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Phylogenetic analysis of *Trichophyton mentagrophytes* isolated from Tinea Patients

Sharma V.[1], Kumawat T.K.[2], Seth R.[3] and Sharma A.[4*]
1. Department of Botany, Mehta PG College and Institute of Technology, University of Rajasthan, Jaipur, INDIA
2. Department of Biotechnology, Biyani Girls College, University of Rajasthan, Jaipur, INDIA
3. Department of Biotechnology, JECRC University, Jaipur, INDIA
4. Department of Botany, Maharshi Dayanand Saraswati University, Ajmer, INDIA
*sharmaanima6@gmail.com

Abstract
In the Asian subtropical region, the dermatophytic infections are important public health issues which are increasing day by day in an insidious manner. Especially in India, about 20-25% of the population has been affected by Tinea or other dermatophytic infections. This study was aimed to explore the molecular characterization of Trichophyton mentagrophytes isolated from clinical specimens of Tinea patients and to analyze the phylogenic relationship between isolates.

The study was extended to examine the effect of environmental factors on the growth of T. mentagrophytes. The culture isolates were identified based on macroscopic and microscopic character and pure culture was confirmed by DNA sequencing by PCR amplification using ITS1 and ITS4 primers. The fungal species were identified as T. mentagrophytes by the nBLAST using fungal DNA sequences against the data available in NCBI/GenBank. Phylogenic trees were constructed using MEGA7 software with the help of reference data retrieved from NCBI GenBank data to study the phylogenic relationship between the isolated fungi.

Keywords: Dermatophytes, Tinea, phylogenic, ITS regions, NCBI.

Introduction

The keratinophiles of earth, a potential source of infection for human beings exist as self-sufficient saprophytes in the soil till the availability of favorable environmental conditions[1,2]. Some of the saprophytes become parasitic by accident and then pathogenic to both humans and animals causing infections and referred to as dermatophytes[3]. On the basis of type and degree of tissue involvement and the host response to the pathogen, dermatophytes broadly cause superficial, cutaneous, subcutaneous, systemic (deep) or opportunistic infections[4,5]. Tinea is a superficial manifestation of dermatophytic infections whose lesions are characterized by circular disposition, desquamation, alopecia and erythematic character of the edges.[6,7]

Epidermophyton, an agent of Tinea, was followed by universal agent *Trichophyton*; latter discovered by Malmsteen(1845) with identification of *T. tonsuran*[8-14]. Other than *T. tonsuran*, *Trichophyton* genera include other 24 members including *T. mentagrophytes*[15-17]. *T. mentagrophytes*, a communicable pathogen, is most ubiquitous around the world and affects both animals and humans alike. It prefers moist, carbon-rich environments and is identified by flat suede-like colonies with a white to cream color and distinctive odor. It grows fairly rapidly and infects in the host on contact with their skin and hair.

The rapid growth of *T. mentagrophytes* generates the highly evolved character in its cellular structures. Nowadays, its accurate aetiological identification is performed by molecular characterization[18-20]. The molecular characterization is the mapping of amplified large rRNA gene or internal transcribed spacer (ITS) regions compared with data at GenBank database[18].

The main objective of this research was to isolate and identify *T. mentagrophytes* by microscopic and molecular characterization and to study their phylogenic relationship with reference to data retrieved from NCBI GenBank. The study was extended to examine the consequence of environmental factors on the growth of *T. mentagrophytes*.

Material and Methods

Collection of clinical specimens: Clinical specimens were collected from 119 clinically suspected patients visiting the out-patient department (OPD) of Skin and Venereal Disease, SMS medical college and hospital, Jaipur. The demographic data and prior manifestations as age, sex, duration of illness, duration of the lesion, clinical picture and prior therapy were obtained with consent of suspected patients. Skin samples were collected on sterile black paper by scraping the infected epidermal scales on the skin with the help of a sterile scalpel blade[21].

