Conventional and molecular characterization of *Trichophyton rubrum*

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Different studies illustrated that *Trichophyton rubrum*, among all species of *Trichophyton*, is the most prevalent and consequently the most important genus. *T. rubrum* as a worldwide filamentous pathogen fungus can infect human keratinized tissue (skin, nails and rarely hair), and causes dermatophytosis. Researchers use two general methods for the identification of dermatophytes namely, conventional methods on the basis of phenotype variations and molecular methods on the basis of molecular differences. Due to some limitations in traditional methods, in the recent years, molecular biological methods are regarded as useful in the exact and rapid recognition of dermatophytes. The present study identified nine clinical isolates and one ATCC as a control strain of *T. rubrum* by using both conventional and molecular methods. The molecular systematics method was used to elucidate genetic diversity among strains of *T. rubrum* and within *Trichophyton* species. Morphological characteristics of all colonies *T. rubrum* quite varies among each other; we revealed that that conventional methods are generally prolonged and may be indecisive. However, molecular studies based on internal transcribed spacer (ITS) sequencing provides a very accurate result, which is more than 96% the similarity of *T. rubrum* among all isolates, and more than 90% similarity within *Trichophyton* spp.

**Key words:** *Trichophyton rubrum*, conventional method, internal transcribed spacer (ITS) regions, identification, dermatophytes.

**INTRODUCTION**

*Trichophyton rubrum* is one of the most commonly encountered dermatophytes that infect human keratinized tissue such as skin, nails and possibly hair. This pathogen causes well-characterized superficial infections, and also produces skin infections in unusual parts of the body in immunodepressed patients (Cervelatti et al., 2004). Nearly 80% of onychomycosis due to *T. rubrum* and 90% of the chronic dermatophyte infections are caused mostly by *T. rubrum*, this pathogen developed mechanisms to avoid or suppress cell- mediated immunity ([Baeza et al., 2006; Baeza et al., 2007]).

Researchers use two general methods for the laboratory identification of various species of dermatophytes: a) identification on the basis of phenotype differences (conventional methods) and b) Identification on the basis of molecular differences. Faggi et al. (2001) mentioned that identification of dermatophyte species by conventional methods requires the examination of colony, particularly with the method of slide culture and microscopic morphological structures. Morphological and physiological features are dynamic. As a matter of fact, outside factors such as temperature variation, medium and chemotherapy, can greatly influence the phenotypic characteristics and consequently can make the identification more difficult.

Molecular biological methods, in the recent years, are regarded as useful in the exact and rapid recognition of
dermatophytes. Sequencing of the Internal Transcribed Spacer (ITS) region of the ribosomal DNA, Sequencing of protein-encoding genes, Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of mitochondrial DNA, Polymerase Chain Reaction (PCR); Random Amplification of Polymorphic DNA (RAPD), Arbitrarily Primed PCR [AP-PCR], and PCR fingerprinting are all instances of molecular techniques which have brought prominent advance in differentiating between species and strains (Faggi et al., 2001; Kanbe et al., 2003; Girgis et al., 2006; Yoshida et al., 2006; Li et al., 2007). In the recent years, quite a few molecular studies have been conducted on the internal transcribed spacer (ITS) region of the rRNA gene. Sequencing analysis of the ITS regions is considered as a useful tool for phylogenetic delineation and the identification of dermatophytes (Yoshida et al., 2006; Li et al., 2007).

Even though about 80 to 90% of all isolated are T. rubrum (Brasch and Hipler, 2008), has been isolated to identify the morphological similarity and the variability among this species, but only a few study has been done about the genetic relationship of Trichophyton.

The aim of this work is to identify ten clinical isolates of T. rubrum by using both conventional methods and molecular method based on universal fungal primers which are internal transcribed spacer 1 (ITS1). T. rubrum (ATCC-10218) was used as a control strain. The molecular systematic method was used to elucidate genetic diversity among strains of T. rubrum and within Trichophyton species.

