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Nannizzia graeserae sp. nov., a new dermatophyte of geophilic clade isolated from vicinity of a barbershop in India

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ABSTRACT

As part of keratinophilic fungal diversity project in Maharashtra state of India, an unusual dermatophyte was isolated by hair baiting technique from soil collected from the vicinity of a village barbershop in Buldana district of Maharashtra. Sequence analysis of the internal transcribed spacer (ITS) and 28S rRNA region of new isolate suggested that it belong to a new species of the recently circumscribed genus *Nannizzia*, forming unusual cylindrical to clavate macroconidia. Sequence similarity search of its ITS region in the CBS dermatophyte database showed a maximum similarity of 91% with *Microsporum* sp. (*A. corniculatum*)-CBS 365.81 and 90.4% with *M. persicolor* (CBS 871.70). Phylogenetically the new species is close to *N. praecox* and *N. persicolor*, forming a monophyletic clade with them in the ITS tree. The present communication describes this new species (*N. graeserae* sp. nov.) based on morphology and sequence divergence in the ITS region. The ITS sequence alignment of dermatophyte species that produce rough walled macroconidia showed certain base positions which have genus-specific nucleotide changes.

Keywords: *Arthrodermataceae*, dermatophyte, ITS, macroconidia, *Microsporum*, *Nannizzia graeserae*, phylogeny.

INTRODUCTION

Dermatophytes are a specialized group of keratin degrading fungi belonging to family *Arthrodermataceae* of the order *Onygenales* (de Hoog *et al*., 2000). Based on their environmental associations they are considered as geophilic (soil associated), zoophilic (animal associated) and anthropophilic (human associated). A strictly geophilic grouping is phylogenetically supported as separate from the lineage containing the zoophilic and anthropophilic species (Gräser *et al*., 2008). Several novel species of dermatophytes have been discovered in recent times mainly through elucidation of sequence variation in the ITS region of rDNA *viz*., *Microsporum aenigmaticum* (current name *Nannizzia aenigmaticum*) Hubka *et al*., 2014), *Microsporum mirabile* (current name *Paraphyton mirabile*) (Choi *et al*., 2012), *Trichophyton eboreum* (current name *Arthroderma eboreum*) (Brasch and Gräser, 2005) and *Trichophyton onychocola* (current name *Arthroderma onychocola*) (Hubka *et al*., 2014).

The internal transcribed spacer (ITS) region is not always sufficient to discriminate among species in some groups of fungi (e. g., *Aspergillus* and *Penicillium*) and in such cases other protein coding genes are required for resolving their phylogeny (Sharma *et al*., 2015a). But as far as dermatophytes are concerned, the phylogeny derived from ITS sequences corresponds well with that obtained from other loci like beta-tubulin (Rezaei-Matekhkolaei *et al*., 2014) and TEF1α (Mirhendi *et al*., 2015). This supports the idea that ITS is the preferred taxonomic marker for dermatophytes. Moreover, the number of ITS sequences from type strains available in database surpasses those representing all other loci. Until recently, all the dermatophytes were classified into the three genera *Epidermophyton*, *Microsporum* and *Trichophyton*. As multilocus phylogenies showed substantial genetic distances among subclades within *Microsporum*, and also showed that *Trichophyton* was polyphyletic, these genera were split into seven, viz., *Arthroderma*, *Epidermophyton*, *Lophophyton*, *Microsporum*, *Nannizzia*, *Paraphyton* and *Trichophyton* (de Hoog *et al*., 2016).

There are about 55 species recognized so far in the seven dermatophyte-related genera (**Fig. 1**). In this new taxonomic scheme (de Hoog *et al*., 2016), distinguishing genera on morphological grounds alone is not possible, due to similar morphology and overlapping characters. At the species level, however, distinction based on morphology can still be done. Also, with the new provisions for naming of fungi laid out by the Melbourne Code (McNeill and Turland, 2011), which accepts only one name for one fungus irrespective of the life cycle stage (asexual or sexual) it is seen in, asexual species can be placed in genera originally defined as sexual (genera typified by sexual morphs) and sexual species can be placed in genera originally based on names of asexual forms. Thus, genera like *Arthroderma* and *Nannizzia*, which were earlier conceived as containing only species forming the sexual morph now contain species that are strictly asexual, such as *Arthroderma phaseoliforme* and *Nannizzia praecox*. The reverse is also true, as seen in the inclusion of the potentially sexual *Microsporum canis* and *Trichophyton simii* in genera once based on asexual forms.