Preliminary identification, isolation and purification of isolated fungal species: The specimens were examined for the presence of hyphae or spores which are characteristics of dermatophyte infection by potassium hydroxide (KOH) method. The skin scrapings or nail clippings were placed on a glass slide and 2-4 drops of 10-20% (w/v) aqueous solution of KOH (based on specimens) were added and gently heated. After 5 min a cover slip was placed on the slide and examined under low and high (10X and 40X) power objective lens to observe for the presence of diagnostic fungal forms[22].

The KOH positive skin scrapings and nail clippings were subjected to culture study by culturing on sabouraud dextrose agar supplemented with chloramphenicol (0.05mg/ml) to inhibit the growth of bacterial contaminants and cycloheximide (0.5mg/ml) to inhibit the growth of saprophytic fungi[23]. After 72-96hrs incubation period, isolated fungal cultures were identified based on macroscopic characteristics (duration of growth, surface morphology, color, texture, pigmentation production on reverse surface, colony morphology) and microscopic examination (presence of hyphae and spore shape) of fungal growth[24].

Genomic DNA extraction: Whole-cell DNA from mycelial growth of isolate was extracted by modified Lee and Taylor method[25]. Seven days old recovered fungal mat was placed in an aseptic mortar and 10-20 ml liquid nitrogen was added. Frozen mycelium mat was quickly ground and transferred to a clean sterile Eppendorf with lysis buffer [100 mM Tris HCl [pH8.0], 50 mM EDTA, 3% SDS]. The mixture was placed for 10-15min at 37ºC to complete lysis process and RNAse was added to digest RNA. It was incubated at 37ºC for 2 hours. To this proteinase K (Sigma St. Louis, MO) was added and incubated at 56°C for 2hrs.

Finally, DNA was extracted by adding an equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) solution and centrifuged it at 13000 rpm for 20min. The cellular debris portion was separated by removing supernatant and chilled absolute alcohol was added to precipitate DNA in basal clean aqueous phase. It was centrifuged at 13000 rpm for 20min. After centrifugation, DNA pellet was obtained in the bottom of Eppendorf washed with 70% alcohol and was dissolved in elution buffer [10 mM Tris-HCl [pH 7.5], 1 mM EDTA].

Quantity and quality determination: The amount of extracted DNA was assessed by using Nanodrop (Thermo Scientific™ NanoDrop 2000C) at 260/280nm. The quality of extracted DNA was monitored by agarose gel electrophoresis using 1.5% agarose gel with ethidium bromide fluorescence. A variety of PCR based methods were utilized for downstream analysis of extracted DNA. The DNA bands were recorded onto gel documentation system (Alpha Innotech) and trans-illuminator.

PCR amplification and DNA sequencing: ITS regions of fungal DNA were amplified using ITS primer such as ITS1 ($^{3'}$TCCGTAGGTGAACCTGCGG$^{5'}$) and ITS4 ($^{5'}$TCCTCCGCTTATTGATATGC$^{3'}$) respectively (Sigma-Aldrich, Bengaluru, India) by Eppendorfs DNA gradient thermal cycler[26]. The PCR products were analyzed by electrophoresis on 1.5% agarose. Sequencing reactions were carried out using Big Dye terminator cycle sequencing kit, version 3.1 (Applied Biosystems, CA, USA) and analyzed by the ABI 3130 genetic analyzer (Applied Biosystems, CA, USA). The resultant sequence of the sequencing reaction product was compared with the GenBank database using NCBI BLAST software (http://www.ncbi.nlm.nih.gov/Genbank/index.html). The sequences showing ≥99% match with database sequences of the reference were taken for species identification. The sequences were aligned with Clustal-ω (omega) computer program in MEGA-7 software. The evolutionary record was calculated using maximum likelihood (ML) method using MEGA7[27].

