Identification of *Trichophyton tonsurans* by Random Amplified Polymorphic DNA

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Background : T. *tonsurans* is an anthropophilic dermatophyte mostly causing tinea capitis and tinea corporis. In East Asian countries, it has rarely been isolated until now. However, it is necessary for researchers in Asian countries to be more accustomed to T. *tonsurans* than before because of frequent international sports exchanges nowadays.

Objectives : This study was performed to identify T. *tonsurans* by random amplified polymorphic DNA (RAPD) analysis.

Methods : Fifteen strains which were tentatively identified as T. *tonsurans* in Brazil were identified again by several conventional mycological tests and RAPD analysis.

Results : Among 15 Brazilian strains, 3 were identified as T. *tonsurans*, 8 T. *mentagrophytes*, 3 T. *rubrum* and 1 T. *raubitschekii* by conventional mycological tests. This result was examined again by RAPD analysis.

Conclusion : RAPD analysis is considered a rapid and reliable method for identification of T. *tonsurans* if the procedure is carefully standardized with adequate primers.

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Key Words : *Trichophyton tonsurans*, Random amplified polymorphic DNA, Dermatophyte, Identification, Fungus

Trichophyton tonsurans is an anthropophilic species mostly causing tinea capitis and tinea corporis[1-3]. It is worldwide in distribution and particularily prevalent in both American continents[1-6]. In East Asian countries, it has rarely been isolated until now[7-10]. Transmission of *T. tonsurans* has been shown to occur through direct human to human contact and by a variety of fomites[1-4]. Athletic events that involve close personal contact between competitors, such as wrestling, carry a high risk of transmission of communicable diseases including dermatophytoses[11-13]. In Korea, since tinea capitis due to *T. tonsurans* was first reported in 1995[8], an epidemic of tinea capitis and tinea corporis caused by this species has occured among amateur wrestlers[14]. Therefore, it is necessary for medical mycologists in Asian countries to be more accus-

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Table 1. Fungi used in this study which were tentatively identified as *T. tonsurans* in brazil

Test code	Original code	Test code	Original code
B1	PE 0001	B9	PE 0007
B2	SP 0001	B10	SP 0003
B3	SP 0002	B11	SP 0004
B4	PE 0002	B12	SP 0005
B5	PE 0003	B13	SP 0006
B6	PE 0004	B14	SP 0007
B7	PE 0005	B15	SP 0008
B8	PE 0006		

tomed to *T. tonsurans* than before because of frequent international sports exchanges these days[8,10,15].

T. tonsurans shows various colony colors and textures and its teleomorph has not been reported yet. So, differentiation of this fungus from other dermatophytes, such as *T. mentagrophytes* or *T. rubrum* is sometimes difficult especially for medical mycologists in the nations unfamiliar to it[8]. Conventional mycological tests such as observation of gross and microscopic morphologies, responses to thiamine supplement and urease test are useful for differentiation of *T. tonsurans* from other dermatophytes but these tests are time-consuming, need experience and sometimes give ambiguous results. Therefore, the development of more rapid and precise identification by DNA-based methods has been long awaited for this species. However, *T. tonsurans* was not well differentiated from other dermatophytes by DNA-based methods[16-19]. There were no differences in patterns between *T. tonsurans* and *T. mentagrophytes* by restriction fragment length polymorphism (RFLP) of mitochodrial DNA[16,17]. Ribosomal DNA of *T. tonsurans* was amplified with a specific primer for that of *T. mentagrophytes*[18]. Its nuclear DNA band pattern was same with those of *T. equinum* or *T. mentagrophytes* by RAPD analysis[19]. Two other papers in which *T. tonsurans* could be differentiated from other dermatophyte species by RAPD analysis described one strain of *T. tonsurans*, respectively[20,21]. Recently, we have standardized the RAPD analysis with three random primers (ATGS, 5'-ATGGATC(G,C)(G,C)C-3'; ATG, 5'-ATGGATCGGC-3'; OPAO-15, 5'-GAAGGCTCCC-3': Operon Technologies, U.S.A.) which was useful for the identification of the most of common dermatophytes such as 7 *Trichophyton* species, 3 *Microsporum* and 3 *Arthroderma*, respectively. This study was carried out to identify 15 Brazilian isolates by RAPD analysis which we have standardized before.