Recent studies on geophilic keratinophilic fungi have yielded several novel forms from India (Sharma *et al*., 2013; Sharma *et al*., 2015b; Sharma and Singh, 2013). During a recent survey of soil in Maharashtra state of India, an unusual dermatophyte was isolated which produced conventional *Microsporum*-like (rough-walled) macroconidia. Detailed morphological study and sequencing of ITS and 28S rDNA regions show that it represents a new species of recently circumscribed genus *Nannizzia*. The present communication describes this new dermatophyte based on morphological study and molecular phylogeny.

MATERIAL AND METHODS

Collection of soil and fungal isolation: Soil samples were collected from the vicinity of an open barbershop in the Buldhana district of Maharashtra (India). Samples were placed, using sterile spatula, into polythene bags that were sealed and brought to the laboratory. The samples were stored at room temperature prior to processing. The hair baiting technique was used to isolate keratinophilic fungi from soil (Vanbreuseghem, 1952; Sharma and Rajak, 2003).

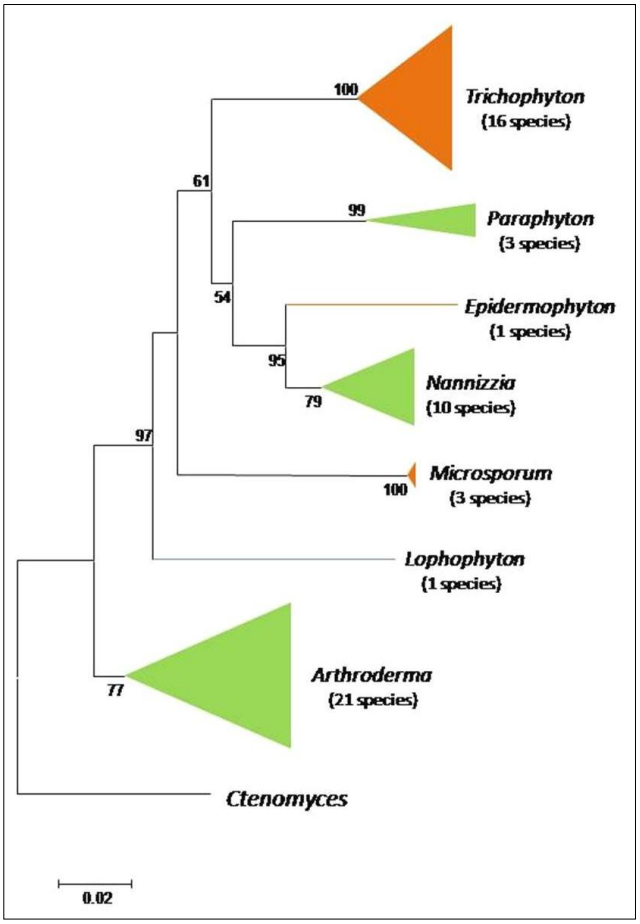

Fig. 1. ITS sequence based phylogenetic tree showing various dermatophytic genera and the number of species known so far within each genus. The dataset comprised a total of 53 ITS sequences belonging to 52 species of 7 dermatophyte genera. The tree was compressed at nodes to represent genera. The phylogenetic tree was constructed in MEGA v. 5.05 using Neighbour-Joining method and Kimura 2 parameter model with bootstrap analysis of 1000 replicates. *Ctenomyces serratus* was used as outgroup. Orange color of clade represent genera with anthropophilic species and green ones represent genera with mostly geophilic species. (After de Hoog *et al.*, 2016)

Approximately 10-15 g of soil was spread onto sterile Petri dishes (Hi-Media, Mumbai, India), moistened with sterile distilled water. Sterile, defatted human hairs were spread onto the moistened soil and incubated at room temperature for 2-3 weeks. Any visible fungal growth on hairs was transferred onto Sabouraud Dextrose Agar (SDA) medium (Hi-Media) using fine needle under a stereomicroscope by direct transfer or using microdilution drop-trail method (Sharma *et al.*, 2002). The ex-type culture of the new species was submitted to the Microbial Culture Collection (MCC), National Centre for Cell Science, Pune, India and Centraal Bureau voor Schimmelcultures (CBS), Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

Morphological Study: Morphology of the fungus was studied on Sabouraud dextrose agar (SDA), Potato dextrose agar (PCA) and Oatmeal agar (OA). Micromorphological features were studied under an Olympus BX53 light microscope (Olympus, Tokyo, Japan) by mounting the fungal material in lactophenol with or without cotton blue stain. Micromorphological measurements were carried out in water mounts. Photomicrographs were taken with a ProgRes C5 camera attached to the microscope (Jenoptik, Jena, Germany). For scanning electron microscopy (SEM) fungal material was directly mounted onto stubs with the help of double sided adhesive tape and coated with palladium using JEOL-JFC 1600 minor coater. Preparations were visualized under a JEOL JSM 6360A microscope (JEOL, Tokyo, Japan) at 10KV accelerating voltage and at varying magnifications. SEM was done at the Department of Physics, S.P. Pune University, Pune.

