Keratinophilic Fungi: Nature's Keratin Degrading Machines!

Their Isolation, Identification and Ecological Role

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Keratinophilic fungi are an ecologically important group of fungi that cycle one of the most abundant and highly stable animal proteins on earth – *keratin*. This article briefly explains how to isolate and identify them, the process of keratin degradation, and the ecological role of this important but unnoticed group of minute keratin cycling machines present in soil. We believe that Indian soil contains many more such fungi which have not been isolated and we hope this article will create interest among students to isolate and study these interesting fungi.

Sometime in the early history of vertebrates, as they evolved from life in water to life on land, they evolved a specialized protein known as *keratin* (from the Greek word for horn). This tough, fibrous, insoluble material provided an outer coat that served to prevent the loss of body fluids. Keratins have since proliferated into a wide variety of substances performing many different functions: the claws and armour of reptiles, the feather and beaks of birds, and the hooves, horns, skin, hair and nails of mammals. Keratin is a scleroprotein and is mechanically hard and chemically unreactive, owing its strength to the numerous cross-links of disulfide bonds, which hold together the molecular chains of this protein.

Due to the strength and stability of keratin, very few organisms are able to break it down and utilize it. Only a few insects, bacteria, actinomycetes and fungi can use keratin as a resource. Humans and other higher vertebrates cannot digest this protein, and if ingested, it just gets accumulated in the form of a lump that remains undigested. A large part of tiger scat and other

carnivore dung contains keratin (mainly hair) apart from bones and other indigestible elements.

Keratinophilic Fungi

The biggest group of organisms that can utilize keratin as the sole source of carbon and nitrogen are the *keratinophilic* fungi. These are minute organisms that cannot be seen by the naked eye, unlike macrofungi like mushrooms. The word keratinophilic means 'keratin loving', and is sometimes misleading in the sense that all fungi that can grow on hair (a common natural keratin substrate) could be considered as keratinophilic. However, hair is not solely made of keratin, and many fungi that grow on natural hair do not actually utilize the keratin, but rather use the non-keratin lipid fraction of the hair. Thus only fungi which actually degrade keratin should be considered as keratinophiles or keratinolytic fungi. In the Kingdom Eumycota (true fungi), two groups – the Deuteromycetes and the Ascomycetes – have keratinolytic members that occur commonly in soil as keratin decomposers. Some species are potential pathogens, and can cause infections in the skin and scalp of mammals (the dermatophytes). These dermatophytes include the genera *Microsporum* and *Trichophyton*. It is thought that dermatophytes were initially saprophytic and lived in the soil, but due to increasing interactions with animals, they gradually evolved a parasitic lifestyle. The dermatophytes have been classified into three ecological groups based on their habitat preference – geophilic (soil loving), zoophilic (animal loving) and anthropophilic (human loving). Molecular studies based on the DNA sequence analysis of the ribosomal ITS region have shown that these three groups are also phylogenetically distinct.

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Ecological Role

The biological function of keratinolytic fungi in the soil is the degradation of keratinized materials such as hides, furs, claws, nails and horns of dead animals (*Box* 1). In the soil, these fungi live in their *teleomorphic* (=sexual) stages in the form of

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Keratinophilic fungi, microbial biodiversity, taxonomy, ecology.

Box 1. Common Habitats of Keratinolytic Fungi

(Almost any place in nature where there is possibility of having keratin)

- Cattle sheds
- Animal burrows
- Bird's nest (collect only abandoned nests)
- Poultry sheds
- Garbage
- Sewage
- Barber's hair dumping area
- Public places like parks, schools, marketplace, etc.
- Herbivore or carnivore dung

