

DIVERSITY OF ARTHRODERMATACEAE COMMUNITIES THAT CREATE HAVOC TO THE OVERALL HEALTH OF HUMAN AND ANIMALS

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Abstract: Keratinous substance rich soil is most conducive for keratinophilic fungi growth and occurrence. Dermatophytes and other related fungi are potential pathogens causing human and animal dermatomycoses. Herein, this study went for screening the presence of dermatophytes and related keratinophilic fungi in various soil samples collected from different locations of Sharkia Governorate, Egypt. A total of 80 soil samples from roadsides, fields and stables were subjected for mycological analysis using modified hair-bait technique with hair of horse, cattle and goat, sheep wool, and chicken feathers as a keratin source for keratinophilic fungi growth. Keratinophilic fungi were identified according to their phenotypical characterization in combination with PCR amplification and sequencing for internal transcribed spacer (ITS) region of rDNA. Keratinophilic fungi were recovered from 73.75% of soil samples (59/80). Field soils yielded a higher positivity rate for keratinophilic fungal isolates (90%) than roadsides (66.67%) and stables (58.82%). The majority of keratinophilic fungi belonged to dermatophytes (57.47%). *Microsporium gypseum* (50.85%) were detected in the majority of sites followed by *Trichophyton mentagrophytes* (30.51%), *Chrysosporium* species (28.81%), *C. keratinophilum* (23.73%), *C. tropicum*, *C. zonatum*, *Arthroderma multifidum*, *Arthroderma benhami*, *Arthroderma fulvum*, *Clonostachys* species, *Simplicillium obclavatum* and *Purpureocillium lilacinum* (1.69%, each). It was found that horse and goat hair were more suitable for isolation of keratinophilic fungi with a percentage of 100% for each, followed by cattle hair (91.66%), sheep wool (87.5%) and chicken feathers (83.33%). This investigation demonstrated that the various soils of Sharkia Governorate might be critical suppliers of certain keratinophilic fungi that may constitute hazards to human and animal health. The genetic-based identification is strongly recommended for a high discriminatory identification of keratinophilic fungi.

Key words: keratinophilic fungi; geophilic dermatophytes; *Arthroderma benhami*; *Arthroderma fulvum*; ITS sequencing

Introduction

Keratinophilic fungi are an exceedingly specific, keratin-corrupting ecological group

of filamentous fungi. They live in natural environments, grow and even reproduce on keratin-rich remains in the soil such as outer cornified layers of the skin, hair, nail, fur, feather, horn, hoof and beak (1). They

comprise fungi known as geophilic dermatophytes and the species of the genus *Chrysosporium* related to them (2). Majority of soil fungi are potential pathogens to both human and animals causing dermatophytosis (ringworm) and are parasites of keratinized tissues (3). Dermatophytosis is common worldwide superficial fungal infections and is of veterinary and public health significance (4,5), but its prevalence can vary extensively, relying on geographical and other epidemiological variables as (temperature, humidity, pH, climate, environmental light and amount of organic substance in soil, etc.) (4,6). Dermatophytes are classified according to the conidial morphology to *Microsporum*, *Trichophyton*, *Epidermophyton* genera (in their anamorphic phase) and *Arthroderma* (in their telomorphic phase). Ecologically, dermatophytes have been classified into zoophilic, anthropophilic or geophilic species on the basis of their natural habitat (animals, humans and soil, respectively) (6). Zoophilic and geophilic dermatophytes often incite a more powerful inflammatory response when they endeavor to attack human skin than anthropophilic dermatophytes (7).

Considering the significance of soil saprophytes as potential human pathogens, many researchers have studied the occurrence of dermatophytes and keratinophilic fungi in soils in many territories around the world (2,8–10). So far, data on the frequency and distribution of keratinophilic fungi in various soils of Sharkia Governorate, Egypt are meager. Thus, in this study the variety of keratinophilic fungi in different destinations frequented by human and animals in Sharkia Governorate, Egypt were investigated and identified using conventional phenotypic and molecular characterization. Keratinophilic fungi were also tested for their potential to utilize hairs of different animals and chicken feathers as keratin substrates.

Material and methods

Soil samples

A total of 80 soil samples were collected from different sites where human and animals

live (33 roadsides, 30 fields, 17 stables and animal habitats) in Sharkia Governorate. The soil samples were gathered from the superficial layer at a depth not surpassing 3-5 cm in sterile polythene bags, taken to the laboratory, stored at 15°C and processed immediately.