DNA extraction and PCR amplification: Genomic DNA was extracted using the method of Edward *et al*. (1991) with slight modifications. Briefly, 50-100 mg of mycelia from a one-week-old colony was taken in an Eppendorf microtube containing 200 µL of Edward's buffer (200 mM-Tris, pH8.0; 200 mM-NaCl, 25 mM-EDTA, 0.5%-SDS) and manually crushed for 5 min with a plastic pestle. The mycelia were crushed further for 5 min after addition of 300 µL of Edward's buffer. The volume was adjusted to 1000 µL by addition of 500 µL of Edward's buffer. The crushed mycelia were vortexed for 15 sec and incubated at 100°C for 10 min and then centrifuged for 10 min at 2000 rpm. Five hundred microlitres of supernatant were transferred to a fresh microtube and centrifuged again at 2000 rpm for 10 min. Four hundred microlitres of supernatant were transferred to a new microtube and 400 µL of ice-cold absolute ethanol were added. The sample was inverted gently several times and incubated at room temperature for 10 min. Thereafter it was centrifuged at 14,000 rpm for 10 min. The pellet was washed with 70% ethanol and air-dried. The pellet was suspended in 100 µL of TE buffer. RNA was removed by addition of 1 µL of RNase enzyme (10mg/mL) followed by incubation at 37°C for 1 hr. The DNA was quantified with the help of NanoDrop spectrophotometer (Thermo Scientific, USA). For PCR, the genomic DNA was diluted to 10ng/ µL and 2.5 µL was used as template per reaction. PCR was performed in 25 µL reaction volume containing 15 µL H_2O, 2.5 µL 10X PCR buffer, 1 µL dNTP mix, 1.5 µL $MgCl_2$, 1 µL of each primer, 0.5 µL Taq polymerase, and 2.5 µL of template DNA (10ng/ µL). For the ITS region, primer pairs ITS1 and ITS4 were used, while for LSU, the amplification primer pairs LROR and LR5 were used. PCR was performed in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Singapore) with the following conditions: 94°C-5min, (94°C for 1min, 55°C for 1min, 72°C for 1 min) repeated 30 times, followed by 72°C for 7 min. For LSU the PCR conditions were same as for ITS except that annealing was at 52°C for 30 sec. The PCR product was checked on a 1.2% agarose gel and was stained with ethidium bromide to allow photography under UV light.

Sequencing and phylogenetic analysis: Sequencing of the ITS and LSU regions was carried out with same primers that were used in amplification. Sequencing was done on a 3130 Genetic Analyzer (Applied Biosystems, CA, USA) at geneOmbio Technologies Pvt. Ltd., Pune. The sequences

obtained in AB1 files were manually checked for inconsistencies using Chromas Lite version 2.1.1 software (Technelysium Pvt. Ltd., South Brisbane, Queensland, Australia). Sequence alignment was done by CLUSTAL W, while analysis to construct phylogenetic trees was performed in MEGA 5 (Tamura *et al*., 2011) by the Neighbour Joining method (NJ) and the Kimura 2 parameter distance model (Kimura, 1980). Testing of phylogeny was done by bootstrap analysis of 1000 replicates.

RESULTS

Soil sample S326 collected from a barbershop in Buldana district yielded an interesting dermatophyte with unique macroconidial morphology. The rough walled, clavate to cylindrical macroconidia did not correspond to those of any known species of dermatophyte. The fungus was tentatively identified as a species of *Microsporum* based on its rough walled macroconidia. A comparison of shape and size of macroconidia was done among all known species that form rough walled macroconidia (**Table 1**). Sequencing of the LSU region of MS326 resulted in an 806bp long segment (GenBank KY290501). A nucleotide BLAST search (NCBI) with the LSU sequence of MS326 indicated that a maximal similarity of 99% lay with *Microsporum persicolor* (CBS 139323, KP636537.1), *M. incurvatum* (CBS 174.64, AY176738.1), *M. gypseum* (CBS 118891, XR001951138.1) and few other species of *Arthrodermataceae*. A NJ phylogenetic tree constructed using eight LSU sequences belonging to four genera (*Nannizzia*, *Paraphyton*, *Lophophyton* and *Microsporum*) positioned the new species in the *Nannizzia* clade.