cleistothecia, whereas in keratinized material (host) they live in an *anamorphic* (=asexual) stage in which they develop only a very simple morphology. When there is ample keratin substrate available in soil, these fungi multiply by asexual means by producing enormous numbers of conidia (aleuroconidia, arthroconidia). When the keratin substrate is depleted, however, the fungi reproduce by sexual means and form characteristic fruiting bodies called ascomata. These ascomata usually have protective peridial appendages that prevent attacks by mites which feed on them. Each ascoma contains numerous asci, which in Onygenales are naked (without covering), usually containing 8 ascospores. These ascospores are propagules for the next generation and can remain dormant until fresh keratin or an alternative source of nutrition becomes available. The ascospores of family Onygenaceae are very small, mostly spherical and variously ornamented. The peculiar shape and surface features including thick walls (exine) of ascospores help the fungus to survive in soil under dry conditions. The members of the order Onygenales are frequently isolated from burrow soils indicating that burrows are excellent habitats for these fungi during hot summers as the animals inside them are a regular source of keratin and moisture. There is a succession of the fungi that grows on natural keratin substrates in nature since these natural substrates are not solely made of keratin but also contain some non-keratin components. First the non-specialists colonize. These species do not actually utilize the keratin portion but rather the non-keratin fatty part of the substrate. Once all the non-keratin portion is exhausted, true keratino-

philic fungi or the keratinolytic species colonize. Finally, mites feed upon these fungi. Thus, apart from few insects, bacteria and actinomycetes these fungi are an integral part of the keratin cycling machinery in the ecosystem, a process which would otherwise have been difficult due to high stability of the keratin protein.

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Isolation of Keratinophilic Fungi

The keratinophilic nature of these fungi makes them easy to isolate using Vanbreuseghem's hair bait method (see *Box* 2). Isolation of keratinophilic fungi can also be done by the other techniques such as the dilution plate method or pour plate method although the hair baiting method is better as the keratinolytic ability is automatically checked if the fungus grows on the de-fatted natural keratin substrate. Once the fungus grows on the keratin substrate in the hair baited plate it can then be transferred onto agar media as these fungi generally can grow on various artificial media (see *Box* 3) (*Figure* 1). Except for some of the unusual strains which have special nutritional requirements, almost all the keratinolytic fungi grow on most artificial media, and Sabouraud's Dextrose Agar is widely preferred for maintaining these fungi in the laboratory. Since some keratinolytic fungi can be pathogenic to humans and other animals, special care is required during their isolation and maintenance. Cultures of these fungi should be autoclaved for 30 min at 121°C before discarding.

Identification of Keratinophilic Fungi

Detection of Keratinolytic Activity

Identifying whether a fungus is keratinolytic is quite easy if one is employing hair bait method for isolation. If the fungus actively degrades keratin in the baits it is keratinolytic. For a fungus to be called keratinolytic there should also be some biochemical evidence. Several workers have also isolated *keratinase(s)*, the enzyme(s) responsible for degrading keratin. However J Kunert who has worked for decades on the enzymes

Box 2. Vanbreuseghem's Hair Bait Technique

The keratinolytic nature of these fungi makes it possible to isolate them from soil by implanting hair, the 'hair baiting' technique initially developed by R Vanbreuseghem, a Belgian mycologist in 1952. Since then, a number of modifications have been developed, but the basic principle remains the same i.e. use of natural keratin substrate as baits to recover these fungi from soil.

Collection of Soil: Soil can be collected in sealed polythene bags using sterile spatula/spoon from habitats where keratin and hence keratinolytic fungi are present e.g. barber's dump, animal burrow, cattle or poultry sheds, sewage and garbage.

Hair Baiting:

I. Half fill sterile Petri dishes with the soil samples.

II. Spread short (2-3 cm) strands of sterilized defatted* human hair or horsehair over the surface of the soil.

III. Add 10-15 ml of sterile water to the soil to facilitate germination of fungal spores. Some antibiotic to prevent bacterial growth may also be added.