Effect of Temperature and pH on Growth of isolated Dermatophytic Fungi: To assess the growth of isolated fungi, sabouraud dextrose broth was used to support the growth. To estimate the effect of pH, different pH (3, 4, 5, 5.6, 6, 7, 9, 11, 13) was adjusted for the broth in different flasks having 100ml of sabouraud dextrose broth. In case of temperature, various temperature (5, 15, 25, 35, 45 and 55ºC) ranges were applied on the growth of isolated fungi. A loopful of 15 days old fungal culture was inoculated in appropriately labeled sterilized flask and then incubated at 28ºC on an orbital shaker with continuous shaking at a speed of 30 revolutions per minute (rpm) for 15 days. On the sixteenth day of inoculation, broth was filtered and mycelium was harvested, dried and finally weighed. The growth was determined in terms of dry mycelia weight[28].

Results

In the present study, the patient samples were found to distribute as 95.96% of skin scraping and 5.04% of nail clipping. Maximum patients were found to be suffering from Tinea cruris followed by Tinea corporis. From a total of 119 samples collected, 49.57% specimens were found to be culture positive and out of these 23.73% specimens were recorded for *Trichophyton mentagrophytes*. Residual samples were found to be affected by other nine predominant fungal species belonging to *Microsporum*, *Trichophyton*, *Histoplasma* and *Chrysosporium*, *Scopulariopsis* genera.

Out of total ten isolated species, *T. mentagrophytes* was chosen for further studies on the basis of its occurrence. Maximum growth of selected *T. mentagrophytes* fungi was observed at 25°C (1.93±0.01 gm). A sharp decline in growth was observed at below and above of 25ºC temperature. *T. mentagrophytes* was also reported to exhibit maximum growth (0.26±0.02 gm) at pH 5 as well as high alkaline pH (Table 1). It was also found that when initial pH was low, it drifted towards neutrality or alkaline range. It was also reversed with a highly increased alkaline medium after 16 days of incubation.

In molecular identification, DNA was isolated by using the phenol-chloroform method and its purity was confirmed to be between 1.9 and 2.6 at 260 nm/280 nm ratios by using NanoDrop™ 2000/2000c Spectrophotometers. The PCR amplification of internal transcribed spacer (ITS) region of the isolate JU3 yielded PCR products of 679 bp respectively (Figure 1). The PCR product with primer pair ITS1 was sequenced for species identification and found to be 90 to 100% similar in the BLAST program to sequences of the ITSI, 5.8S rRNA gene and ITSII regions of respective fungi.

On basis of BLAST program results, ITS1 sequence of clinical isolate was determined as *Trichophyton mentagrophytes* (KU5781026) which was similar to morphological identification.

After a preliminary identification, a sequence of *T. mentagrophytes* was deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) with the accession numbers KU578106. The isolates were also deposited at the National culture collection of pathogenic fungi (NCCPF), PGIMER, Chandigarh, India as NCCPF-ILK980.

The multiple sequence alignment analysis of isolated *T. mentagrophytes* was found to be similar with reference HQ014707.1 and KJ606098.1 at Genbank data. Phylogenetic trees were prepared by the Neighbor-joining (Figure 2) and Maximum Likelihood (Figure 3) methods. The data of seven dermatophytic fungal sequences including one test sequence and six references sequences were used in Phylogeny construction.

The aligned sequences of their ITS1 regions are presented in figure 4. In the phylogenic trees, *T. mentagrophytes* was found to associate with *T. mentagrophytes, Arthroderma vanbreuseghemi, T. interdigitale, A. benhamiae* and *T. krajdenii* members in ITS1 homology group (Figure 2). In the Neighbor-Joining tree of isolated *Trichophyton* strain, the branch length was calculated by the method of Saitou and Nei[29] and it was obtained with the sum of 23.92 branch lengths.

In NJ tree, *T. mentagrophytes* (KU5781026) and *A. benhamiae* (EF631618.1) were found to form cluster A and all other *Trichophyton spp* were recorded to form Cluster B including to *A. vanbreuseghemii* with 100% bootstrap support. The members of the dermatophytes in cluster A were classified into two groups with ITS1 homology (groups a and b) according to their ITS1 DNA sequences. The group b was found to have very close relationships between sequence KU5781026 and HQ014707.1. The phylogenetic relationships mentioned above were also supported by the ML tree.

