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Isolation of keratinophilic fungi from selected soils of The Gir Forest National Park and Wildlife Sanctuary, Gujarat, (India)

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ABSTRACT

Eighty samples were collected from six different sites in the vicinity of **Gir Forest National Park** and screened for the presence of keratinophilic fungi using hair baiting technique for isolation. Seventy three isolates were recovered and identified. The cultures were identified using macro- and micro morphological features. Their identification was also confirmed by the BLAST search of sequences of the ITS1-5.8S-ITS2 rDNA region against the NCBI/Genbank data and compared with deposited sequences for identification purpose. Ten species of seven genera were isolated, viz. *Aphanoascus durus* (2.50 %), *Aphanoascus fulvuscence* (5.00 %), *Arthrographis kalrae* (2.50 %), *Auxarthron conjugatum* (1.25 %), *Chrysosporium indicum* (16.25 %), *Chrysosporium tropicum* (6.25 %), *Chrysosporium zonatum* (3.75 %), *Chrysosporium* state of *Ctenomyces serratus* (7.50 %), *Microsporium gypseum* (12.50 %), and *Trichophyton mentagrophytes* (3.75 %). This study indicates that the soils of **Gir Forest National Park** may be significant reservoirs of certain keratinophilic fungi.

Key words: India, keratinophilic fungi, Gir Forest National Park, soil fungi,

INTRODUCTION

The Gir Forest National Park and Wildlife Sanctuary is a forest and wildlife sanctuary in Gujarat, India. Established in 1965, with a total area of 1412 km² (about 258 km² for the fully protected area (the national park) and 1153 km² for the Sanctuary), the park is located 43 km in the north-east from Somnath, 65 km to the south-east of Junagadh and 60 km to south west of Amreli. It is the sole home of the Asiatic Lions (*Panthera leo persica*) and is considered to be one of the most important protected areas in Asia due to its supported species. Gir National Park and Sanctuary does not have a designated area for tourists, however, to reduce the tourism hazard to the wildlife and to promote nature education, an Interpretation Zone has been created at Devalia within the sanctuary. Within its chained fences, it covers all habitat types and wildlife of Gir with its feeding-cum-living cages for the carnivores and a double-gate entry system. Its unique location, tropical climate and geographic diversity make it a potentially interesting area to study the distribution of keratinophilic fungi. We therefore undertook this study and report the results obtained.

METHODOLOGY

Collection and processing of soil samples

Eighty samples were collected from 6 sites in the Gir Forest National Park and Wildlife Sanctuary in the month of November 2013 in sterile tightly closed polythene bags. These sites were Forest soil, Grass land soil, barren land, the raw road (pagdandi), near water reservoirs and resting areas of animals. The samples were collected from the superficial layer, depth not exceeding 35 cm, with a plastic spoon and transferred to sterile polythene bags, brought to the laboratory, stored at 15 °C and processed immediately. The hair bait technique of Vanbreuseghem, (1952) was used to isolate keratinophilic fungi. For this purpose, sterile Petri dishes were half filled with the soil samples and moistened with water and baited by burying sterile human hairs in the soil. These dishes were incubated at

room temperature (25 ±1 °C) and examined for fungal growth over a period of four weeks.

Isolation and identification of keratinophilic fungi

After observing the growth under a stereoscopic binocular microscope it was cultured on Sabouraud's dextrose agar supplemented with chloramphenicol (50 mg/l) and cycloheximide (500 mg/l). The slopes /plates were incubated at room temperature for five to ten days following which the cultures were microscopically checked for purity and subcultured to get pure cultures. These fungi were identified based on the various available monographs (Sigler and Carmichael, 1976; Oorschot, 1980; Currah, 1985; Arx, 1986; Cano and Guarro, 1990).