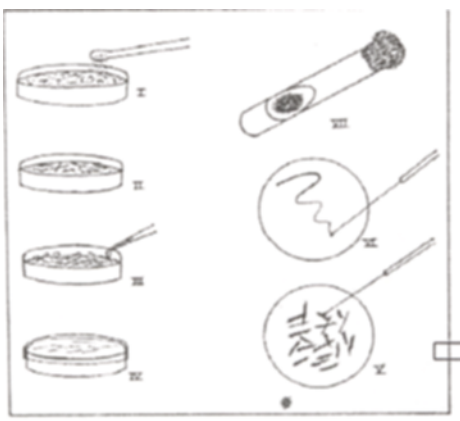
IV. Incubate the preparations at room temperature (20-25° C) in the dark, for 4-6 weeks. Examine the plates periodically for the development of mycelium using a Stereo binocular microscope.

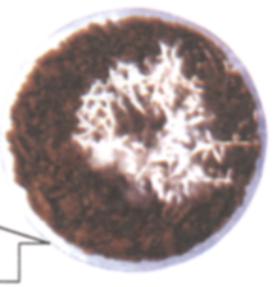
Note: while opening the plates make sure that there is no free moving air, because the spores are dispersed by air and many keratinolytic fungi are pathogenic.

V. Remove hairs with fungus growth or take inoculum and place it on plate of Sabouraud's dextrose agar.

VI. After one or more week, check the colonies and identify the fungus. Pure cultures can now be prepared.

*Defatting can be done by soaking the hair for 24 hrs in either diethyl ether or in a chloroform/methanol (1:1) mixture. Later rinse 4-5 times with distilled water and air dry.





Positive soil plate with fungal growth on hair.

Box 3. Growth Media for Keratinolytic Fungi

Sabouraud's Dextrose Agar (SDA)

Peptone	10 gms
Dextrose	40 gms
Agar	20 gms
Demineralised water	1000 ml

(Most commonly used medium for keratinolytic fungi)

Corn Meal Agar (CMA)

Cornmeal	15 gms
Agar	20 gms
Demineralised water	1000 ml

Oatmeal Agar (OA)

Oatmeal	40 gms
Agar	20 gms
Demineralised water	1000 ml

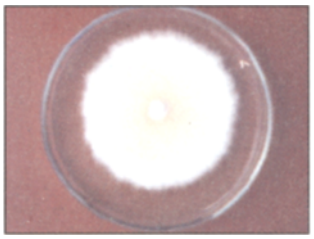
(Excellent medium for perfect or sexual states on agar medium)

All these media can be prepared by autoclaving at 121° C for 15 min

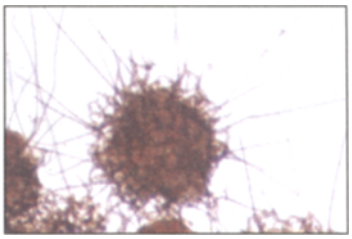
of keratinolytic fungi believes that there are no true keratinases. According to him, a number of enzymes involved in keratin degradation are primarily *proteases,* which act upon the substrate once it is denatured. The difference between keratinolytic fungi and non-keratinolytic fungi may be quantitative and not qualitative i.e. both can produce keratin degrading enzymes except that the former produce these in higher amounts. The keratinolytic fungi denature the substrate by the process of *sulphitolysis,* which is absent in non-keratinolytic fungi. In this process, the fungus initially releases some sulphide responsible for the breakdown of disulphide bonds of cysteine present in keratin protein. The proteolytic enzymes released by the fungus then cleave this partially denatured protein.

To ascertain the keratinolytic ability of a fungus there are two ways – (i) *morphological assessment* and (ii) *biochemical assessment.* In the first case, one can use small pieces of guinea pig hair/ child

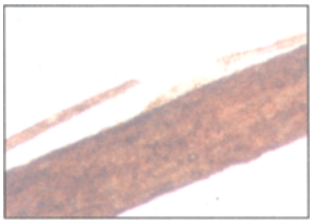
Figure 1.