MATERIALS AND METHODS

Isolates
Nine isolates of T. rubrum which are T. rubrum (1138), T. rubrum (1059), T. rubrum (1164), T. rubrum (1208), T. rubrum (1160), T. rubrum (1008), T. rubrum (1298), T. rubrum (1044) and T. rubrum (2970), were obtained from the culture collection of clinical isolates preserved at the laboratory of Medical Mycology Department in Tehran University of Medical Sciences, Iran for study; and T. rubrum (ATCC-10218) was used as a control strain. All clinical isolates were kept in sterile saline (0.85%) v/v NaCl at 4°C until required for bioassays.

Conventional method
All isolates of T. rubrum were cultured on Sabouraud dextrose agar media (Difco Laboratories, Detroit, Michigan) at 28°C for 14 days. Then, slide cultures of isolates were prepared and identified under light microscope (Carl Zeiss, Germany).

Molecular method
All isolates of T. rubrum maintained on Sabouraud’s dextrose agar medium and stored at 4°C. Then fungus was cultured in Sabouraud dextrose broth, and incubated at 28°C for 14 days. 200 to 300 mg of mycelia was harvested and centrifuged at 1600×g for 10 min, then washed twice with ice-cold sterile phosphate buffered saline (PBS) and finally stored at −70°C.

DNA extraction
Fungal genomic DNA from T. rubrum was isolated according to Rezaie et al. (2000) with slight modification. 200 to 300 mg of mycelia was ground with liquid nitrogen to powder form. 500 μl of DNA extraction buffer (50 mM Tris-HCl pH 8.0), 50 mM EDTA, 25 μl 20% SDS, and 10 μl of proteinase-K, was added and mixed gently. Then, incubated at 65°C for 60 min and centrifuged at 3000×g for 15 min. 25 μl Rnase H (10 mg/ml) was added to supernatant and incubated again at 37°C for 30 min. Then mixed with 500 μl of phenol:chloroform:isoamyl alcohol (25:24:1) and and centrifuged at 10000×g for 10 min and the supernatant were collected and transferred to new steril eppendorff tubes. Then mixed again with 500 μl of chloroform:isoamyl alcohol (24:1) and centrifuged at 10000×g for 10 min, and the supernatant were collected and transferred to new steril eppendorff tubes. DNA was precipitated by adding 500 μl isopropanol and 30 μl 3 M sodium acetate followed by centrifugation at 15000×g for 30 min and the supernatant was discarded. DNA pellet was rinsed twice or more with 200 μl of 70% cold ethanol and centrifuged at 10000×g for 10 min. The pellet was air-dry and resuspended DNA pellet in 30 μl of distilled water at 37°C for 60 min and stored at -20°C.

PCR amplification
Internal transcribed spacer 1 and 4 (ITS1 and ITS4) (AI-TBiotech, Singapore) were designed as ITS1 forward primer is 5’TCC GTA GGT GAA CCT GC-3’ and the ITS4 reverse primer 5’TCC TCC GCT TAT TGA TAT G-3’ (Shehata et al., 2008; Yang et al., 2008 ).

PCR reaction mixtures were prepared in a 25 μl volume containing 2.5 μl of 10× reaction buffer, 1.5 μl of 25 mM MgCl2, 0.5 μl of 10 mM dNTPs, 0.5 μl of 0.2 mM of each ITS 1 primer and ITS 4 primer, 0.5 μl of genomic DNA and 0.5 μl of 1 U Go Taq DNA polymerase (Promega Corporation, USA), and 18.5 μl of distilled water. PCR reactions were carried out on a thermal cycler (MJ Research, Inc. USA) with the following conditions: 1 cycle in an initial step of 94°C for 5 min and then subjected to 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s. After the last cycle, this was followed by a final extension step at 72°C for 10 min. Then, 5 μl of PCR product was loaded on 1% agarose in 1X Tris–Acetic Acid–EDTA buffer and stained with 0.5 mg/ml ethidium bromide at 80 V for 40 min and visualised with UV transilluminator (Alpha Innotech, USA), compared with a standard DNA size marker; 100 bp DNA ladder (Fernemenats, USA), and photographed in UV light.