Molecular identification of keratinophilic fungi

Molecular characteristics of the cultures were studied by determination of their DNA sequences of the ITS1-5.8S-ITS2 region. Genomic DNA was extracted by the mini prep protocol of Lee and Taylor (1990). The ITS1-5.8S-ITS2 rDNA was amplified using ITS1 and ITS4 as the forward and reverse primers, respectively, as described by White *et al.* (1990). Amplification was performed in 100µL reaction volumes containing 10X buffer 10µl, MgCl₂ (25mM) 2µl, dNTP (10mM) 2µl, ITS1 primer (20pm) 2µl, ITS4 primer (20pm) 2µl, Taq Polymerase (2.5U) 1µl, DNA Sample (5µg/ml) 3µl, and Milli Q Water 78µl. The PCR reaction was carried out using a Thermal Cycler (M.J. research, PTC 200) with conditions as follows: denaturation for five minutes at 94 °C, 34 cycles of (30 sec at 94 °C, 30 sec at 55 °C, 1 min at 72 °C) extension for four minutes at 72 °C and storage at 4 °C. Negative controls were used in each set of reactions. The final products were analyzed by electrophoresis on 1.2% agarose (Sigma). The PCR products were purified using Qiagen Gel extraction kit (CAT No. 28704) and the PCR products of expected size were sequenced using ITS1 and ITS4 primers in an Applied Biosystem 3730 DNA analyzer at GenOmbiotech, Pune, India.

Table 1. Distribution of keratinophilic fungi from Gir Forest National Park soil samples

Source of soil samples		Forest soil	Grass land soil	bare land	The raw road (paved and i)	Near water reservoirs	Resting areas of animals	Total	% Distribution
Number of samples examined		15	15	10	10	15	15	80	
Number of samples positive		8	7	7	6	12	8	48	
Distribution (%)		53.33	46.66	70.00	60.00	80.00	53.33	60.00	
1	<i>Aphanoascus durus</i>	1	1	-	-	-	-	2	2.50
2	<i>Aphanoascus fulvuscence</i>	1	1	-	-	1	1	4	5.00
3	<i>Arthrographis kalrae</i>	-	-	-	-	-	1	1	1.25
4	<i>Auxarthron conjugatum</i>	-	-	1	-	-	-	1	1.25
5	<i>Chrysosporium indicum</i>	2	2	2	2	4	1	13	16.25
6	<i>Chrysosporium tropicum</i>	1	1	1	1	1	-	5	6.25
7	<i>Chrysosporium zonatum</i>	-	1	1	1	-	-	3	3.75
8	<i>Chrysosporium state of Ctenomyces serratus</i>	1	-	1	-	3	1	6	7.50
9	<i>Microsporium gypseum</i>	2	1	1	2	2	2	10	12.5
10	<i>Trichophyton mentagrophytes</i>	-	-	-	-	1	2	3	3.75
Total		8	7	7	6	12	8	48	60.00

Phylogenetic analysis

Similarity analysis of the nucleotides was performed by BLAST searches against sequences available in GenBank (Altschul *et al.*, 1990). For phylogenetic tree construction, multiple sequences were obtained from GenBank and the alignments were performed using MEGA6 (Tamura *et al.*, 2013).

RESULTS

The results of the isolations are presented in **Table 1**. They show that out of 80 samples only 48 yielded keratinophilic fungi which were categorized into 10 species of 7 genera, namely *Aphanoascus durus* (2.50 %), *Aphanoascus*

fulvuscence (5.00 %), *Arthrographis kalrae* (2.50 %), *Auxarthron conjugatum* (1.25 %), *Chrysosporium indicum* (16.25 %), *Chrysosporium tropicum* (6.25 %), *Chrysosporium zonatum* (3.75 %), *Chrysosporium state of Ctenomyces serratus* (7.50 %), *Microsporium gypseum* (12.50 %), and *Trichophyton mentagrophytes* (3.75 %) (**Fig. 1**). All the ten strains yielded unique PCR amplification. The sequences of the ITS1- ITS2 rDNA region for the ten strains were from 527 bp to 677 bp. *Chrysosporium state of Ctenomyces serratus* and *Trichophyton mentagrophytes* were the smallest and the largest, respectively. The other species showed a product size of approximately 600 bp. There was considerable