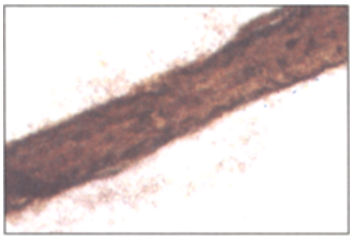
Colony of *Microsporum gypseum* on SDA medium after 15 days of incubation



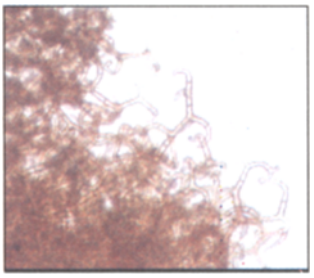
Globose ascoma of *Auxarthron umbrinum* with elongate appendages (X100)



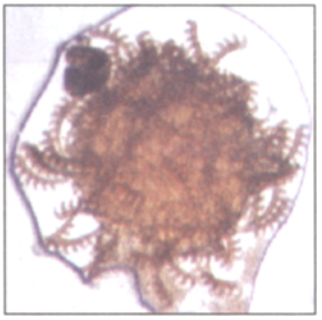
Cuticle eruption of human hair ue to attack of *Chrysosporium indicum*, a keratinophilic fungus (X400)



Extensive degradation of human hair by *Chrysosporium indicum* (X400)



Part of ascoma of *Gymnoascus reesii* showing boat-hook shaped peridial appendages(X200)



Ascoma of *Ctenomyces serratus* with comb-like characteristic peridial appendages (X100)

Rahul Sharma

hair sterilized with chloroform or by tindallization (but not autoclaved to prevent substrate denaturation), which can be inoculated with the fungal material, and incubated at 25°C for 2-4 weeks for degradation to occur. If the fungus is keratinolytic, it will grow into the hair by forming special structures (boring hyphae), and hydrolyze it. But if the fungus is non-keratinolytic, even after 4 weeks of incubation, the hair should remain intact. For biochemical assessment, the fungus could be grown (2-4 weeks) in a flask containing sterile basal salt solution along with

the sterilized hair (as the sole source of carbon and nitrogen). The sterilization procedure should not denature the keratin substrate. The filtrate should be assayed for the degradation products of keratin, like – cysteine, S-sulphocysteine, cysteic acid, cysteine and inorganic sulfate. If these compounds are not detected in the culture filtrates, then the fungus does not possess the ability to degrade keratin. *Keratin azure* (Sigma) can also be used for the detection of keratinolytic ability in fungi. Keratin azure is a blue compound that upon cleavage turns colorless, thereby indicating the hydrolysis of keratin.