PCR purification
DNA PCR products were purified according to the QIAquick PCR Purification Kit (Qiagen, Germany) and send for sequencing (1st Laboratories, Seri Kembangan, Malaysia).

RESULTS AND DISCUSSION

Morphological characteristics of colonies T. rubrum
This study used both conventional and molecular methods to diagnose ten isolates of T. rubrum. Studies revealed that colonies characterization of all isolates quite varies among each other. Of these isolates, isolate
numbers 1138 and 1059 are white and cottony or fluffy but isolates number 1164, 1160, 1008, and 1298 are cream, flat and downy, but the others are cream with a carmine and woolly or granular type (isolates numbers 1208, 1044, 2970 and 10218) (Figure 1). The microscopic features of the isolates also varies, which are macroconidia and microconidia of isolates numbers, 1138, 1008, 1298, 1044, 2970 and 10218 more abundant than isolates number 1059, 1164, 1208, and 1160. However, the shape of the macroconidia and microconidia of all isolates are almost similar, which is cyclindrical to cigar shaped (Figure 1).

Isolation, identification and molecular characterization of ITS1 of T. rubrum

Figure 2 showed that ITS1 of all isolates T. rubrum had been amplified and then were isolated and sequenced. The length of nucleotides sequence of all isolates are not similar which is T. rubrum (1138) 658 bp, T. rubrum (1059) 715 bp, T. rubrum (1164) 722 bp, T. rubrum (1208) 713 bp, T. rubrum (1160) 614 bp, T. rubrum (1160) 614 bp, T. rubrum (1008) 719 bp, T. rubrum (1298) 668 bp, T. rubrum (1044) 658 bp, T. rubrum (2970) 660 bp and T. rubrum (ATCC-10218) 633 bp. Nucleotide sequence of all isolates of T. rubrum and ATCC-10218 are shown in Figure 3. Previous studies by Rakeman et al. (2005) and Shehata et al. (2008) also revealed that the universal fungal primers amplified the ITS regions (ITS1-5.8S-ITS2) of the ribosomal DNA nearly 690 bp for T. rubrum isolates.

Nucleotide sequence of ten isolates of T. rubrum shown in Figure 3. All nucleotide sequences of T. rubrum isolates were analyzed using online software CLUSTALW (www.Pir.georgetown.edu/pirwww/search/multialn.shtml) to reveal the similarities among isolates. Figure 4 showed that the similarities among nine isolates of T. rubrum are higher, which is more than 96% identities.

Nucleotide sequence of isolates T. rubrum were analyzed using online software CLUSTALW (www.Pir.georgetown.edu/pirwww/search/multialn.shtml) to reveal the similarities among isolates T. rubrum and other species of Trichophyton which are Trichophyton raubitschekii strain NOMH 789 (GenBank accession no. AF170469), T. rubrum strain UAMH 8547 (GenBank accession no. AF170471), T. kanei (GenBank accession no. AF170460), T. rubrum strain WM 06.348 (GenBank accession no. EF568093), T. rubrum strain 05-287-3929 (GenBank accession no. EU200395), T. rubrum 5.8S rRNA (GenBank accession no. AJ270808), T. soudanense strain UAMH 8548 (GenBank accession no. AF170474), T. rubrum strain NCPF 295 (GenBank accession no. EU181449), T. megninii strain ATCC 12106 (GenBank accession no. AF170464), and T. rubrum strain ATCC 28188 (GenBank accession no. AF170472). The similarities of all isolates of T. rubrum and other species of Trichophyton is also higher than 90% as shown in Figure 5, CLUSTAL 2.0.12 multiple sequence alignment.