Keratin substrates and hair-bait technique

Samples were cultured on fragments of sterile hairs (approximately 2-3 cm long) from healthy animals such as hair of horse, cattle and goat, sheep wool, and chicken feathers as a keratin source for growth of keratinophilic fungi in modified hair-bait technique (11). For this purpose, five Petri dishes were used for every soil sample, one each for different baits. Each Petri dish was half-filled with soil samples and fragments of sterilized hairs or feathers were scattered on their surface. Soil samples were wetted with about 15 ml of sterile distilled water and 10 drops from Dermasel selective supplement (Oxoid Thermo Fisher Scientific, Basingstoke, Hants, UK) dissolved in 3 ml ethyl alcohol. Plates were incubated at room temperature for 2-3 weeks. Soil samples were examined regularly for the development of mycelium on the baits and moistened with distilled water with some drops of Dermasel selective supplement every four day to prevent the drying till fungal growth appeared.

Isolation and identification of keratinophilic fungi

When mycelial growth was visible on the hair, wool or feather, parts of each with fungal growth were put on a slide and examined microscopically for macroconidia, microconidia or septated hyphae after staining with lactophenol cotton blue. Invaded hair, wool or feather was inoculated onto slopes of Sabouraud's Dextrose Agar (SDA) with Dermasel selective supplement containing cycloheximide 500mg/l and chloramphenicol 50 mg/l (Thermo Fisher Scientific / Oxoid) for 5-10 days at 25°C. Isolates were further subcultured for 14 days on SDA with Dermasel selective supplement at 25°C and growths were recognized phenotypically to genus and species level based on colony morphology and

microscopic examination. Morphology of dermatophyte isolates was examined from all aspects as regard to texture of colonies, rate of growth, surface and reverse colour on Bromocresol Purple Milk Dextrose Agar (BCP), Rice Lactritmal Agar (RLA) and Rice grain (RG) media (12).

Extraction of genomic DNA

Total DNA was extracted from each isolate using Thermo Scientific Gene JET Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific; Dreieich, Germany). One hundred milligrams of mycelia were weighed, placed into liquid nitrogen and then grinded thoroughly with a mortar and pestle. Grounded mycelia powder was transferred immediately into a 1.5 ml microcentrifuge tube containing Lysis Buffer A of the kits and other steps were performed according to the manufacturer's recommendations. The quality of extracted DNA was evaluated using gel electrophoresis.

PCR amplification, sequencing and Phylogenetic analysis

The internal transcribed spacer region of rDNA encompassing ITS1, 5.8S and ITS2 genes was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer pairs (13). PCR reactions were performed in a final reaction volume of 25 µl containing 5 µl templates DNA, 1 µl from each primer (20 pmol), 12.5 µl of Dream Taq™ Green master mix (2x) (Thermo Fisher Scientific; Dreieich, Germany) and 5.5 µl nuclease free water using Biometra T3 thermal cycler (Göttingen, Germany). PCR Cycling condition: An initial denaturation step at 95°C for 5 min was followed by 35 cycles of denaturation at 95°C for 5 min, annealing at 55°C for 1 min and extension at 72°C for 1 min with a final extension step of 72°C for 10 min. Amplicons were electrophoresed in ethidium bromide-stained 1.5% agarose gels (Gellyphor, Euroclon, Italy) and estimated by correlation

with markers in the Gene Ruler™ 100-bp DNA Ladder (MBI Fermentas, Vilnius, Lithuania). Gels were photographed utilizing a digital documentation system (Gel Doc 2000, BioRad, UK).

Amplified products of eight genomic DNA samples were each refined utilizing Gene JET PCR purification kit (Fermentas, Vilnius, Lithuania) and afterward sequenced using the Taq DyeDeoxyTerminator Cycle Sequencing Kit (v.2, Applied Biosystems) in an automated sequencer (ABI-PRISM 377). The ITS1, 5.8S and ITS2 sequences were then determined using ITS1 and ITS4 primers. The nucleotide sequences were additionally compared with published sequences of fungi available in the National Center for Biotechnology Information (NCBI) database using BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). The nucleotide sequence data for *Arthroderma benhami*, *Arthroderma multifidum*, *Clonostachys* species, *Arthroderma fulvum*, *Chrysosporium tropicum*, *Simplicillium obclavatum*, *Purpureocillium lilacinum* and *Chrysosporium zonatum* reported in this paper have been deposited in the GenBank database under accession numbers KR265107, KR265108, KY129987, KY129988, KY129989, KY129990 and KY290545, respectively.