Sequencing of the ITS region of MS326 resulted in a 597 bp-long segment (GenBank-KY290502) comprising 244 bp of ITS1, 151bp of the 5.8S, and a 200bp portion of the ITS2. A BLASTn search with ITS sequence showed a maximal identity of 94% with *Nannizzia persicolor* (IHEM 3450; KP132834.1; 96% query coverage; CBS 139323; KP636536.1; 96% query coverage). At lower similarity were *Nannizzia duboisii* (CBS 349.49; NR077141.1; 96% query coverage), with 93% identity, and several other species of *Nannizzia*. Pairwise sequence comparison of the ITS for MS326 with sequences in the CBS dermatophyte database showed maximum similarity of 91% with *Nannizzia corniculata* (CBS 365.81 & CBS 364.81), followed by 90% with *Nannizzia gypsea* (CBS 170.64) and *Nannizzia persicolor* (CBS 871.70), 89.1% with *Nannizzia duboisii* (CBS 349.49) and 88.2% with *Nannizzia praecox* (CBS 673.89). For clarity, new names as per de Hoog *et al*. (2016) have been used here instead of the ones in GenBank. The ITS sequence of *N. graeserae* differs from that of its closest neighbour, *N. praecox*, at 45 positions (15 substitutions and 30 indels) out of 569 aligned sites. It differs from *N. persicolor* at 50 positions (32 substitutions and 18 indels) out of 602 aligned sites. The next nearest neighbour *N. corniculata* differs at 53 positions (40 substitutions and 13 indels) out of 594 aligned sites. The remaining species of *Nannizzia* show greater numbers of differences in the ITS region.

An NJ tree constructed using reference ITS sequences from GenBank placed *Nannizzia graeserae* in a distinct clade along with *N. praecox* and *N. persicolor* (**Fig. 2**).

Table 1: Comparative morphology of macroconidia* of different species of dermatophytes (previously belonging to genus *Microsporum/Arthroderma*) which form rough-walled macroconidia (Grey shaded portion within a column shows similarity with *N. Graeserae*)

S. No.	Species Name**	Wall	Shape	Size (in µm)		Septation
				Length	Width	
1	*Nannizzia aenigmaticum****	-	-	-	-	-
2	*N. duboisii*	Thick	Ovoid	18-34	16-18	3 (mostly)
3	*N. corniculata*	Thin	Cigar shaped	23-54	4.6-9	1-4(8)
4	*N. fulva*	Thin/ Thick	Broadly fusiform to clavate	25-60	7-12	(4-)5-6(-7)
5	*N. gypsea*	Thin	Fusiform	25-60	8.5-15	3-5(-7)
6	*N. incurvata*	Thin	Ellipsoid to fusiform	25-58	8.5-15	Up to 5
7	*N. nana*	Thin	Obovoidal to pyriform	12-18	5-7.5	1
8	*N. persicolor*	Thin	Cigar- shaped	40-60	4-8	3-6
9	*N. praecox*	Thin	Lanceolate with narrow apex	Up to 65	9	5-8
10	*N. graeserae*	Thin	Club-shaped	26-65	10-15	(2-)3-4(-5)

* compiled from de Hoog *et al*., 2000 ;Currah, 1985; Takshio and de Vroey, 1982.
** *N-Nannizzia*.
*** This species does not produce conidia.