Table 2. Fungi used in this study as reference strains

Species	Test code	Source	Isolation region
T. tonsurans	Tt1	IFM 5275	Japan
	Tt2	IFM 5276	Japan
	Tt3	IFM 5274	U.S.A.
	Tt4	IFM 45545	Taiwan
	Tt5	IFM 45546	Taiwan
	Tt6	IFM 41127	Brazil
	Tt7	IFM 41128	Brazil
T. mentagrophytes	Tm1	clinical isolate*	Korea
	Tm2	clinical isolate*	Korea
	Tm3	clinical isolate*	Korea
T. rubrum	Tr1	clinical isolate*	Korea
	Tr2	clinical isolate*	Korea
T. raubitschekii	Tra1	IFM 45577	Japan
	Tra2	IFM 45578	Japan

* Isolates from skin lesions of patients in Taegu, Korea
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MATERIALS AND METHODS

Organisms

Twenty-nine strains of dermatophytes were used in this study. Fifteen strains which were tentatively identified as *T. tonsurans* in Brazil (Table 1) were included. Seven strains of *T. tonsurans* isolated from 2 Japanese, 1 American, 2 Taiwanese, 2 Brazilian patients and preserved in IFM culture collection were used as references. Three strains of *T. mentagrophytes* var. *interdigitale*, 2 *T. rubrum* and 2 *T. raubitschekii* were also included as references (Table 2).

Gross and microscopic morphologies

Gross morphologies were observed after culturing on Sabouraud dextrose (2%) agar at 25℃ for 28 days. Colonies of *T. tonsurans* were classified into 3 types - powdery to suedelike type which is the typical morphology of this species, cottony to velvety type, and glabrous type. Microscopic morphologies were observed by slide cultures on potato dextrose agar at 25℃ for 14 days.

Thiamine requirement test

Responses to thiamine supplement were observed by comparing the growth on vitamine-free casamino acids agar (Trichophyton agar #1, Difco, U.S.A.) and that on thiamine-supplemented casamino acids agar (Trichophyton agar #4, Difco, U.S.A.) after culturing at 25℃ for 7 and 14 days.

Red pigment production on potato dextrose agar (PDA)

Pigment production was observed after culturing on potato dextrose agar (Difco, U.S.A.) at 25℃ for 2 and 4 weeks. The color was compared to blood-red pigment produced by *T. rubrum*.

Urease production

Urease production was examined by culturing on Christensen Urea Agar (Eiken Chem. Co., Japan) and in Christensen Urea Broth (Difco, U.S.A.) at 25℃. Color change was observed after 7 and 14 days. Bright pink was considered as positive and yellow as negative.

***In vitro* hair perforation**

Hair obtained from a 2-year-old female Japanese child was autoclaved (15 psi/15 min) and dispersed into sterile, 0.05% yeast extract solution (Difco, U.S.A.). A small amount of fungal mycelium and conidia was inoculated into the solution and the hair was observed after culturing at 25℃ for 7 and 14 days. Formation of deep, narrowly wedge-shaped perforation in hair shafts, so called perforation body, was considered as positive.

Preparation of fungal DNA

Genomic DNA was extracted by the method described by Makimura et al.[22]. Brifely, a small amount of fungal mycelium was harvested into an Eppendorf tube with 600μl of extraction buffer (200 mM Tris-HCl, pH 7.5 with 25 mM EDTA, 0.5 w/v % sodium dodecyl sulfate and 250 mM NaCl). After being crushed with a pellet pestle (Kontes, U.S.A.), the mixture was incubated at 100℃ for 15 min, kept at -20℃ for 1 hr and centrifuged at 12,000 rpm for 10 min. Supernatant was transferred into new tubes and DNA was precipitated as usual. Precipitated DNA was solubilized and diluted to 10 ng/μl in distilled water.

