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Phenotypical and Molecular Characterization of *Microsporum canis* Strains in North-Tunisia

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Abstract

In this study, 40 *Microsporum canis* isolates were obtained from different patients from the Mycology Unit of the Hospital La Rabta (Tunis) during a 3 month period. The phenotypic identification was done by morphological characterization and biochemical tests. Molecular analysis was performed by amplification of the ITS region of rDNA, the amplified region was subjected to enzymatic digestion and sequenced to evaluate phylogenetic relationships. The morphological analysis showed a considerable diversity of colonies as well as different morphologies of conidia and we have noted variability in the assimilation of the nitrogen and carbon sources. The PCR-RFLP results showed only one restriction pattern for each enzyme. The phylogenetic tree proves that all the strains from Tunisian patients are clonal and related with other strains from different origins. The classical methods used in the mycological laboratories are time-consuming, the PCR-RFLP analysis of the ITS is a reliable tool for the identification of *M. canis* strains. *M. canis* from infected Tunisian patients are clonal, although the isolates had different phenotypic characteristics.

Key words: *Microsporum canis*, assimilation of carbon, assimilation of nitrogen, PCR-RFLP, Sequencing

Introduction

Microsporum canis constitutes the main species spreading from animals to humans and causing especially *tinea capitis.* Sometimes it can reach the nails, and in exceptional cases the pubic area in the form of *tinea circinata*. Several epidemiological studies undertaken in Tunisian areas revealed the re-emergence of *M. canis*, (Meziou *et al*., 2011) which did not exist in Tunisia before 1950 and of which the frequency has been increasing. There is not much information available on the molecular epidemiology of *M. canis* isolated from patients in Tunisia. Only classical identification was performed in the mycological laboratories in Tunisia.

Furthermore, identification at the strain level might be necessary for specification of the etiologic agent. Different molecular methods were used, such as restriction fragment length polymorphism analysis of mitochondrial (Kawasaki *et al.,* 1996) , sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA (Graser *et al*., 2000; Sharma *et al*., 2006), sequencing of protein-encoding genes (Jung *et al*., 2014; Kano *et al*., 2000), random amplification of polymorphic DNA (Mochizuki *et al*., 1997) arbitrarily primed PCR (Liu *et al.,* 1997; Liu, *et al*., 2000) and PCR fingerprinting (Graser *et al*., 1998), have brought important progress in distinguishing between species and strains. In addition, the ribosomal regions consisting of the internal transcribed spacers (ITS-1 and -2) and their intermediary 5.8S rDNA are highly variable regions of fungal DNA and exhibit adequate variability to determine the phylogeny of closely interrelated filamentous fungi (Turin *et al*., 2000). These results indicate that PCR-RFLP targeting the ITS regions is a valuable tool for species identification of dermatophytes (Toshio, 2008). The present study aimed to characterize by means of phenotypic and molecular techniques a collection of 40 clinical isolates established in Tunisia from patients with *M. canis* infection. The assimilation of different nitrogen and carbohydrate sources was the preliminary biochemical tool used with the on the total collection.

This study aims to characterize a collection of *M. canis*, established from Tunisian patients, by using phenotypic and molecular tools.

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Experimental

Materials and Methods

Origin of fungal strains. A total of 40 clinical isolates of *M. canis* was obtained from patients seen at the Unit of Mycology in the La Rabta Hospital of Tunis (Tunisia). All patients originated from the North of Tunisia, in the majority (95%) affected with *tinea capitis* and only two human cases (5%) with *sycosis pubien* and *onychomycosis*. *M. canis* was detected in children from 2 to 19 years old with 77.5% males and 22.5% females. The disease originated in the majority of cases from contact with animals (cat, dog and hamster) while few had an anthropophilic origin. Each isolate was transferred to Sabouraud dextrose agar (SAB) medium and then incubated at 25°C for 10 days and identified by macroscopic and microscopic characteristics of the culture strains.