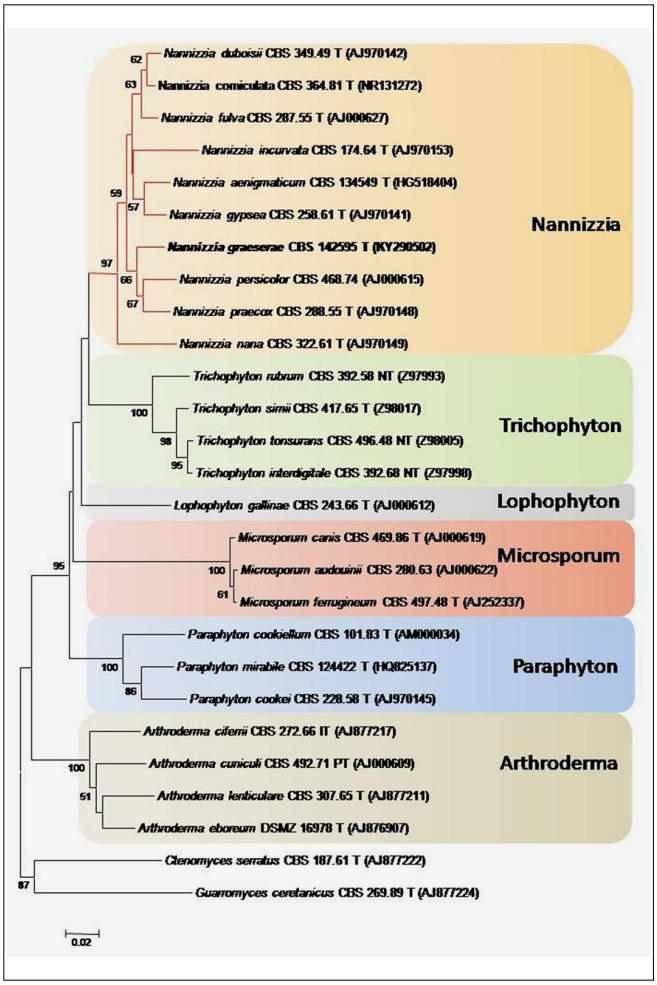

Fig. 2. A Neighbour Joining tree constructed using ITS sequences of all known species of *Lophophyton*, *Microsporum*, *Nannizzia* and *Paraphyton* along with representative species of genus *Arthroderma* and *Trichophyton*. The new species is positioned close to *N. praecox* and *N. persicolor* in a distinct clade. *Ctenomyces serratus* and *Guarromyces ceretanicus* were used as outgroup. Designations T, NT and PT after strain ID represent sequence from type, neotype and paratype strains, respectively.

TAXONOMY

Nannizzia graeserae Rahul Sharma & Shouche **sp. nov.** **Figs. 3-5**

Mycobank Mb819778

Diagnosis: The presence of clavate to cylindrical, rough-walled, 3-4-septate macroconidia measuring 26-65x10-15 µm in size,which are broader at the apex and narrower at the base, distinguish this species from the other related species.

Etymology: The species is named in the honor of Prof. Yvonne Gräser (Humboldt University, Berlin) for her contribution to the molecular phylogeny of family *Arthrodermataceae*, especially the dermatophytes.

Description: Colonies fast growing at 28°C on SDA (5 cm/12 d), evenly circular in outline with radial ridges at centre, initially white turning to pale tan, with dense leathery growth, reverse pale yellow; on PCA (6 cm/12d), circular with sparse growth on surface, white, turning pale tan with age; on OA (4.5 cm/12 d) circular with sparse growth on surface, white to pale tan at centre, reverse uncoloured (**Fig. 3 A-C**). Growth on SDA at 37°C, 3.7 cm/12 d. Vegetative hyphae thin walled, hyaline, 2-4 µm wide. Thallic macroconidia abundant, borne directly on hyphae or on short conidiophores, hyaline to pale brown, rough-walled clavate to cylindrical, mostly broader at apex and narrower at base, rounded at apex, 26-65x10-15 µm, mostly 3-4 septate. Microconidia rare, smooth walled, ovoid to cylindrical, 2.5-5x3-18 µm.

Sexual morph: Not seen.

Type: **INDIA**: Maharashtra: Deolgaon Raja, Buldana district, isol. ex soil sample from barber's shop, 2 June 2016, *R. Sharma*, (**Holotype**-MCC H1005; CBS 142595=MS326=MCC 1311-cultures ex-type).

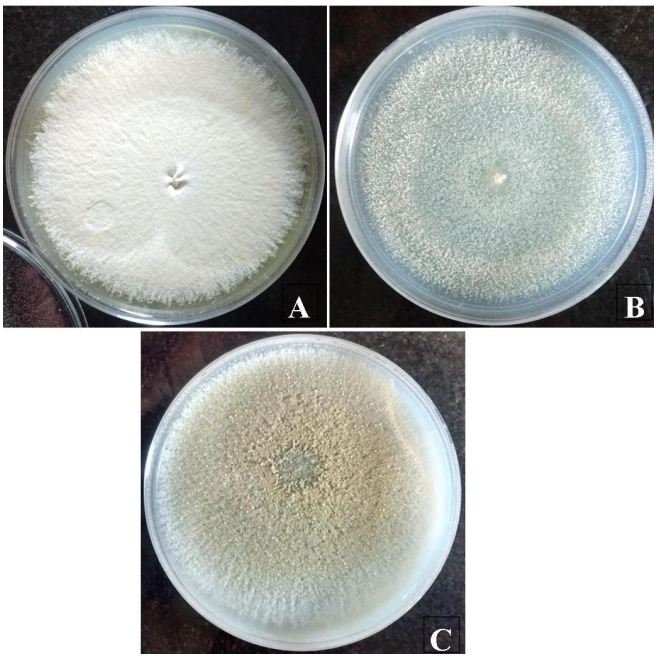

Fig. 3. Colony of *Nannizzia graeserae* (MCC 1311^T) on different media after 25 d of incubation. **A**. Sabouraud dextrose agar, **B.** Potato carrot agar, **C.** Oatmeal agar.