RAPD analysis

RAPD analysis was performed in a total reaction volume of 100μl, containing 5μl of genomic DNA (50 ng), 8μl of dNTP (2.5 mM of each of dATP, dCTP, dGTP and dTTP), 0.5μl of 5 U/μl of Taq polymerase (Perkin Elmer, U.S.A.), 10μl of 10× PCR buffer, 2.5μl of 20 pM of primer, and 74 μl of distilled water. $MgCl_2$ was contained in 10X PCR buffer and the final concentration of $MgCl_2$ of the reaction mixture was 1.5 mM. Three random primers (ATGS, 5'-ATGGATC(G,C)(G,C)C-3'; ATG, 5'-ATGGATCGGC-3'; OPAO-15, 5'-GAAGGCTCCC-3': Operon Technologies, U.S.A.) were used. Primer ATGS is a mixture of 4 components, 5'-ATGGATCCGC-3', 5'-ATGGATCGCC-3', 5'-ATGGATCCCC-3' and primer ATG. The reaction was performed in PCR Thermal Cycler MP (Takara, Japan) according to the following protocol: one cycle of denaturation at 94℃ for 2 min, 40 cycles

Table 3. Results of mycological tests of preserved strains of *T. tonsurans*

Strain	Morphology		Thiamine requirement	Red pigment on PDA	Urease production	*In Vitro* hair perforation
	Gross*	Microscopic				
Tt1	glabrous	*T. tonsurans*	+	−	+	−
Tt2	powdery	*T. tonsurans*	+	−	+	−
Tt3	suedelike	*T. tonsurans*	+	−	+	+
Tt4	suedelike	*T. tonsurans*	±**	−	−	−
Tt5	suedelike	*T. tonsurans*	±**	−	+	−
Tt6	powdery	*T. tonsurans*	+	−	+	−
Tt7	powdery	*T. tonsurans*	+	−	+	−

* Gross morphologies were observed after culturing on Sabouraud dextrose (2%) agar at 25℃ for 28 days.

** The mark '±' means that the strain grew considerably on vitamine-free casamino acids agar that its requirement for thiamine was not strict.

Table 4. Results and identification concluded from mycological tests of 15 Brazilian isolates

Strain	Morphology		Thiamine	Red pigment	Urease	*In Vitro* hair	Identification
	Gross	Microscopic	Requirement	on PDA	production	perforation	
B1	suedelike	*T. tonsurans*	+	−	+	−	*T. tonsurans*
B2	cottony	*T. tonsurans*	±**	−	+	−	*T. tonsurans*
B3	suedelike	*T. tonsurans*	+	−	+	−	*T. tonsurans*
B4	cottony	*?T. menta**	−	−	+	+	*T. menta*
B5	suedelike	*Trichophyton* sp.	−	−	+	+	*T. menta*
B6	cottony	*?T. menta*	−	−	+	+	*T. menta*
B7	powdery	*T. menta*	−	−	+	+	*T. menta*
B8	cottony	*T. menta*	−	−	+	+	*T. menta*
B9	cottony	*?T. menta*	−	−	+	+	*T. menta*
B10	suedelike	*T. menta*	−	−	+	+	*T. menta*
B11	powdery	*T. menta*	−	−	+	+	*T. menta*
B12	powdery	*?T. rubrum*	−	+(brown)	−	−	*T. rubrum*
B13	velvety	*?T. rubrum*	−	+(pink)	−	−	*T. rubrum*
B14	suedelike	*?T. rubrum*	−	+(pink)	−	−	*T. rubrum*
B15	suedelike	*?T. rubum*	−	+(pink)	+	−	*T.raubitschekii*

* *T. menta* : *T. mentagrophytes*

** The mark '±' means that the strain grew considerably on vitamine-free casamino acids agar that its requirement for thiamine was not strict.