The neighbor-joining method that applied to DNA distance matrices was calculated according to Kimura two-parameter model was used for construction of the phylogenetic tree. The confidence values of branches were determined by a bootstrap analysis using MEGA 6 program version 6 (14).

Data Analysis

Data were expressed as cross tabulation using Statistical Package for Social Sciences version 25.0 (SPSS, IBM Corporation, Armonk, NY, USA); where rows and columns represent the isolated Keratinophilic fungi from the examined soil samples and different soils, respectively.

Results

Isolation and identification of dermatophytes and related keratinophilic fungi from soil samples

Eighty seven keratinophilic fungal isolates were recovered by modified hair-baiting technique from 59 positive soil samples. Within 2-3 weeks, hairs, wool or feathers distributed on the surface of soil samples were overgrown with white or brownish white mycelia as shown in (Fig. 1). The potential of keratinophilic fungi to utilize hairs of different animals and chicken feathers as keratinous substrate for growth were tested. It was found that horse and goat hair were more suitable for isolation of keratinophilic fungi with a percentage of 100% for each, while cattle hair (91.66%), sheep wool (87.5%) and chicken feathers (83.33%). Fungal growth with part of invaded hair was subcultured on SDA supplemented with chloramphenicol and cyclohexamide and subjected for identification by macroscopic and microscopic examination which was differentiated to keratinophilic fungi namely *M. gypseum*, *T. mentagrophytes* and *Chrysosporium* species and *C. keratinophilum*. Macroconidia of *M. gypseum* were produced in great numbers as broadly spindle shaped with moderately thick walls and 4 to 6 septa with rounded ends. Hyphae are branched, septated and hyaline as in Figure (2a and b). Conidia of *Chrysosporium* species were clavate to ovoid with thick walled as in Figure (2c and d).

Amplification of the entire ITS (ITS1, 5.8S rDNA, and ITS2) region of nineteen representative isolates using ITS1 and ITS4 primers yielded unique amplified products ranging from 590 to 740 bp where *Arthroderma multifidum* is smallest and *M. gypseum* is largest in size as shown in (Fig. 3).

DNA sequence analysis was done for eight amplified products from *M. gypseum*, *T. mentagrophytes*, *Chrysosporium* species, *Simplicillium*, *Purpureocillium* and *Clonosotychs* species which formally identified by phenotypic methods and confirmed by PCR for ITS region. Nucleic acid sequence revealed 100% identity to the reference

sequences in NCBI Genbank database. A phylogenetic tree was developed with the sequences of closest kind strain in light of ITS region of rDNA gene sequences (Fig.4). The phylogenetic tree showed different clusters for each species, indicating variation in their sequence.

Incidence of Keratinophilic fungi in different soils

Keratinophilic fungi were recovered from 73.75% of the examined soil samples (59/80). The most elevated rate of positive samples was accounted for fields (90%) then roadsides (66.67 %), and stables (58.82%).

Dermatophytes were the predominant keratinophilic fungi isolated from soil samples, represented 57.47% (50/87) of the total isolates. *M. gypseum* (50.85%, 30/59 positive soil samples) were detected in the majority of the sites followed by *T. mentagrophytes* (30.51%, 18/59), *Chrysosporium* species (28.81%), *C. keratinophilum* (23.73%), *C. tropicum*, *C. zonatum*, *Arthroderma multifidum*, *Arthroderma benhami*, *Arthroderma fulvum*, *Clonostachys* species, *Simplicillium obclavatum* and *Purpureocillium lilacinum* (1.69%, each). An association of two to three fungi was observed in 30.51% of the positive soil samples. The most incessant fungal combination was *M. gypseum* & *C. keratinophilum* (61.11%), *M. gypseum* & *T. mentagrophytes* (22.22%) and *T. mentagrophytes* & *Chrysosporium* species (22.22%). Based on the occurrence frequency, the other species detected by ITS sequencing including *Arthroderma fulvum*, *Arthroderma benhami*, *Arthroderma multifidum* (the perfect state of *M. fulvum*, *T. mentagrophytes* and *Chrysosporium* species, respectively), *C. tropicum*, *C. zonatum*, *Clonostachys* species, *Simplicillium obclavatum* and *Purpureocillium lilacinum* were sporadically occurring.

As depicted in Table (1), among the three types of examined soils, roadsides and fields soils have the highest frequency of keratinophilic fungal isolates (41.4%). That is, a large frequency was for *Chrysosporium* species (47.1%) from roadsides and *C. keratinophilum* (42.9%) from fields.