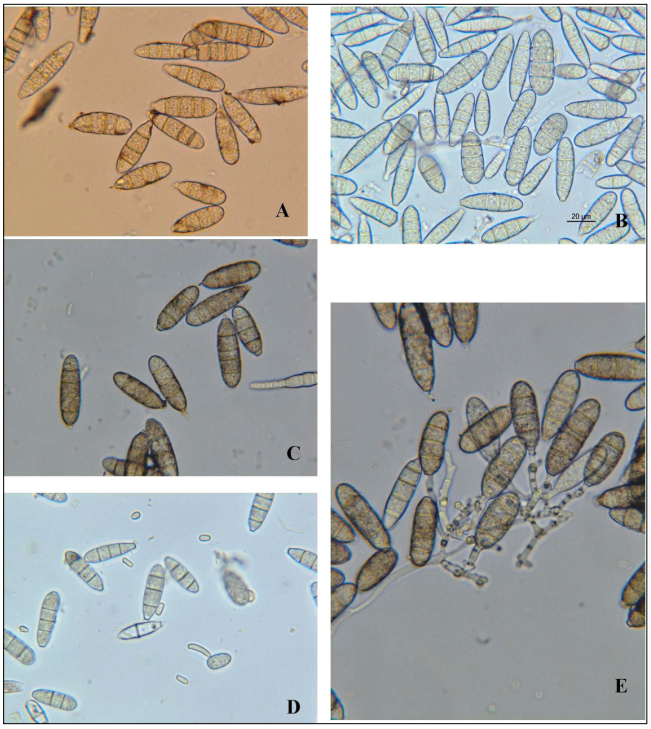

Fig. 4. *Nannizzia graeserae* (MCC 1311^T)- Macroconidia formed on different media, **A**. Oatmeal Agar, **B**. Sabouraud dextrose agar, **C**,**D.** Potato carrot agar, **E.** Microconidia on SDA (arrow).

Substratum: Isolated on defatted human hair from soil.

Distribution: Known only from type locality, Buldhana, Maharashtra, India.

DISCUSSION

The genus *Microsporum* traditionally comprised of 14 species all of which formed rough walled macroconidia except *M. aenigmaticum* (which lacks sporulation and was identified via sequence analysis) (Hubka *et al*., 2014). **Table 2** lists the traditional *Microsporum* species and their new affiliations. As mentioned above, there is no clear-cut

Table 2: New affiliations to the traditional *Microsporum* species/*Arthroderma* asexual states (having rough-walled macroconidia) along with recently recognized new species*. (as per de Hoog et al., 2016)

S. No.	Genus (number of species)	New Name/ Valid name**	Old name
1	*Arthroderma* (1)	*Arthroderma amazonicum*	*Microsporum amazonicum /A. borellii*
2	*Lophophyton* (1)	*Lophophyton gallinae*	*M. gallinae*
3	*Nannizzia* (10)	*Nannizzia aenigmaticum*	*M. aenigmaticum*
4		*N. corniculata*	*Microsporum sp./A. corniculatum*
5		*N. duboisii*	*M. duboisii*
6		*N. fulva*	*M. fulvum/A. fulvum*
7		*N. graeserae*	-
8		*N. gypsea*	*M. gypseum/A. gypsea*
9		*N. incurvata*	*M. gypseum/A. incurvatum*
10		*N. nana*	*M. nanum/A. obtusum*
11		*N. persicolor*	*M. persicolor/A. persicolor*
12		*N. praecox*	*M. praecox*
13	*Microsporum* (3)	*Microsporum audouinii*	*M. audouinii*
14		*M. canis*	*M. canis/A. otae*
15		*M. ferrugineum*	*M. ferrugineum*
16	*Paraphyton* (3)	*Paraphyton cookei*	*M. cookei*
17		*P. cookiellum*	*Arthroderma cookiellum*
18		*P. mirabile*	*Microsporum mirabile*

* *Microsporum aenigmaticum*, *M. mirabile* and *Nannizzia graeserae*.
** Authority names are omitted here for clarity.