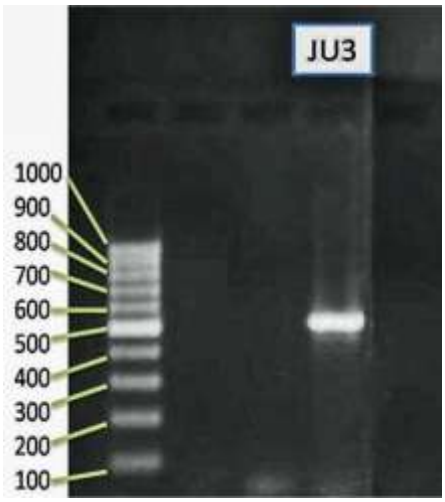

Figure 1: DNA Bands Visualized by the UV Transilluminators

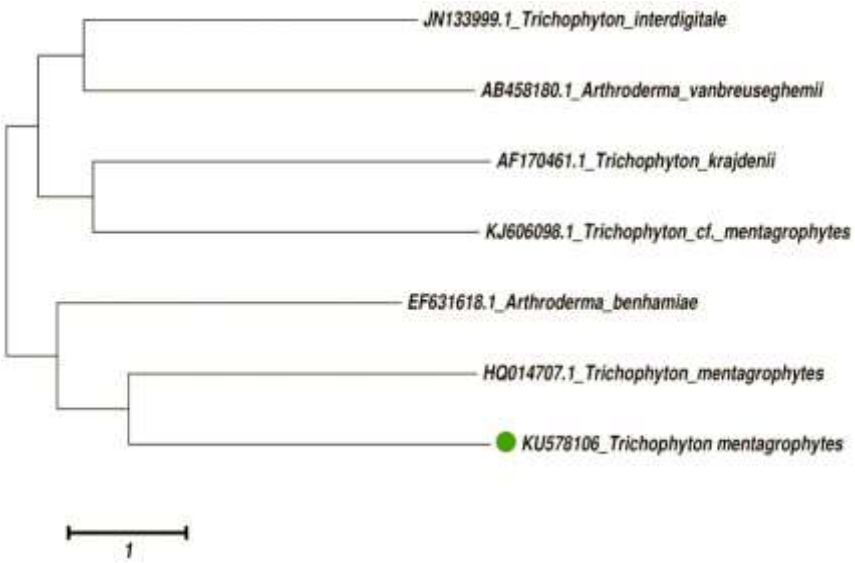

Figure 2: Neighbor joining tree of isolated *Trichophyton mentagrophytes* (KU5781026) with reference data.

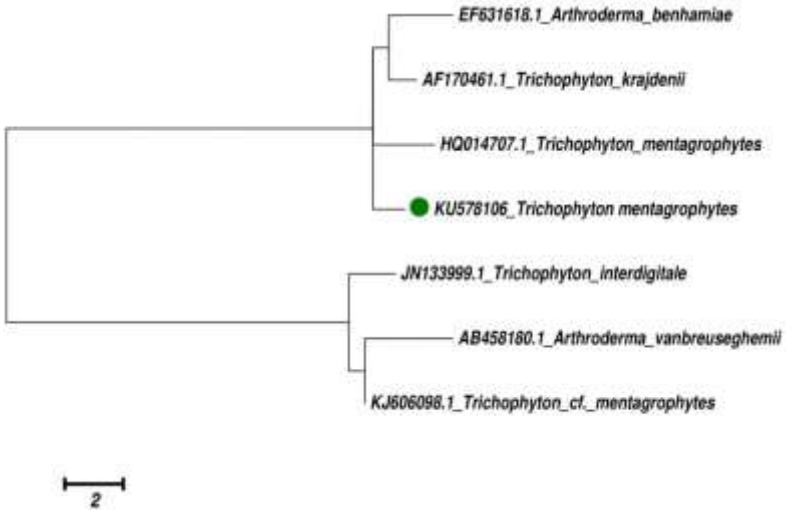

Figure 3: Maximum Likelihood tree of isolated *Trichophyton mentagrophytes* (KU5781026) with reference data