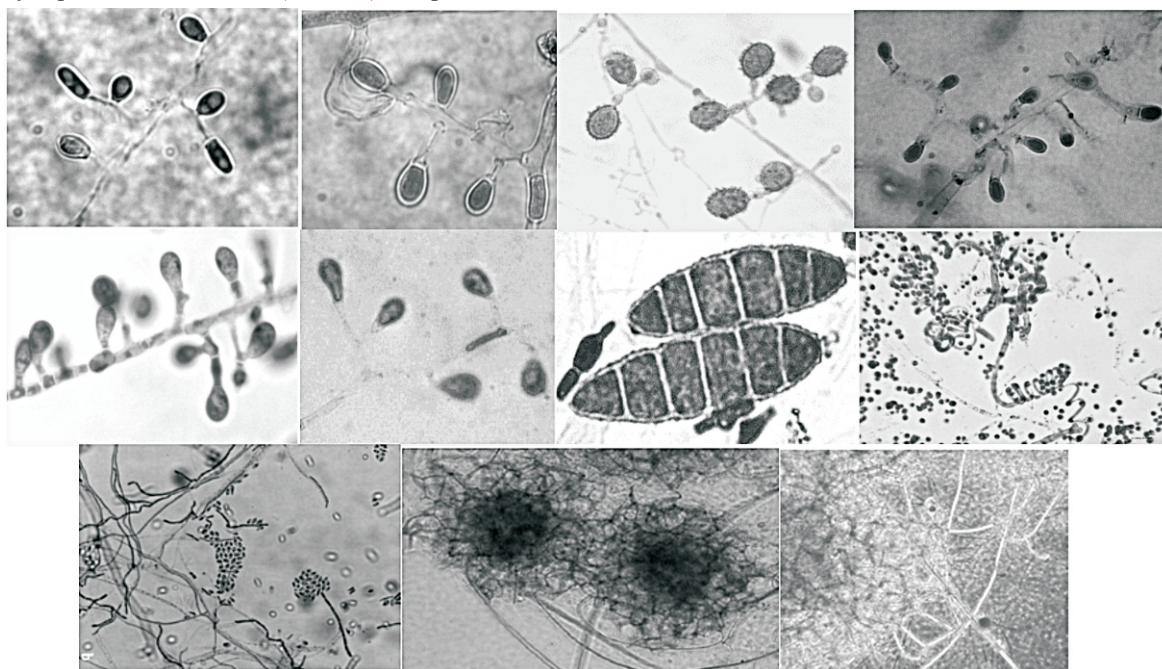


Fig. 1. Microscopic structures of some of the keratinophilic fungi from The Gir Forest National Park and Wildlife Sanctuary, (India). A. *Chrysosporium indicum*, B. *Chrysosporium tropicum*, C. *Chrysosporium state of Ctenomyces serratus*, D. *Chrysosporium zonatum*, E. Conidial stage of *Aphanoascus fulvuscence*, F. Conidial stage of *Aphanoascus durus*, G. *Microsporium gypseum*, H. *Trichophyton mentagrophytes*, I. *Arthrographis kalrae*, J. *Auxarthron conjugatum* (Ascocarp) and K. *Auxarthron conjugatum* (Mature peridial hyphae).

difference in the sequence data of the ten strains analyzed. The data were also compared with sequences deposited in the NCBI/Genbank for identification purposes and a tree was constructed with the nearest type strain sequences based on rRNA gene sequences (ITS region) using the Maximum Composite Likelihood Method (Fig. 2). The Phylogenetic tree indicates different clusters for each isolate showing their sequence variation. The sequences obtained show greater than 96% similarity to the reference

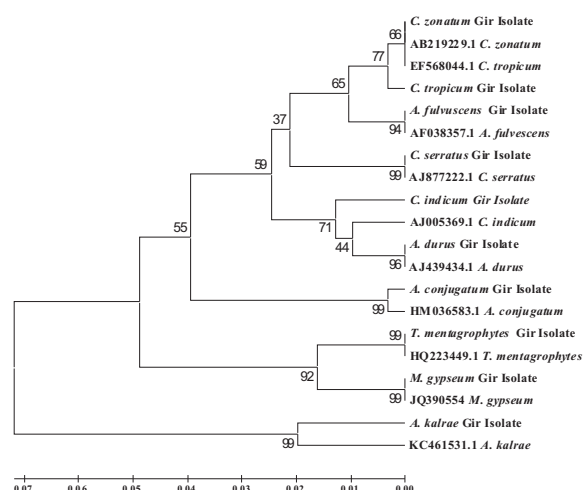


Fig. 2. Phylogenetic tree based on ITS1-5.8S-ITS4 region sequences for isolated 10 keratinophilic fungi isolated from Gir Forest National Park with reference strains. Numbers at the respective nodes are percentage of 1000 bootstrap replicates. Bar indicates genetic distance due to sequence variation.

sequences available in NCBI Genbank database.

