globosa* were the two most prevalent species isolated from dandruff patients of northern India having moderate to severe lesions while *M. furfur*, *M. restricta* and *M. globosa* were either isolated singly or in mixture from patients from southern India. In Japan and USA, *M. globosa* is most frequently isolated from dandruff cases followed by *M. restricta*[13]. Whereas *M. furfur* and *M. slooffiae* are isolated from scalp of healthy individuals and are considered as commensal species. In the present study, the sample collection

Table II. Distribution of *Malassezia* spp. among dandruff patients according to geographical location, hair fall and severity

Malassezia species	Culture positive		Hair fall				Severity of dandruff			
	Northern India (37)	Southern India (47)	Northern India		Southern India		Northern India		Southern India	
			Mild (30)	Moderate to excess (20)	Mild (29)	Moderate to excess (21)	Mild (13)	Moderate to severe (37)	Mild (22)	Moderate (28)
M. furfur	3	28*	1	2	12	4	1	2	3	13*
M. globosa	14	18	10	4	1	4	3	11	3	2
M. restricta	15	26*	8	7	1	-	1	14*	-	1
M. slooffiae	2	-	1	1	-	-	-	2	-	-
M. sympodialis	3	-	2	1	-	-	1	2	-	-
M. furfur + *M. restricta*	-	-	-	-	8	4	-	-	5	7
M. globosa + *M. restricta*	-	-	-	-	4	9*	-	-	9	4*

*$P \leq 0.05$. For statistical analysis, moderate and excess/severe category were combined and compared with mild category

Table III. Isolation of *Malassezia* spp. among dandruff patients and healthy individuals

	Dandruff (n=100)	Healthy Individuals (n=20)
Malassezia isolated	84* (84)	6 (30)
Cleansing agent		
(i) Soap	62 (62)	14 (70)
(ii) Shampoo with active ingredients	13 (13)	1 (5)
(iii) Shampoo without active ingredients	25 (25)	5 (25)
Oil usage		
Northern India (n=50)		
(i) Mustard	35 (70)	5 (50)
(ii) No oil	15 (30)	5 (50)
Southern India (n=50)		
(i) Coconut	45 (90)	9 (90)
(ii) No oil	5 (10)	1 (10)

Values in parentheses are percentages; *$P<0.05$ compared to healthy individuals

was carried out during the winter season (usually dry) from northern part of India and in summer from coastal region of southern India, which is humid (~ 65-90%) all through the year. According to Pierard-Franchimont *et al*[14] the severity of dandruff may fluctuate with seasons and often worsens in winter. High humidity may be the reason for occurrence of less severe cases from coastal part of southern India as compared to dry weather during winter in northern part of India. In addition, application of coconut oil in majority of the southern India patients (90%) might have prevented severe cases.

The isolation rate of *Malassezia* spp. is known to be highest in the age group of around twenty years when sebaceous glands activity is maximum[15]. Similar to the findings of Lee *et al*[16], in the present study no significant age dependent variation of *Malassezia* species was noticed. This could be due to the relatively small number of the participants in our study.

Recovery of *Malassezia* species in culture usually depends on the culture medium and techniques used for its isolation. In a culture dependant study from Canada, *Malassezia* recovery rate was 82 per cent[17]. Similar studies from Sweden and Japan showed far less recovery rate. *M .restricta* was not isolated from any of these studies[18,19]. Tajima *et al*[20] by using culture independent molecular technique showed that *M. globosa* and *M. restricta* were predominantly associated both with lesional and non-lesional area of Japanese SD/D patients. In the present study, isolation rate of *Malassezia* spp. was 74 per cent in northern India and 94 per cent from southern India patients.

Though association of dandruff is related to the species of *Malassezia* implicated, presence of this yeast in high number is also related in causation of dandruff. In a culture dependant study, Gupta *et al*[17] showed higher number of *Malassezia* colony at non-lesional site than on the lesional site. But, in DNA based study *Malassezia* found at lesional site was higher in density than on non-lesional site[20]. In a few reports the quantity of yeasts was not found to be correlate positively with the severity of the disease[4,21]. In the present study, the higher colony count positively associated with the severity of dandruff. It is also important to note that accurate quantification of *Malassezia* on the scalp is difficult to perform as the *Malassezia* resides along with sebum at infandibulum of the hair follicle[22,23]. Hence possibly culture based technique is better than DNA based non-culture technique, as in the later only the surface of the skin is sampled without including hair infundibulum. Another disadvantage of DNA based non-culture technique is that the DNA from both viable and nonviable cells are amplified.

In our study, *M. restricta* was recovered from individuals having any type of hair (oily and dry). Severe hair loss was associated more with *M. restricta* and *M. globosa* than other *Malassezia* species, but the difference was not significant. This finding was in agreement with the study of Nematian *et al*[24] where increased hair loss was shown to be associated with *Pityrosporum ovale (M. globosa)*. *Malassezia* may cause hair loss by utilizing the lipids present in different strata of epidermis and dermis leading to weakening of hair root and hair fall[25,26].

The major difficulty while conducting epidemiological studies on *Malassezia* associated disease is its isolation due to slow growing nature of this agent. A few species like *M. globosa, M. restricta* and *M. obtusa* take longer than a month to grow in sufficient amount to extract the DNA by phenol-chloroform extraction technique. In the present study, we used the rapid method of template preparation from the *Malassezia* colonies using microwave irradiation[12]. This technique is rapid, reliable, cost-effective, and simple. This technique could also be applied to amplify the DNA from a single isolated colony during the co-existence of multiple *Malassezia* species in a given culture.

In conclusion, *M. globosa* and *M. restricta* seemed to be the predominant species causing dandruff in Indian population and high density of these species on the scalp was related with the severity of dandruff. Small sample size was a major limitation of the present study. Such studies need to be done at various geographic locations in India with large carefully drawn sample.

Acknowledgment

Authors acknowledge the Indian Council of Medical Research, New Delhi, India for financial assistance. Authors also acknowledge Dr P.V.M. Lakshmi, Department of Community Medicine, PGIMER, Chandigarh and Shri Kapil Mukesh for their help in analysis and sample collection respectively.

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