Identification of Species

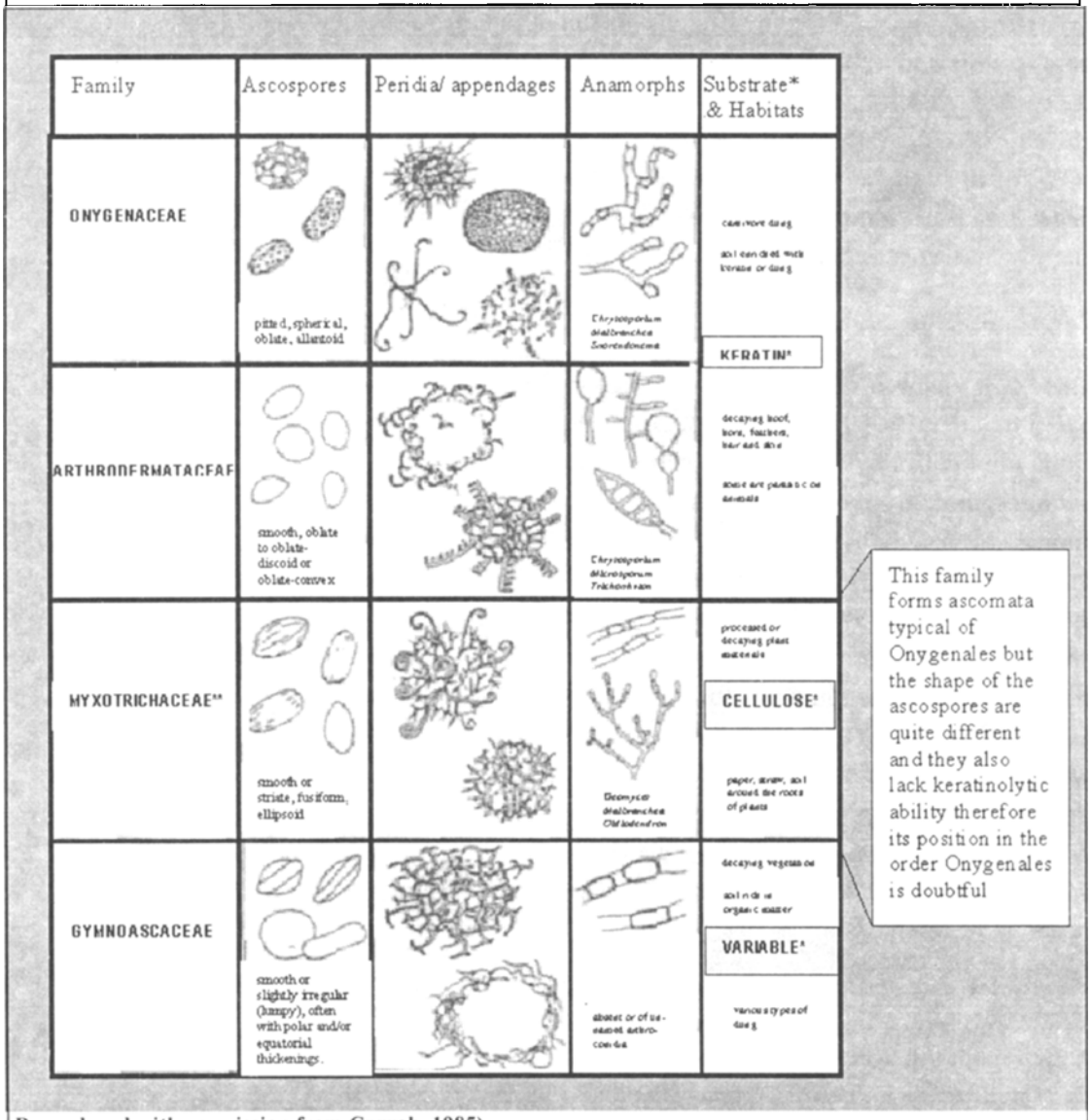
Once it is ensured that the fungus is keratinolytic, it becomes easier to identify its genus or species (since very few known fungi are keratinolytic). Here we concentrate on the two major groups of ascomycetous and the deuteromycetous keratinolytic fungi. The order Onygenales of the Ascomycetes consists of four families: Arthrodermataceae, Gymnoascaceae, Myxotrichaceae and Onygenaceae (*Box* 4) with 37 genera having more than 100 species. The taxonomic position of the family Myxotrichaceae is not certain, as none of its members are keratinolytic, and their ascospore shapes are somewhat different from the rest of the Onygenales. Detailed identification down to species level can be done with the help of authoritative descriptions, figures and dichotomous keys provided by Currah [1, 2]. However, for species discovered after 1985, more recent specific literature needs to be referred to.

Order Onygenales

* Genera in the order are recognized by a sudden discontinuity, first in *ascospore shape and ornamentation*, and second, in *peridial morphology*.

* Families in Onygenales are groups, which share degradative capabilities and a common type of ascospore sculpturing (including shape). Peridial morphology is not given much importance in delimiting families.