DISCUSSION

Traditional method such as investigation of macroscopic and microscopic features of cultures of fungi had been applied since early 19th century. However, these methods seem to be difficult to amplify due to the polymorphic feature of these characters, besides increased by differences in media compounds, temperature variations, and other variables of cultivation. Furthermore, in some cases, the dermatophytes fail to make reproductive organization in culture (sterile mycelia) that makes it impossible for final identification (Malinovschi et al., 2009). Besides that, conventional method is often difficult due to abnormal microscopic or macroscopic morphology (Li et al., 2008). Currently, molecular studies become crucial and necessary for identification of pathogenic fungi (Borman et al., 2008; Malinovschi et al., 2009). The internal transcribed spacer (ITS) regions of the fungal ribosomal DNA (rDNA) had been used as one of techniques for species identification because it is faster, accurate species determination, specific, and are less feasible to be affected by exterior effects such as temperature changes and chemotherapy (Girgis et al., 2006; Kong et al., 2008). Studies revealed that morphological characteristics of colonies of all isolates T. rubrum are similar to T. rubrum isolated from tinea cruris, tinea pedis, and tinea capitis of human (Graser et al., 2000). Colonies of T. rubrum are fluffy to cottony and white to cream in colour. Macroconidia are sparse or abundant and microconidia are present in all isolates.

In this studies, the length of ITS1 of all isolates is about 690 bp, 10 clinical isolates of T. rubrum were collected from the Clinical Mycology Laboratory at Westmead Hospital, Sydney, and the Women’s and Children's Hospital, Adelaide, Australia also have almost the same length of ITS1, which is 666 bp (Kong et al., 2008). Consequently, the results of our study are in agreement with these studies and showed that molecular method based on ITS sequencing is a reliable and useful method for the identification of dermatophytes as well as for confirmation of diagnosis of the conventional methods.

In this study, the molecular method was also used to clarify genetic diversity among strains of T. rubrum and within Trichophyton species. The results of this study regarding nucleotide sequence of isolates of T. rubrum demonstrated that the similarities among ten isolates of T. rubrum are more than 96% identities. It also showed the similarities among ten isolates of T. rubrum and ten isolates of other genus of Trichophyton are higher than 90%. The results of this study are in agreement with Graser et al. (2000) who showed that the Trichophyton species are supported by high similarities with value of
more than 86% among isolates of *T. rubrum* and isolates of other genus of *Trichophyton*. Our results are also in agreement with Li et al. (2008), who revealed that percentage identity of *Trichophyton* species with
Figure 2. PCR amplification of isolates of *T. rubrum* on 1% agarose gel electrophoresis. *T. rubrum* ATCC-10218 as positive control strain also showed DNA amplification at 690 bp.

> *T. rubrum* (1138)
> 50     NNNNGGGAGAGCGTAAGTGGGCTGCCACTATAGAGGACCGGACATTCCAT   
> 100   ...   
> 700 CAAAAGGGGGGAGGAAGAGGGGGGCCCCCCATAGGGGCCCCCCCCTTTTT 
> 722 TTTTGGGGTAGCGAGAAGGGGGGG

> *T. rubrum* (1059)
> 50     NCCAGTAACCGTAGGTGACCTGCGCATATCATAAGCGGAGGACTCCGTG   
> 100   ...   
> 700 CAAAAGGGGGGAGGAAGAGGGGGGCCCCCCATAGGGGCCCCCCCCTTTTT 
> 722 TTTTGGGGTAGCGAGAAGGGGGGG

> *T. rubrum* (1164)
> 50     CNNNNAGACCGTACGTTGCGCTGCGCATATCAGATAACCGGACATGACAT  
> 100   ...   
> 700 CAAAAGGGGGGAGGAAGAGGGGGGCCCCCCATAGGGGCCCCCCCCTTTTT 
> 722 TTTTGGGGTAGCGAGAAGGGGGGG

Figure 3. Nucleotide sequences of 9 isolates of *T. rubrum* and ATCC-10218. Nucleotide sequence numbering is shown on the left.
> *Trypanosoma rubrum* (1298)