Table 5. Four species of dermatophytes used in this study and their bands (kbp) by RAPD analysis with primers ATGS, ATG and OPAO-15

Species	ATGS	ATG	OPAO-15
T. tonsurans	1.70, 0.82, 0.56, 0.31	1.34, 0.98, 0.92, 0.87	1.28, 0.94
T. mentagrophytes	1.70, 1.12, 0.82, 0.60, 0.48, 0.31	1.87, 0.98, 0.92, 0.87, 0.75, 0.68	1.42, 1.27
T. rubrum	0.60, 0.36, 0.28	1.35, 1.18, 0.99, 0.88, 0.69	1.03, 0.75
T. raubitschekii	0.60, 0.36, 0.28	1.35, 1.18, 0.99, 0.88, 0.69	1.03, 0.75

of denaturation at 94℃ for 1 min, annealing at 34℃ for 1 min, extension at 72℃ for 2 min, and finally, extension at 72℃ for 10 min. The PCR products were visualized by UV light after separation by electrophoresis on 1.2% agarose gel and staining with ethidium bromide. As a size marker, a mixed solution of Hind III digest of lamda DNA and Hae III digest of φ X174 DNA was used.

RESULTS

Gross morphologies on Sabouraud dextrose (2%) agar

Among 7 preserved IFM strains of *T. tonsurans*, 6 showed powdery to suedelike colonies and 1 glabrous colony (Table 3). Among 15 Brazilian isolates, 9 were powdery to suedelike, 6 were cottony to velvety (Table 4).

Microscopic morphologies

Seven IFM strains showed typical microscopic findings of *T. tonsurans*, pear- or club-shaped microconidia borne on the tips of conspicuous lateral stalks occasionally with spherical balloon forms (Table 3). Three of 15 Brazilian strains (B1, B2, B3) showed the typical microscopic findings of *T. tonsurans*. Seven strains (B4, B6, B7, B8, B9, B10, B11) were considered as *T. mentagrophytes* by ob-