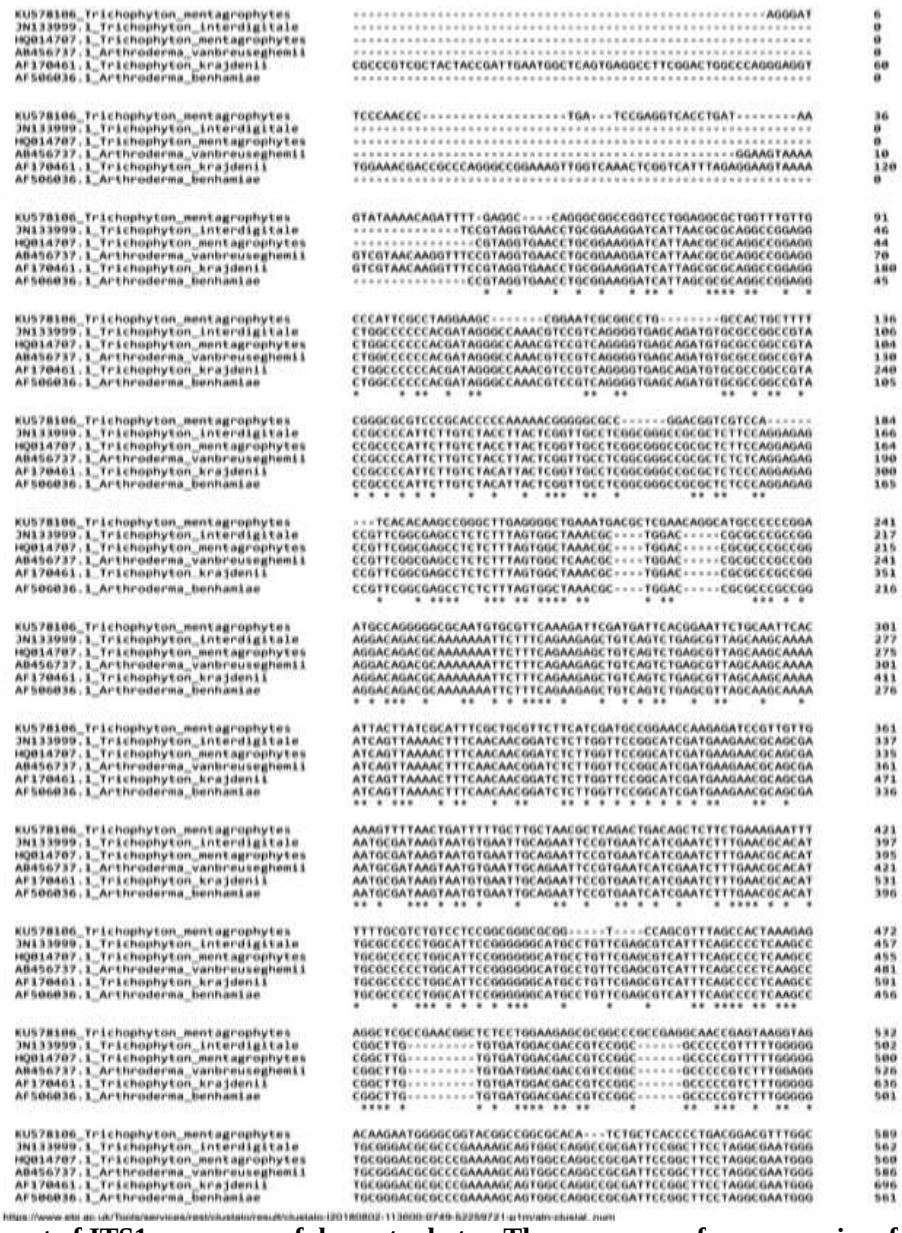

Figure 4: Alignment of ITS1 sequences of dermatophytes. The sequences of seven species of dermatophytes [one test (KU5781026) and six references sequences] were aligned by using the Clustal W (1.60) multiple sequence alignment.

Table 1
Effect of temperature and pH on the growth of *T. mentagrophytes*.