50 TNNACCGAGCCTACGTGGGCTGGCTGGAAATATCGGGAACGGACATTCTCCGTGG
100 GGGTTCTGCCGCCTCGGCCTCGGGCCGCTACGCCCATCCTTCTGCTTACCCTACC
150 GGCCCTCTTGCTGGGCTGCTGGCAATACCGCGGAGGCACGGCGCGCCCGGAGAGCGACA
200 CCAAAAGAAATTCTCTAGAGAGACTGCGTGGTACGTTAGTGGTTATAGGAAGCA
250 AAACTTCTTTAAACATTCTTCAACAAAGGATCTTCTTGGTCTCCGCGCATCGATG
300 AGAAGCGAGCGAAATGCGGCTAAGTTGAATTTGGAATTGGAAGACCGCAATTACCG
350 AGGCCTGCGCTTCTCGCTTCTCGGCGGCAATCGTGCATAATAGGATAGGATAGGAT
400 AGGTTGCAGCCCTGCTTGACAGCCCAGGAGCCAGCGCAGCGCAGCGCAGCAGCGCAGC
450 GTGGACGACCGTCCGGCCCCTCCCTTCGGGGGCGGGACGCGCCCGAAAAGCAGTGCG
500 TGGGCCGCGCTTCTCTGGGAGCCTCGAGCCGGACCGCGCCCGCCGGAGGACAGAC
550 GTCCGGCGGGCCCCTTCTGGGAGCCTCGAGCCGGACCGCGCCCGCCGGAGGACAGAC
600 CATCGAATCTTTGAACGCACATTGCGCCCTCTGGCATTCCGGGGGGCATG
650 ACAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAA
700 AAGAACGCGGTTGGTCTCGGCGGGCCGCGCTCCCCCTGCCAGGGAGAGCCGTCCGG
750 GTGGACGACCGTCCGGCCCCTCCCTTCGGGGGCGGGACGCGCCCGAAAAGCAGTGCG

> *Trypanosoma rubrum* (1160)

50 TNNACCGAGCCTACGTGGGCTGGCTGGAAATATCGGGAACGGACATTCTCCGTGG
100 GGGTTCTGCCGCCTCGGCCTCGGGCCGCTACGCCCATCCTTCTGCTTACCCTACC
150 GGCCCTCTTGCTGGGCTGCTGGCAATACCGCGGAGGCACGGCGCGCCCGGAGAGCGACA
200 CCAAAAGAAATTCTCTAGAGAGACTGCGTGGTACGTTAGTGGTTATAGGAAGCA
250 AAACTTCTTTAAACATTCTTCAACAAAGGATCTTCTTGGTCTCCGCGCATCGATG
300 AGAAGCGAGCGAAATGCGGCTAAGTTGAATTTGGAATTGGAAGACCGCAATTACCG
350 AGGCCTGCGCTTCTCGCTTCTCGGCGGCAATCGTGCATAATAGGATAGGATAGGAT
400 AGGTTGCAGCCCTGCTTGACAGCCCAGGAGCCAGCGCAGCGCAGCGCAGCAGCGCAGC
450 GTGGACGACCGTCCGGCCCCTCCCTTCGGGGGCGGGACGCGCCCGAAAAGCAGTGCG
500 TGGGCCGCGCTTCTCTGGGAGCCTCGAGCCGGACCGCGCCCGCCGGAGGACAGAC
550 GTCCGGCGGGCCCCTTCTGGGAGCCTCGAGCCGGACCGCGCCCGCCGGAGGACAGAC
600 CATCGAATCTTTGAACGCACATTGCGCCCTCTGGCATTCCGGGGGGCATG
650 ACAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAA
700 AAGAACGCGGTTGGTCTCGGCGGGCCGCGCTCCCCCTGCCAGGGAGAGCCGTCCGG
750 GTGGACGACCGTCCGGCCCCTCCCTTCGGGGGCGGGACGCGCCCGAAAAGCAGTGCG