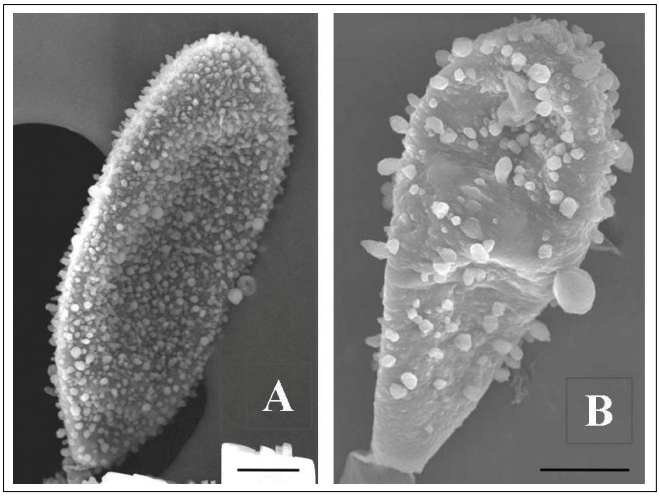

Fig. 5. *Nannizzia graeserae* (MCC 1311[T])- Scanning electron microscopy (SEM) of macroconidia showing rough-wall nature. **A.** Mature conidium, **B.** Young conidium. Scale bar-5µm.

morphological difference distinguishing these newly circumscribed genera; they can only be distinguished phylogenetically (**Fig. 6**). The genera *Microsporum*, *Nannizzia* and *Paraphyton* have unique apomorphies at otherwise conserved loci whereas *Lophophyton* and *Microsporum* have shared apomorphies at two loci, suggestive of common ancestor.

The new species *N. graeserae* differs from its closest neighbours *N. persicolor* and *N. praecox* by more than 7% in the ITS region, while other species are even more distant. Among the dermatophytes, sequence divergence in the ITS region generally corresponds well with species delimitation. Just some anthropophilic *Microsporum* and *Trichophyton* species show a high degree of ITS sequence similarity (99%); the remaining dermatophytes and their relatives have at least a 3% difference in the ITS region.

The morphology of the macroconidia (**Fig. 4 A-E**), an important character among the geophilic species is distinct in the new species. These conidia are cigar shaped in *N. persicolor*, while in *N. praecox* they are lanceolate and narrowed at the apex. **Table 1** contrasts the macroconidial morphologies of dermatophyte species forming rough walled macroconidia. It shows that the macroconidial shape of *N. graeserae* (**Fig. 5A-B**) is similar to that of *N. fulva* and *N. gallinae*, two relatively phylogenetically distant species. The