Assimilation of carbon and nitrogen sources. The assimilation of carbon and nitrogen sources was tested according to Maia *et al.* (2001). For carbohydrate assimilation, yeast nitrogen base (Biolife, Italia) was prepared 6.7 g/l in distilled water patched in the plugged tube and then autoclaved at 121°C for 15 min. A stock solution of 15% for carbohydrates (mannitol, maltose, mannose, sorbitol, dextrin, dulcitol, lactose, xylose, galactose) was used. Each solution was sterilized by filtration through a filter (Millipore). A control without carbohydrate was included in each test to give a final concentration of 10 g/l. For nitrogen assimilation the basal medium consisted of yeast carbon base (Biolife, Italie) 11.7 g/l in distilled water, and autoclaved as described above. A stock solution of 15% for nitrogen sources were prepared and added to medium to give a final concentration of nitrogen equivalent to 2 g/l sodium nitrate, 2.6 g/l asparagines, 2.4 g/l ammonium sulphate and 0.225 g/l urea. The strains were cultivated on HL-agar medium (sodium chloride 5 g/l, dipotassium hydrogen phosphate 0.3 g/l, peptone 2 g/l and agar 15 g/l) for 2 weeks at 25°C, and then a small portion of each mycelium was inoculated into each tube. The growth of each mycelium strain has been observed after 5 days of inoculation. Isolates were grouped using the cluster analysis UPGMA (Unweighted Pair Group Method with Arithmetic Average) (Nei *et al.*, 1979).

Preparation of monosporic culture of *Microsporum canis*. The conidial suspension of each pure culture was prepared by flooding 10-day-old cultures of *M. canis* on SAB dishes with 4.5 ml of sterile distilled water and dislodging the spores with a glass rod. After serial dilution, 0.05 ml aliquots were spread-plated onto SAC plates and incubated at 25°C during 48 h. Germinated conidia visualized with an optic microscope were picked individually with a sterile needle and then incubated at 25°C during 10 days. A mycelia disk of 10 mm diameter of each culture was inoculated in a 250 ml Erlenmeyer flask containing 100 ml of Sabouraud Broth medium. The liquid culture was then incubated at 25°C for 10 days on a rotary shaker (110 rpm) and the cultivated mycelium was recuperated, filtered and then frozen before being lyophilized.

DNA fungal extraction. Fungal DNA was prepared according to the method described by Sadfi *et al.* (2009). The cetyltrimethyl ammonium bromide (CTAB) method was used to extract DNA from isolates. Liquid nitrogen was added to 0.1 g of lyophilized mycelium in a mortar, and the cells were ground finely with a pestle. Genomic DNA was extracted as described by Doyle and Doyle (1987) with a few modifications. In brief, the mycelium powder was transferred to an Eppendorf tube and 700 µl lysis buffer (2% CTAB) supplemented with 0.2% (w/v) β-mercaptoethanol (Biomatic) was added. The mixture was incubated at 65°C for 40 min. An equal volume of chloroforme/isoamyl alcohol [24:1 (v/v)] was added. After homogenization of the mixture and centrifugation at 13000 rpm for 10 min at +4°C, the supernatant was recuperated into sterile Eppendorf tube. The DNA was precipitated with two volumes of cold 2-propanol supplemented with 10% (w/v) sodium acetate buffer (3 M, pH 8) at –20°C for 2 hours, washed twice 500 µl of 70% ethanol, air dried, and resuspended in 100 µl TE buffer (40 Mm Tris/HCl pH 8.0, 2 mM EDTA). The final DNA was RNase treated with incubation at 37°C for 30–60 min. DNA was eluted in a final volume of 200 µl, quantified on a Nanodrop ND-1000 (Nanodrop Technologies) and diluted to a concentration of 50 ng/µl in PCR grade water. The DNA samples were stored at –20°C for further use.

PCR amplification of internal transcribed spacer (ITS) regions of rDNA. The ITS1-5.8S-ITS2 region of the nuclear rDNA gene cluster was amplified using primers ITS1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS4 (5'-CCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) for isolates. PCR was performed in a total reaction volume of 25 µl containing 5 µl of 5X buffer (Promega), 1 µl of dNTP (20 mM), 1.5 µl of $MgCl_2$ (25 mM), 0.25 U (5 U/µl) *Taq* DNA polymerase, 2 µl of primer (20 pmoles/µl), and 2 µl of genomic DNA (50 ng/µl). The amplification program included an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 98°C (15 s), annealing at 59°C (60 s), extension at 72°C (120 s) and a final extension period of 10 min at 72°C in a Biometra thermal cycler (Germany). Following amplification, PCR products were electrophoresed on 1.5% agarose gels buffered with 0.5X TBE (4.5 mM Tris, 4.5 mM boric acid and 1 mM EDTA, pH 8), stained with ethidium bromide, and photographed.

RFLP. PCR amplicons of 40 isolates of *M. canis* were digested with restriction enzymes *Eco*RI, *Hinf*I and

*Dde*I (Promega) in 20 µl reaction mixtures consisting of 5 µl PCR product, restriction enzyme (10 units) with the corresponding buffer (10X), and 12.8 µl H_2O. The activation of each enzyme was used according to the manufacturer's instructions, and the results were separated on 8% polyacrylamide gels, stained with ethidium bromide and visualized by UV light.