Figure 1: Hair baits culture of roadsides soil showed fungal growth (white or brownish white mycelia) within 3 weeks

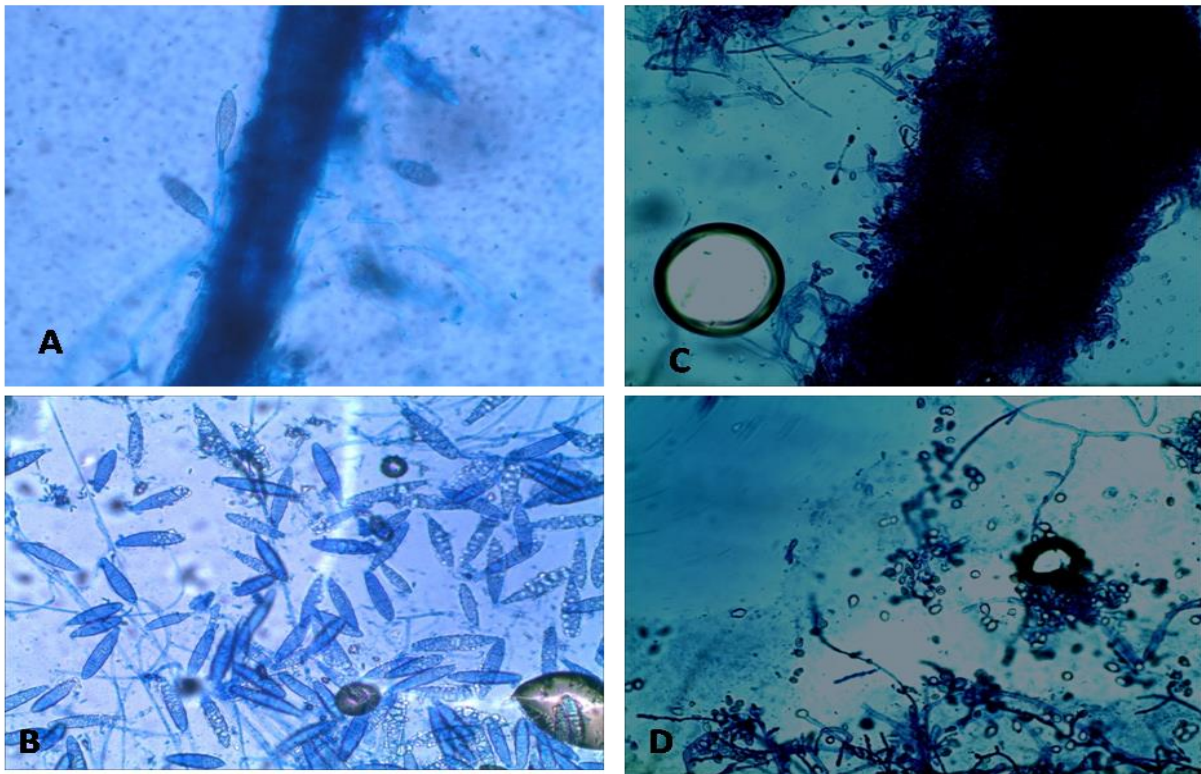


Figure 2: Microscopical examination of *M. gypseum* and *Chrysosporium* species on horse hair from hair baits technique and from culture stained with lactophenol cotton blue (400x). A: *M. gypseum* on hair showing microconidia on short conidiophores. B: *M. gypseum* macroconidia showing: branched septated hyaline hyphae and few microconidia and spindle shaped macroconidia with rounded end. C: *Chrysosporium* species on hair showing one oval cell microconidia on short conidiophores. D: *Chrysosporium* species showing clavate to ovoid thick walled conidia