Box 4. The four families of order *Onygenales* with ascospores types, ascomata and their appendages including the type of anamorphs formed



Reproduced with permission from Currah, 1985)

Key to Order Onygenales

Ascomata are bright colored, composed of loosely intertwined hyphae, often with thick walled, branched and/or ornamented

hyphal appendages; asci never in chains; ascospores very small, brightly colored, usually spherical or lenticular, often ornamented; anamorph usually with thallic proliferation; often keratinophilic (Onygenales) — 1

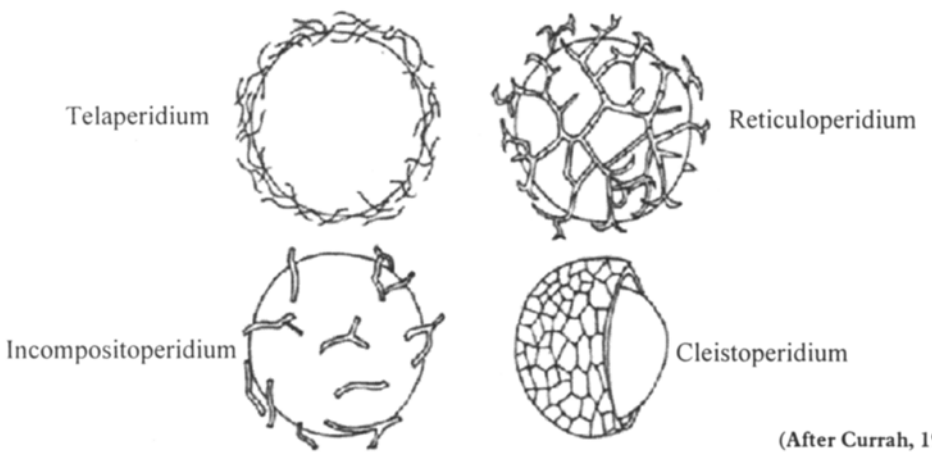
1. Ascospores fusiform to ellipsoidal, sometimes striate; cellulolytic — *Myxotrichaceae*
   Ascospores spherical or flattened (oblate or lenticular) smooth or variously ornamented — 2

2. Ascospores usually flattened, pitted or reticulate; always keratinolytic — *Onygenaceae*
   Ascospores smooth walled; nutrition variable— 3

3. Ascospores oblate to discoid, without equatorial thickenings, always keratinolytic — *Arthrodermataceae*
   Ascospores usually flattened with equatorial thickenings, keratinolytic or cellulolytic — *Gymnoascaceae*

Identification of keratinolytic fungi (Onygenalean ascomycetes) primarily requires the *study of ascomatal structure* (*Box* 5), *peridial hyphae and appendages, ascospore shape, size and ornamentation and*

Box 5. Peridium type: An Important Feature in Species Identification



(After Currah, 1985)

Molecular analysis of fungal genomes with the use of PCR based technique of RAPD (random amplification of polymorphic DNA) has become very useful for taxonomy. DNA based identification techniques are especially useful for those fungi which are difficult to distinguish morphologically.

anamorphic stages. These structures give important information as to which family, genera or species a fungus belongs. For example – family Onygenaceae contain members with reticulate or punctate ascospores, or both, but never smooth whereas family Arthrodermataceae contains only two members, one produces peridial hyphae which have *ossiform cells* (*Arthroderma*) and the other with *ctenoid* appendages (*Ctenomyces*) which are not found in any other member of the order. In taxonomy one has to keep track with the developments taking place all around the world as the field is highly dynamic and taxonomic changes are very frequent because of new information. The major difficulty in fungus identification comes when a fungus exists in two stages (sexual and asexual) as both the stages can be morphologically quite dissimilar. For the non-specialist it becomes very difficult to assign proper genera or species without the knowledge of both the stages. It becomes more difficult in fungi that are heterothallic [i.e. they have different mating types (+ and – strains) and sexual stage (which results in ascospores)], as they form sexual stages only when opposite mating types cross.

Molecular analysis of fungal genomes with the use of PCR based technique of RAPD (random amplification of polymorphic DNA) has become very useful for taxonomy. DNA based identification techniques are especially useful for those fungi which are difficult to distinguish morphologically. Another reason to use molecular tools for species identification is that many fungi do not produce characteristic spores which are key in fungal species identification. In RAPD analysis, genomic DNA from fungi is first isolated in pure form and amplified using either arbitrary or specific primers (short DNA segment of known sequence and length) on the basis of the presence or absence of that particular sequence in the fungal genome. The PCR products (or amplified DNA) are separated on an agarose gel, which reveals the similarity or the differences at the genetic level (*Box* 6). The method is widely used to identify particular species/strain using species-specific oligonucleotide primers, and is used also to study phylogeny.