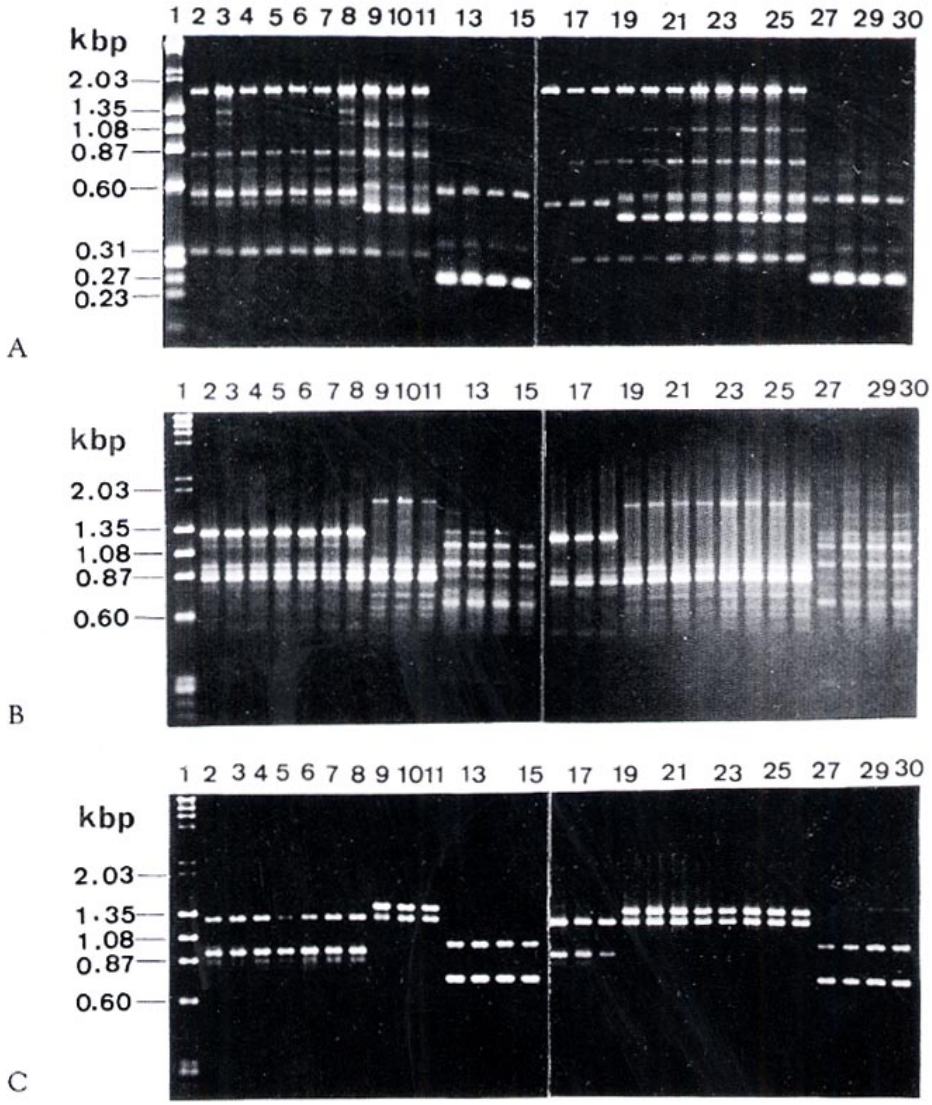

Fig. 1. Band patterns of reference strains of dermatophytes and 15 Brazilian isolates.
Fig. 1A, RAPD analysis with primer ATGS, 5'-ATGGATC(G,C)(G,C)C-3'; Fig. 1B, with primer ATG, 5'-ATGGATCGGC-3'; Fig. 1C, with primer OPAO-15, 5'-GAAGGCTCCC-3'. Lane 1, size marker; lane 2 to 8, control strains of *T. tonsurans* (Tt1 to Tt7); lane 9 to 11, *T. mentagrophytes* (Tm1 to Tm3); lanes 12 and 13, *T. rubrum* (Tr1 and Tr2); lanes 14 and 15, *T. raubitschekii* (Tra1 and Tra2); lane 16 to 30, Brazilian isolates (B1 to B15). For abbreviations, see Tables 1 and 2.

serving spherical, grouped microconidia with or without macroconidia, and spiral bodies. Four strains (B12, B13, B14, B15) were considered as *T. rubrum* by observing peg-shaped, sessile microconidia (Table 4).

Response to thiamine stimulation

Among 7 IFM strains of *T. tonsurans*, 5 grew barely on the vitamine-free casamino acids agar and their growth was strongly stimulated on thiamine-supplemented agar. Two strains (Tt4, Tt5) grew considerably on vitamine-free casamino acids agar, so their requirement for thiamine was not so strict (Table 3). Three Brazilian strains were stimualted by thiamine supplement, 2 (B1, B3) strongly and 1 (B2) weakly. The other 12 strains did not show any requirement for thiamine (Table 4).

Red pigment production on PDA

Four Brazilian isolates produced reddish pigment on PDA. B12 showed brownish red color, diffused into media. The color of the other 3 (B13, B14, B15) was pink, lighter than typical blood-red pigment produced by *T. rubrum* or *T. raubitschekii*.

Urease production

All IFM strains of *T. tonsurans* except for one strain (Tt4) changed the color of both urese test media to pink(Table 3). All Brazilian strains changed the color of both media to pink except for 3 strains (B12, B13, B14) (Table 4).

***In vitro* hair perforation**

One IFM strain (Tt3) of *T. tonsurans* produced perforating bodies abundantly, indistinguishable from those of *T. mentagrophytes*. Eight Brazilian strains (B4 to B11) produced perforating bodies abundantly (Table 4).