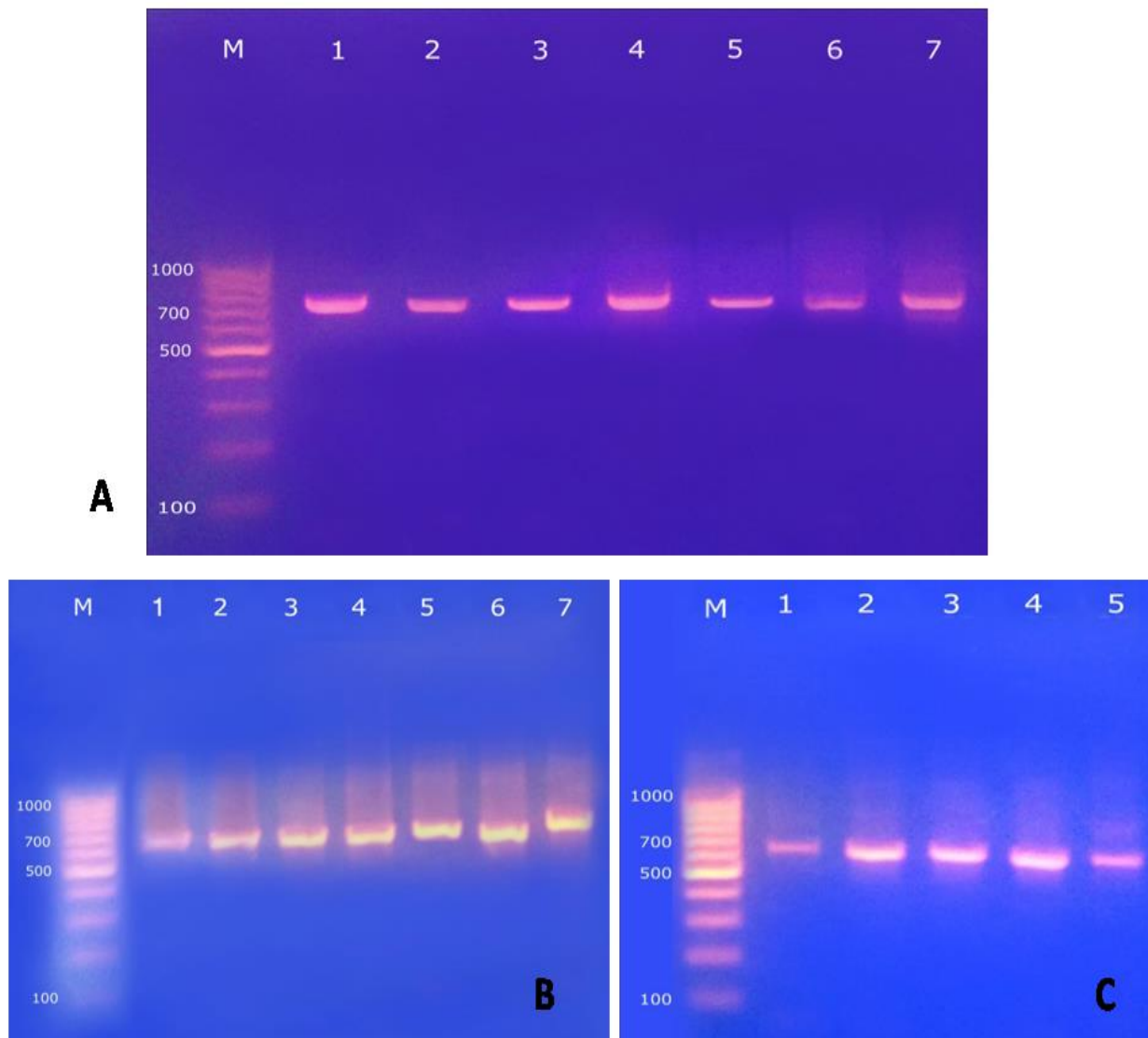


Figure 3: Agrose gel electrophoresis for amplified products of ITS1-5.8S-ITS2 rDNA region of dermatophytes and other keratinophilic fungi. A: lane M, molecular size marker, lane 1: amplified products of *Purpureocillium lilacinum* at 770 bp, lanes 2 to 7: *M. gypseum* at 740 bp, lane 7 identified by DNA sequences as *Arthroderma fulvum*. B: Lanes 1-4, 6: *T. mentagrophytes* at 635 bp. Lane 5: *T. mentagrophytes* var *quinckeanum* at 683 bp and lane 7: *T. mentagrophytes* at 700 bp (identified by DNA sequences as *Arthroderma benhami*). C: lane 1: *Simplicillium obclavatum* at 670 bp, lane 2: *Chrysosporium tropicum* at 620 bp, lane 3: *Chrysosporium zonatum* at 610 bp, lane 4: *Clonosotychs* species at 600 bp and lane 5: *Chrysosporium* species at 580 bp (identified by DNA sequences as *Chrysosporium* state of *Arthroderma multifidum*)