**Figure 3.** Cotnd.
Figure 3. Cotnd.

Figure 4. Comparison of nucleotide sequence between *T. rubrum* ITS1 orthologues. Nucleotide sequences that are present in all ITS1 are shaded in blue colour. Nucleotide sequence numbering is shown on the right.
Figure 4. Contd.
**Figure 4.** Contd.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence</th>
<th>Position</th>
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<tr>
<td>T. rubrum (1138)</td>
<td>CATATGAAAG-</td>
<td>727</td>
</tr>
<tr>
<td>T. rubrum (1164)</td>
<td>TTTCCTTTGCGGAAACCAAATGGG-</td>
<td>727</td>
</tr>
<tr>
<td>T. rubrum (1208)</td>
<td>TCCTCTTTTGCGGAAACCAAATGGG-</td>
<td>727</td>
</tr>
<tr>
<td>T. rubrum (1044)</td>
<td>--</td>
<td>727</td>
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<tr>
<td>T. rubrum (ATCC-10218)</td>
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<td>727</td>
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<tr>
<td>T. rubrum (2970)</td>
<td>--</td>
<td>727</td>
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<tr>
<td>T. rubrum (1059)</td>
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<td>727</td>
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<td>T. rubrum (1160)</td>
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<td>727</td>
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<tr>
<td>T. rubrum (1298)</td>
<td>--</td>
<td>727</td>
</tr>
<tr>
<td>T. rubrum (1008)</td>
<td>AGGGTGTGGAGACCGCGCCGC-</td>
<td>727</td>
</tr>
</tbody>
</table>

**Figure 5.** Comparison of nucleotide sequence between *T. rubrum* ITS1 orthologues. Nucleotide sequences that are present in all ITS1 are shaded in black colour. Nucleotide sequence numbering is shown on the right.
Figure 5. Contd.
**Figure 5. Contd.**

T. raubitschekii strain NOMH 789

T. rubrum strain NOMH 789

T. megnini strain ATCC 12106

T. saudanese strain ATCC 12106

T. rubrum strain UAMH 8547

T. saudanese strain UAMH 8547

T. rubrum strain ATCC 28188

T. kanei

T. rubrum 5.8S rRNA gene

T. rubrum strain WM 06.348

T. rubrum strain NCPF 295

T. rubrum strain 05-287-3929

T. rubrum (1138)

T. rubrum (1168)

T. rubrum (1298)

T. rubrum (1008)

T. rubrum (1059)

T. rubrum (1208)

T. rubrum (1264)

T. rubrum (2970)

T. rubrum (ATCC-10218)

T. rubrum (1044)

T. raubitschekii strain NOMH 789

T. megnini strain ATCC 12106

T. saudanese strain ATCC 12106

T. rubrum strain UAMH 8547

T. rubrum strain ATCC 28188

T. kanei

T. rubrum 5.8S rRNA gene

T. rubrum strain WM 06.348

T. rubrum strain NCPF 295

T. rubrum strain 05-287-3929

T. rubrum (1138)

T. rubrum (1164)

T. rubrum (1298)

T. rubrum (1008)

T. rubrum (1059)

T. rubrum (1208)

T. rubrum (1264)

T. rubrum (2970)

T. rubrum (ATCC-10218)

T. rubrum (1044)

T. raubitschekii strain NOMH 789

T. megnini strain ATCC 12106

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T. rubrum (1264)

T. rubrum (2970)

T. rubrum (ATCC-10218)

T. rubrum (1044)
Figure 5. Contd.
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T. rubrischekii strain NOMH789
T. megnini strain ATCC 12106
T. saudanese UAMH 8548
T. rubrum strain UAMH 8547
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T. rubrum (2970)
T. rubrum (ATCC-10218)
T. rubrum (1044)

**REFERENCES**


Figure 5. Contd.

Concluding remarks

By conventional characterization, colonies of all isolates quite varies; however, the shape of macroconidia and microconidia are similar. Beside that, molecular characterization also revealed that all isolates of *T. rubrum* show high similarity among them and with other *Trichophyton* species.

**ACKNOWLEDGEMENT**

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reference sequence in GenBank (BLAST search) ranged from 85.9 to 100%.

**Conclusion**

By conventional characterization, colonies of all isolates quite varies; however, the shape of macroconidia and microconidia are similar. Beside that, molecular characterization also revealed that all isolates of *T. rubrum* show high similarity among them and with other *Trichophyton* species.