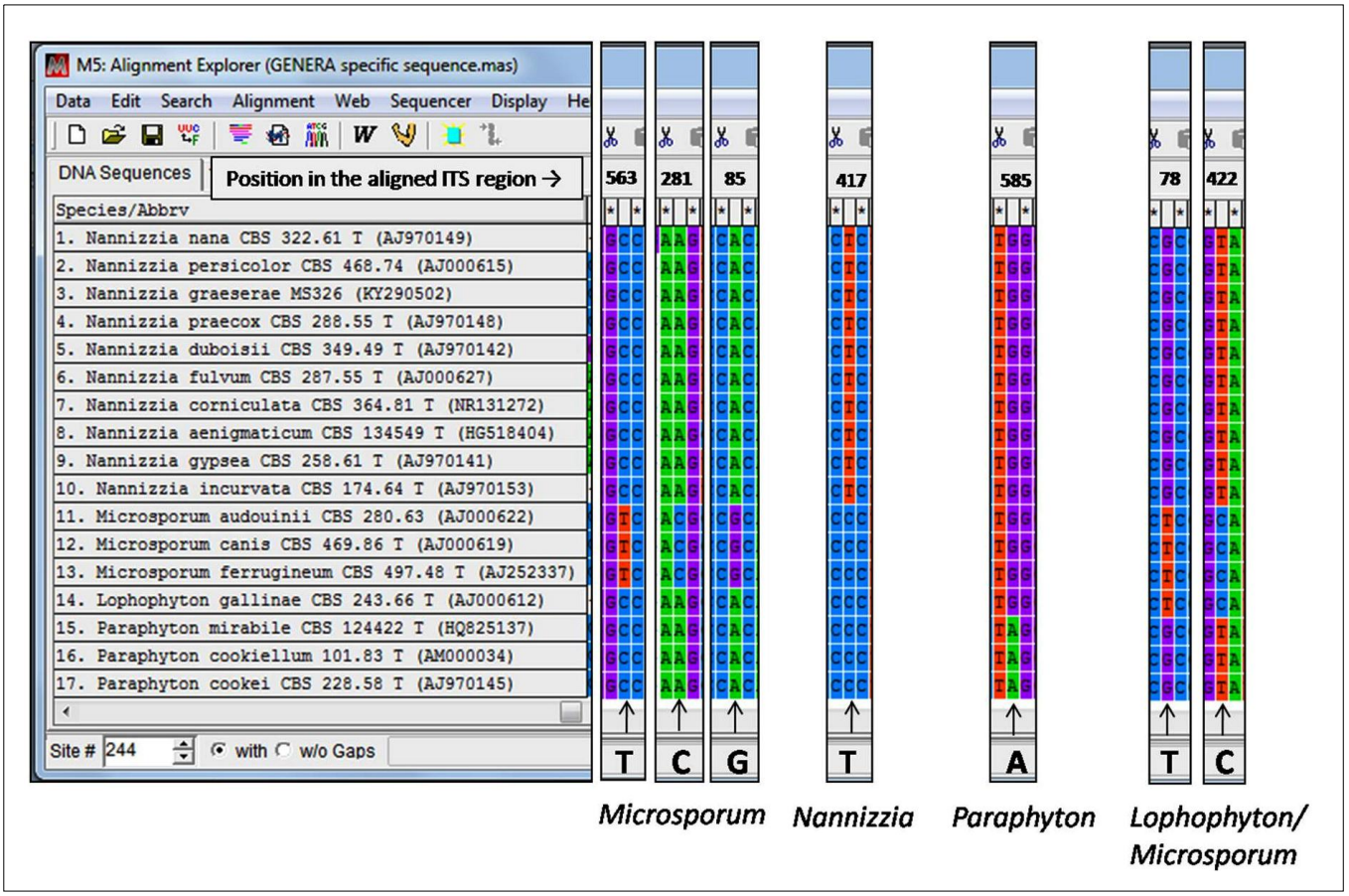

Fig. 6. Genus specific nucleotides changes at various loci in the ITS1-5.8S-ITS2 region of rRNA among dermatophyte genera producing rough walled macroconidia (except *Arthroderma amazonicum*). Only those nucleotide positions (loci) are considered which have conserved sites on both sides. Total aligned sites 646; ITS1-2 to 297; 5.8S-298 to 448; ITS2-449 to 646. All sequences are from type strains except for *M. audouinii* and *N. persicolor* for which sequences from authentic strains have been used. The alignment was carried out by CLUSTAL W using MEGA 5.0 software.

clavate to cylindrical macroconidia of *N. graeserae* are abundantly produced in all media tested. Microconidia are also produced but very sparsely (**Fig. 4 A-E**). An LSU phylogenetic tree places the new species in the *Nannizzia* clade. The ITS phylogeny shows *N. graeserae* in a clade with *N. persicolor* and *N. praecox*. *Nannizzia persicolor* is conventionally considered a zoophile due to its association with bank voles (English, 1966; 1967) mostly in UK, but an extensive study from soil in central India (Sharma *et* al., 2008) showed the geophilic nature of the species (evident from high degree of recombination among the populations studied as seen using microsatellite markers). The other species in the clade, *N. praecox*, is rare and was originally isolated from a human. Although the new species has been isolated from soil, its ability to grow well at 37°C suggests it may have potential to infect humans or animals. The new species seems to be rare as it was observed in only one sample out of more than 580 soil samples analyzed so far from 24 districts of Maharashtra (unpublished, Sharma and Shouche, 2016) as part of bigger project of studying keratinophilic fungal diversity (including dermatohytes) of Maharashtra.

Soil sample S326 also yielded in another *Nannizzia* species, *N. corniculata*, which was more abundant than *N. graeserae*; the latter species colonized only two hair strands while most of the hairs were colonized by the other *Nannizzia* species. We could detect *N. graeserae* in the hair baited plate due to its slightly different growth pattern on hair and distinct macroconidia.

Evolutionarily, the anthropophilic and zoophilic dermatophytes are derived from the ancestral geophilic *Arthroderma* clade (**Fig. 1**) which is most diverse in containing 38% of all known species of dermatophytes. The overall geophilic group (*Arthroderma*, *Nannizzia*, and *Paraphyton*) comprises the majority of (>60%) of dermatophyte species. In recent years, more new species have been described in the geophilic clades than in the anthropophilic or zoophilic clades. The latter clades groups are extensively investigated in connection with medical and veterinary cases while scanning of soils for dermatophytes is seldom carried out in systematic manner. De Hoog *et al*. (2016) suggested that numerous species are yet to be discovered among the geophilic and zoophilic group from under-sampled habitats. *N. graeserae* represents one such species which escaped discovery during a previous investigation of soil in the region (Deshmukh and Verekar, 2014). This is the first time that a new dermatophyte has ever been recorded from India.

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