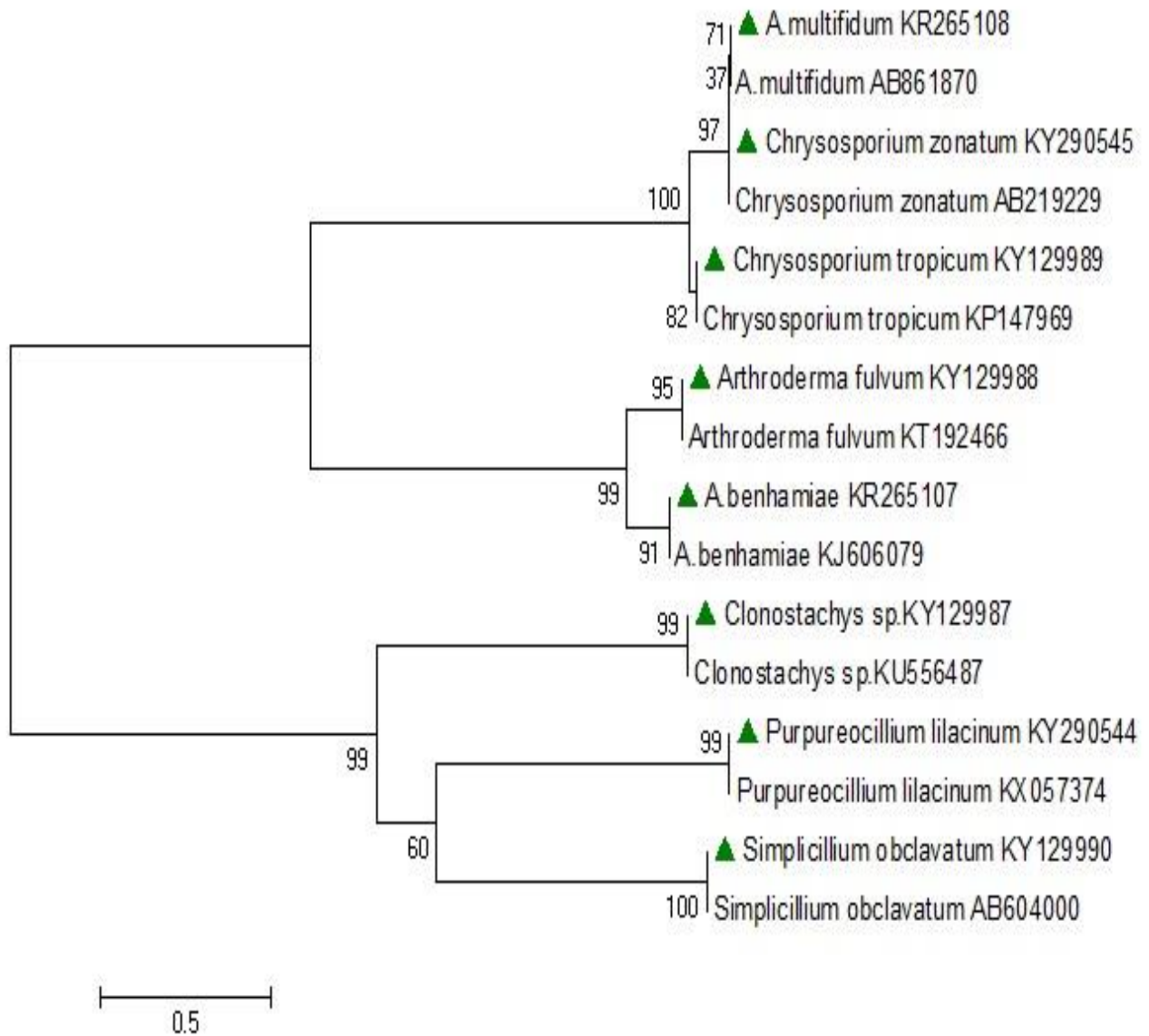


Figure 4: Phylogenetic tree based on ITS region of rDNA gene sequences for Keratinophilic fungi obtained in this study with their reference strains. Bar indicates two base changes per 1000 nucleotide position. Numbers at the respective nodes are percentage of 1,000 bootstrap replicates

Table 1: Frequency and distribution of keratinophilic fungi in different types of soil in Sharkia Governorate, Egypt