Initial pH	Final growth weight of mycelium (in gm)	Final pH of growth medium after growth
3	0	3.5
4	0	4.3
5	0.26±0.02	4.5
6	0.14±0.07	4.8
7	0.12±0.01	7
9	0.26±0.03	6.8
11	0.23±0.04	6.9
13	0	13

Initial Temp (in C°)	Final growth weight of mycelium (in gm)	Final pH of growth medium after growth
5	0.20±0.026	6.8
15	1.42±0.045	6.5
25	1.93±0.015	6.8
35	1.42±0.040	6.9
45	1.31±0.020	5.8
55	0.44±0.032	8.2

In NJ tree, *T. mentagrophytes* was shown to be closely allied with the simian-parasitizing species *T. mentagrophytes* but in turn, on a well-supported branch that contains another major lineage with anamorphs traditionally included in the *A. benhamiae* lineage. However, in case of ML tree, the reference sequence of *T. mentagrophytes* (KJ606098.1) was found to be closely allied with *A. vanbreuseghemi*, another anamorph while *T. mentagrophytes* (HQ014707.1 and KU5781026) was closely allied to *A. benhamiae* lineage. In ML tree results of *T mentagrophytes*, it was found to have a phylogenetic relationship within ITS1 homology groups by forming 02 major clusters. The tree was found with the highest log likelihood of -6547.1425.

Discussion

Dermatophytes are an opportunistic group of keratinophilic and keratinolytic allied molds which get an opportunity and affect host body as the parasite. They cause slow infectious diseases in human beings representing geographical limit[30]. In the present study, the *Trichophyton mentagrophytes* was found to be most predominant species which was also reported to be involved in different Tinea manifestations on various body areas of patients. The study was found to agree with the several researchers studies[30-34] who reported the *T. mentagrophytes* as predominant species followed by *T. rubrum, T. violaceum, T. tonsurans, T. simii* and *C. tropicum*.

The higher prevalence of *T. mentagrophytes* could be due to the fact that *Trichophyton* is a keratinophilic filamentous fungus with a high ability to invade keratinized tissue due to possession of several enzymes such as acid proteinases, elatinases, keratinases and other proteinases[20].

According to the present study, the growth of dermatophytes was found to be influenced by optimum temperature and pH that also may support to parasitism. The latter phenomenon has been studied earlier[32,35,36]. The ideal growth of test fungi was found to prefer 25°C temperature and pH 5 as well as high alkaline pH. Due to increasing incidences of dermatophytosis, the need for rapid and accurate identification of the etiological agent is responsible for the disease[18]. Molecular characterization provides a rapid and sophisticated method for identification of fungal species.

Results of the present study are in agreement with findings of Kumar and Shukla[37] who recorded the DNAs between 1.7 and 1.8 at the 260 nm/280 nm ratios. Ferrer et al[38] conducted a study using the ITS1, ITS4 and ITS86 primers to identify fungal strains which is in support of our results. The outcomes of present study are in agreement with findings of Hsiao et al[39] who also amplified the ITS region using fungal universal primers ITS1 and ITS4.

Ramaraj et al[40] obtained the amplicon size of 650-800bp for amplified regions (ITS-1 and ITS-2) of dermatophytes using primers ITS-1 and ITS-4 that is very close with our findings. These primers are skillful in annealing at different regions of the ribosomal gene.

Conclusion

The phylogenetic relationship based on the ITS1 DNA sequence alignment of meiosporic (perfect) and mitosporic (imperfect) states of the strains agreed with the proposed taxonomic connection in their sexual compatibility. In conclusion, *T. mentagrophytes* was found to show maximum growth in alkaline pH at 25ºC temperature which reveals that *T. mentagrophytes* might be an adaptive pathogen on the keratinous surface of humans and animals in preference of alkaline sweat and humidity.

Henceforth, the closest similarity of *T. mentagrophytes* with other allied geophilic-dermatophytic members in ITS homology group also anticipated that soils contaminated with keratinaceous debris have a huge reservoir of propagules of geophilic-dermatophytic fungi which prove to be pathogenic by accident or opportunistic attitude and also cause infections in human beings and animals.

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