Box 6. *Molecular Taxonomy* (DNA based species identification)

DNA based identification is primarily done at two levels – one at phylogenetic level (tracing phylogenetic position) and at identifying individual species or strains. At both levels, one needs to study a specific location in the fungal genome eg. ribosomal operon which contains both highly conserved and variable regions.

Major breakthroughs in phylogenetic systematics came with the sequencing of the larger and more complex small (18S) and large (25S) subunits of ribosomes. More recently, taxonomists have used sequences from the non coding spacer regions between the small and large subunits to assess genetic variation among closely related species and between different populations of the same species.

The variable rDNA regions are valuable for species identification among fungi, and in some cases for inference of intra-specific variations, also. Each fungal species might have its own 'signature' in these regions, and internal transcribed spacers (ITS)/inter generic region (IGR) could be suitable to design species-specific oligonucleotide primers for fungus identification. DNA based identification is facilitated by PCR technology. The basic principle is that the DNA from different fungi are isolated and multiplied using automated PCR machines (in which different conditions for multiplying DNA is controlled) using oligonucleotide primers. Typical band pattern (shown below) is generated due to presence or absence of a particular sequence in the genomes studied, which is scored and analyzed using various computer programs that help estimate the similarity or differences among fungi/strains at the genetic level. The amplified DNA is then sequenced and these sequences are aligned for confirmation of the genetic divergence or similarity.

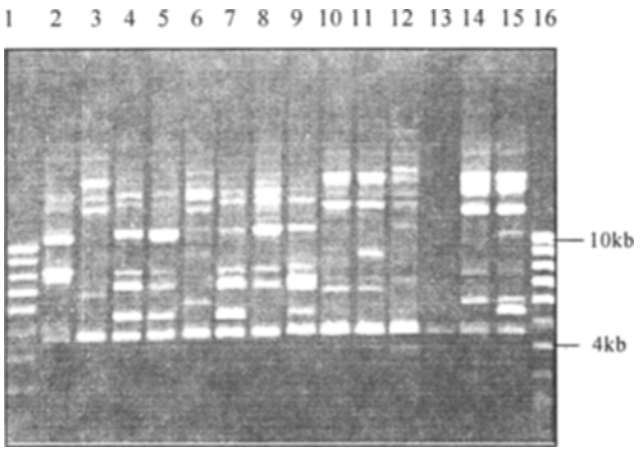


Figure shows typical band pattern generated when genomic DNA of 14 isolates of *Microsporum gypseum* was multiplied by primer OPA09 in a thermal cycler and then separated on a 1.2 % agarose gel. These *M. gypseum* isolates were obtained from soils of various districts of Central India.

Conclusion

Keratin is one of the most abundant animal proteins on earth as it forms a part of the exoskeleton of reptiles, birds and mammals. Among the microbes that cycle this protein in nature, keratinophilic fungi are very common and the most diverse. During the course of evolution, many of the soil-associated keratinophilic fungi have adopted a pathogenic life cycle and are now potential agents of fungal diseases in humans and animals. If keratinophilic fungi were not there to cycle this highly stable protein (keratin), then one can imagine the quantity of keratin that would have accumulated on earth, since a vast quantity of keratin is shed by the vertebrates. Indian soils contain many more keratinophilic fungi than those presently recorded, and there is need for further taxonomic and ecological studies of this interesting group of organisms.

Suggested Reading

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"By changing what man knows about the world, he changes the world he knows; and by changing the world in which he lives, he changes himself. Herein lies a danger and a hope; a danger because random changes of the biological nature are likely to produce deterioration rather than improvement; a hope because changes resulting from knowledge can also be directed by knowledge."

Theodosius Dobzhansky