		Soil fungi * Soil samples Cross tabulation				
		Types of soil samples examined			Total	
		Roadsides	Fields	Stables		
Fungal isolates	<i>M. gypseum</i>	Number	13	12	5	30
		*Frequency % within spp.	43.3	40.0	16.7	100.0
	[†] <i>Arthroderma fulvum</i>	Number	1	0	0	1
		Frequency % within spp.	100.0	0.0	0.0	100.0
	<i>T. mentagrophytes</i>	Number	7	7	4	18
		Frequency % within spp.	38.9	38.9	22.2	100.0
	[‡] <i>Arthroderma benhami</i>	Number	0	1	0	1
		Frequency % within spp.	0.0	100.0	0.0	100.0
	<i>Chrysosporium</i> species	Number	8	7	2	17
		Frequency % within spp.	47.1	41.2	11.8	100.0
	<i>C. keratinophilum</i>	Number	4	6	4	14
		Frequency % within spp.	28.6	42.9	28.6	100.0
	<i>C. tropicum</i>	Number	0	1	0	1
		Frequency % within spp.	0.0	100.0	0.0	100.0
	<i>C. zonatum</i>	Number	1	0	0	1
		Frequency % within spp.	100.0	0.0	0.0	100.0
	[§] <i>Arthroderma multifidum</i>	Number	1	0	0	1
		Frequency % within spp.	100.0	0.0	0.0	100.0
	<i>Clonostachys</i> species	Number	0	1	0	1
		Frequency % within spp.	0.0	100.0	0.0	100.0
<i>Simplicillium obclavatum</i>	Number	1	0	0	1	
	Frequency % within spp.	100.0	0.0	0.0	100.0	
<i>Purpureocillium lilacinum</i>	Number	0	1	0	1	
	Frequency % within spp.	0.0	100.0	0.0	100.0	
Total	Number	36	36	15	87	
	Frequency % of all isolated sp.	41.4	41.4	17.2	100.0	

† The perfect state of *M. fulvum*, ‡ the perfect state of *T. mentagrophytes*, § the perfect state of *Chrysosporium* species *Frequency % within spp.= number of isolated spp. from one soil type/total number of spp. from three soil types.

Discussion

Studying of keratinophilic fungi from the environment is of hygienic and epidemiological significance because they constitute a risk of human and animal infection. Therefore, in this study soil samples from different destinations frequented by human and animals in Sharkia Governorate, Egypt were screened for dermatophytes and other related fungi distribution. Dermatophytes and other keratinophilic fungi were recovered from 73.75% of the examined soil samples. In comparison with other soil surveys recorded in the literature, this frequency is similar to those reported in Iran which reached 73.5% (15),

India (73.27%) (16) and Mumbai, India (75.0%) (17), but obviously lower than those recorded in the vicinity of Cairo, Egypt (40%) (18), Bahrain (45%) (19) and Assam, India (50%) (10). Moreover, in the soils of Djerba, Tunisia a lowest isolation rate (20.6 %) was reported (20) and a higher rate (100%) was reported in Slovakia (21). This could be ascribed to the climatic conditions and the soils nature.

As affirmed by various investigations, the keratinophilic fungal community varies from that observed for a very populated and strolled territory. With increase in the soils contamination, the indices of fungal occurrence, that correlates with pH, humidity,

organic matter, nitrogen activity, alkalinity and others, are increased (16,20). Subsequently, field soils yielded a higher rate of positive samples for keratinophilic fungi (90%) than from roadsides (66.67%) and stables (58.82%). Furthermore, the highest frequency of keratinophilic fungal isolates (41.4%) was recovered from roadsides and field soils. This is in consistence with Anane et al. (2) who declared a higher rate of keratinophilic fungal isolates from muddy soils than from sandy soil (56.0% and 39.9%, respectively). On the contrary, a lower isolation rate was detected in roadsides and industrial areas in Djerba, Tunisia (20). They attributed that to the high concentration of different pollutants in these regions that could repress the survival of keratinophilic fungi.

Different investigations (22–24) have recognized the internal transcribed spacers region (ITS1 5.8S, and ITS2) of nuclear rDNA as well as the 28S rDNA genes (25) as significant markers for elected yeasts and filamentous fungal species. ITS sequence analysis was conducted for confirmatory identification of eight representative isolates. This approach permitted distinguishing species that were rarely isolated in soils as the geophilic fungal species *Arthroderma fulvum* that causes human dermatophytosis and is one of the *M. gypseum* complex (26). Moreover, the frequent zoophilic dermatophyte *Arthroderma benhamiae*, which gives rise to inflammatory dermatophytosis in human (tinea capitis, tinea corporis, tinea manus and tinea faciei) was identified. Sequencing of the ITS region of rDNA has been affirmed as culture confirmatory test for *Trichophyton* species of *A. benhamiae* (27). Furthermore, *A. multifidum* has been recorded from soil and from bird combs as the dominant species in the Nansei islands in Japan (28).