DISCUSSION

Chrysosporium indicum (16.25 %) was found to be the most dominating species followed by *Microsporum gypseum* (12.0 %). *Chrysosporium indicum* was reported from India's plains in various studies and dominates Indian soil mycobiota because it is adapted to warmer conditions (Randhawa and Sandhu, 1965; Deshmukh and Agrawal, 1983; Deshmukh and Verekar, 2011). *Microsporum gypseum*, a well-known geophilic dermatophyte, had shown a frequency of 12.5 %, it has also been reported from Indian plains (Garg, 1966; Singh *et al.*, 2009; Jain and Sharma, 2011). *Chrysosporium* state of *Ctenomyces serratus* frequency followed that of *M. gypseum* at 7.50 % distribution. *Chrysosporium* state of *Ctenomyces serratus* was reported from various parts of India (Randhawa and Sandhu, 1965; Garg, 1966; Deshmukh and Verekar, 2011). Amongst the *Chrysosporium* species isolated, *Chrysosporium tropicum* was 6.25% in distribution. It is a cosmopolitan species and has been reported from various parts of India (Randhawa and Sandhu, 1965; Deshmukh and Agrawal, 1983; Jain and Sharma, 2011). The other species, *Chrysosporium zonatum* (3.75%) was next dominating species after *Chrysosporium indicum* and is reported from various part of India (Katiyar and Kushwaha, 2000; Singh and Kushwaha, 2010).

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The teleomorphic stages of *Aphanoascus durus*, *Aphanoascus fulvusces*, *Arthrographis kalrae* and *Auxarthron conjugatum*, were 2.50, 5.00, 1.25 and 1.25 % in distribution. *Aphanoascus durus* has been reported from chicken yard and hedgehog in Ivory Coast (Cano and Gurrao, 1990) and also from half decomposed feathers or dropped off feathers of the birds from Lonar crater (Deshmukh and Verekar, 2006). *Aphanoascus fulvusces* was reported from soils of Italy and also from soils of Bahrain (Deshmukh *et al.*, 2008; Caretta *et al.*, 1992). *Arthrographis kalrae* is reported in soils from various parts of world (Sigler and Carmichael, 1976). Recently we have recorded *Arthrographis kalrae* from public park soils of Mumbai (Deshmukh and Verekar, 2012). *Auxarthron conjugatum* is reported from Indian soils by Deshmukh and Verekar (2011), Jain and Agrawal (1979), and Deshmukh and Agrawal (1983).

Trichophyton mentagrophytes was 3.75 % in distribution. *Trichophyton mentagrophytes* has been reported from Indian soils in various studies (Randhawa and Sandhu, 1965; Garg, 1966; Guignani *et al.*, 2007; Singh *et al.*, 2009; Sharma and Sharma, 2010).

There are several reports of distribution of keratinophilic fungi from Indian soils. The predominant keratinophilic fungi reported include *Chrysosporium* sp., (mainly *C. indicum*, *C. tropicum*, *C. keratinophilum*, *C. zonatum*) and the dermatophyte *Microsporum gypseum*. Other dermatophyte species known to occur sporadically in soil are *M. cookei*, *M. canis*, *M. vanbreuseghemii*, *Trichophyton terrestre* and *T. gloriae*. *Trichophyton simii*, *T. mentagrophytes*, and *Microsporum nanum* are the well-known zoophilic dermatophytes, which have also been frequently recovered from soil in some countries (Deshmukh and Agrawal, 1998; Deshmukh and Verekar, 2010). To our knowledge there are several publications on occurrence of these fungi from soils. We are reporting for the first time the presence of keratinophilic fungi from Gir

Forest National park.

There are reports on various types of infections caused by species of *Chrysosporium* in man and animals particularly in immuno-compromised patients (Anstead *et al.*, 2012). For example *Chrysosporium zonatum* was reported to cause disseminated infection in a patient with chronic granulomatous disease (Roilides *et al.*, 1999). In Japan, *C. zonatum* strains were isolated from bronchial lavage from a female in Chiba and from a male in Kyushu. Both patients presented pulmonary cavity sites (Sigler *et al.*, 1998). *Chrysosporium tropicum* was reported from comb lesion in two different breeds of chicken in India (Saidi *et al.*, 1994).

CONCLUSION

Our study indicates the presence of these fungi in Gir National Park. The occurrence of these fungi in these areas is an indicative that they may be a source of superficial infections to man and animals on one hand. Whereas on the other hand these fungi may be playing an important role in decomposing the keratinic matter added to the soil by human and animal activity

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