RAPD analysis

Reference strains of *T. tonsurans*, *T. mentagrophytes*, *T. rubrum* and *T. raubitschekii* showed distinct band patterns with three random primers ATGS (Fig. 1A), ATG (Fig. 1B) and OPAO-15 (Fig. 1C), and the yielded bands were summarized in Table 5. With primers ATGS and ATG, some bands of *T. tonsurans* were at the same positions with those of *T. mentagrophytes*, but these two speices were differentiated from each other by other different bands. Moreover, these two species differentiated more clearly by using primer OPAO-15. *T. rubrum* and *T. raubitschekii* generated same bands with all three primers, so they were not differentiated. Among 15 Brazilian strains, 3 generated identical bands with those of *T. tonsurans*, 8 *T. mentagrophytes*, 4 *T. rubrum* (or *T. raubitschekii*) with three primers (Fig. 1 A, B, C).

DISCUSSION

Recently, we have standardized the RAPD analysis with three random primers ATGS, ATG and OPAO-15 to identify the common dermatophytes. In this study, we applied our RAPD analysis method to confirm the identification of 15 strains which were tentatively identified as *T. tonsurans* in Brazil. The gross morphologies of 15 Brazilian isolates were very confusing. For example, 3 strains (B13, B14, B15) which were confirmed as *T. rubrum* or *T. raubitschekii* later, were very similiar to *T. tonsurans* because they showed yellowish, sudedelike colonies and their reverse were yellow in color. One strain which was identified as *T. tonsurans* (B2) looked like *T. rubrum* because it had a whitish colony with a cottony, centrally mounded surface. Even by microscopic observations, only half of them were identified. So, several physiological tests were required to complement the morphological observations.

It is well known that the growth of *T. tonsurans* is suppressed by thiamine depletion and strongly stimulated by its supplement[1-3]. However, 2 of 7 preserved IFM strains of *T. tonsurans* were only slightly suppressed on the thiamine-free agar. The tests were repeated three times with care not to carry-over isolation media[2]. Although the thiamine requirement test is most helpful for the diagnosis of *T. tonsurans*, it is necessary to realize that there is a little variation in the degree of requirement. About red pigment production on PDA, 4 Brazilian isolates produced brownish red or pink pigment, different from typical blood-red color of *T. rubrum* or *T. raubitschekii*. Later, these 4 strains produced typical blood-red color on PDA after decontamination of bacteria. So it was supposed that these 4 strains were confused with *T. tonsurans* because of their red color production were inhibited by bacterial contamination[2]. *T. tonsurans* is usually positive in urease test and usually negative but sometimes positive in *in vitro* hair perforation test[2]. So, these two tests are helpful to rule out *T. rubrum* but do not differentiate *T. tonsurans* from *T. mentagrophytes*. Putting together all of the results of mycological tests, among 15 Brazilian strains, 3 were identified as *T. tonsurans*, 8 *T. mentagrophytes*, 3 *T. rubrum* and 1 *T. raubitschekii*. One Brazilian strain(B15) was identified as *T. raubitschekii* due to urea hydrolysis[2,3].

The identification by conventional mycological tests was confirmed by RAPD analysis which we have standardized in our previous study. Three Brazilian strains showed the identical band patterns with that of *T. tonsurans*, 8 *T. mentagrophytes*, and 4 *T. rubrum* (or *T. raubitschekii*). The latter 2 species showed identical band patterns with all 3 primers, so they could not be separated as two different species. *T. rubrum* and *T. raubitschekii* were also considered as same species in a study by

RFLP of mitochondrial DNA[17]. To clarify the relationship between *T. rubrum* and *T. raubitschekii*, further studies are needed. For the consistent identification of *T. tonsurnans* by using conventional mycological tests, it takes at least 2-3 weeks after isolating a causative fungus by culture. In case of RAPD analysis, identification could be obtained within a day - 3 hours for extraction of DNA from cultured mycelium, 3 and a half hours for PCR, and 2 hours for electrophoresis and staining.

In conclusion, for the identification of *T. tonsurans*, several physiological tests are necessary in addition to gross and microscopic morphologic findings. RAPD analysis is considered a rapid, reliable and easy method for identification of *T. tonsurans* if the procedure is carefully standardized with adequate primers. RAPD analysis may be especially helpful for the medical mycologists in Asian countries who are not accustomed to *T. tonsurans*.

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