Distribution of dermatophyte species as *M. gypseum*, *Microsporium fulvum* teleomorph (*Arthroderma fulvum*), *T. mentagrophytes* & its teleomorphs *Arthroderma benhamii* and the other keratinophilic fungi including *C. keratinophilum*, *C. tropicum*, *C. zonatum*, *Chrysosporium* species and its perfect state *Arthroderma multifidum* in various soil samples

revealed that they mainly survive on keratinous substrate in soil in all habitats. In addition, the detection of their teleomorphic phase in soil is indicative to long-term survival. Hence, soil acts as store for primary infection, at least for some pathogenic fungi, and furthermore for those which are possibly pathogenic to human and animals. Singh et al. found the same results (29).

Our results revealed the majority of keratinophilic fungi belonged to dermatophytes (57.47%) with *M. gypseum* as the predominating species (50.85%) isolated. It was additionally reported as the first or second most prevalent geophilic dermatophyte recovered from soils in previous literatures (2,20,30,31). Therefore, this is vital in light of the fact that *M. gypseum* causes dermatophytosis in both human (tinea capitis and tinea corporis) and animals. Similar to other studies (2,20), *M. gypseum* was frequently present in soils rich in organic matters.

In this study, *Chrysosporium* genus was represented by *Chrysosporium* species (28.81%), *C. keratinophilum* (23.73%), *C. tropicum*, *C. zonatum* and *Arthroderma multifidum* (1.69%, each). A large frequency was for *Chrysosporium* species (47.1%) from roadsides and *C. keratinophilum* (42.9%) from fields soils. These well-known soil fungi were isolated with a various recurrence from soils in numerous areas of the world (2,18,20,30,31). Since *Chrysosporium* species occur in different types of soil, it is essential to perceive their pathogenic potential. For instance, *Chrysosporium* species are occasionally isolated from human and animals and have been recorded to cause skin infections or onychomycosis, sinusitis, endocarditis, osteomyelitis, endophthalmitis and pulmonary disease (32–34). Recently, Cook et al. (35) diagnosed a disseminated *Chrysosporium* species infection in a dog. In the human literature, *Chrysosporium* infections either local or invasive are very rare, affect mainly immunocompromised host and can be fatal. To date only thirteen cases were diagnosed (36).

Noteworthy, *T. mentagrophytes* was the second most frequently isolated (30.51%) species from soil samples and it was frequently

recovered from roadsides and fields (38.9%). This is consistent with Jain and Sharma who isolated *T. mentagrophytes* (24.88%) from various soil samples in Jaipur city, India. They found that roadside and garden soils were the most appropriate destinations for almost all keratinophilic fungi (16). As it has been accounted for in past investigations, *Clonostachys* species and *Simplicillium obclavatum* were isolated from soil samples (37,38).

Among the isolated fungal species, *Purpureocillium lilacinum* was isolated from field soils (1.69%), as it has been previously declared (2,9). *Purpureocillium lilacinum* is an emerging, opportunistic pathogen that presents in the soil. It is a new genus for the medically significant *Paecilomyces lilacinus*. It is also known to cause various infections in immunocompromised and immunocompetent hosts and other vertebrates (39,40). Moreover, a case of nasal septal perforation and maxillary sinusitis in immunocompetent patient due to *P. lilacinum* infection (40,41) were reported.

The present work donates the superiority of modified hair-bait technique proposed by Orr (11) in which cyclohexamide was added, for isolation of Arthrodermataceae and other keratinophilic fungi from soil. It was found also that horse and goat hairs are better than cattle hair, sheep wool and feathers of birds when used in hair-bait technique indicating the tendency of Keratinophilic fungi to utilize horse and goat hairs than other sources as keratin bait for fungal growth. Hence the obtained results could be explained with the findings of Suh (42) that each keratinaceous substrates could be utilized as bait, however some were the best and the other were poor baiting materials. Some keratinophilic fungi were well baited on the body hairs of horses, and on the beard and body hairs of goats.

Conclusion

It could be inferred that most of the isolated keratinophilic fungi were either well-known mycotic agents or have been recouped from various animal and human lesions. Therefore, soil acts as a repertoire for primary infection of

human and animals, at least for some pathogenic fungi, and furthermore for possibly pathogenic species. The genetic-based identification is strongly recommended to allow a highly discriminatory identification of keratinophilic fungi.

Conflict of interest

None of the authors have any conflict of interest to declare.

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