Sequencing. The PCR products were purified with enzymatic methods using *ExoI* and *SAP* and then the sequences were determined by cycle sequencing using the Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems; HTDS, Tunisia) and fragment separation in an ABI PrismTM 3130 DNA sequencer (Applied Biosystems; HTDS, Tunisia). The sequence analysis was perfomed by the Chromas Pro program.

Phylogenetic analysis. The phylogenetic analysis of isolates sequences and the *M. canis* sequences available in the GenBank database was conducted using MEGA4.0. A neighbor-joining phylogenetic tree was produced using *Hypocrea virens* AN73 (HQ292945) as an out-group. The nucleotide sequences reported in this paper have been deposited in the GenBank Database under accession No JQ922440 until JQ922479 for E1 until E40, respectively. The reference strains used are *Arthroderma otae* IFM45853 (AB193632); *M. canis* CHUS65602 (EF581129.1), *A. otea* WM 05.15(EF568060) *M. canis* LM 2174 (EU181444), *M. canis* CHUS14303 (EF581130); *A. otae* IFM 46803 (AB193649); *A. otae* IFM 5286 (AB193610); *M. canis* strain ATCC 23828 (AY213657); *M. canis* strain ATCC MYA-4605 (GU291265); *M. canis* strain ATCC 36299 (FJ545254); *A. otae* isolate 574 (JN134123); *M. canis* strain 04-049-1860 (EU200371); *M. canis* strain 04-047-2395 (EU200368); *A. otae* isolate 268 (JN134110); *A. otae* strain SD100902 (HQ328946); *A. otae* (HQ223448); *A. otae* strain SD100915 (HQ395072); *M. canis* strain CBS566.80 (AJ252330)/*M. canis* strain CBS190.57 (AJ252329)/; *A. otae* strain A6 (HM016897); *A. otae* strain A11 (HM016898); *M. canis* (EU590655).

Results

Macroscopic and microscopic morphology. On Sabouraud's dextrose agar, the isolates matured within 6 to 10 days producing colonies that were flat, spreading, white to cream colored, with a dense cottony, granular to coarsely fluffy to hairy surface which might show some radial grooves. Colonies usually had a bright golden yellow to brownish yellow reverse pigment (Fig. 1). In microscopic observation, the strains produced septate hyphae, macroconidia, and few or rare microconidia. Macroconidia were typically long spindle-shaped, with 5–15 cells, verrucose, thick-walled and often had a terminal knob. The septal walls were thin. Microconidia were rare, unicellular and clavate to pyriform in shape. Raquet hyphae, nodular bodies, and chlamydospores might be present. Some strains were not pigmented, for example, while other, say "dysgoniques" produced distorted macroconidia and few, or did not produce them, and had a macroscopic aberrant (Fig. 2).

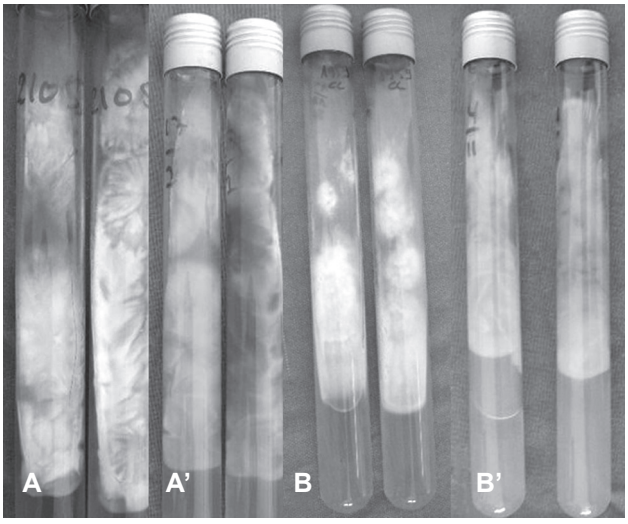

Fig. 1. Macroscopic morphology of *M. canis* isolates (A, A': strain E3; B, B': strain E12)
(A, A') colonies fluffy, white, reverse red-brown; (B, B') colonies fluffy, white; reverse yellow

Assimilation of carbon and nitrogen sources. For the nitrogen sources all isolates assimilated sodium nitrate, asparagines and ammonium sulphate but 92.5% were able to assimilate urease. For the carbon sources, all isolates assimilated glucose, mannose and sorbitol but no assimilation was detected for dulcitol and lactose. For the other carbohydrates variable results for assimilation were obtained (95% for maltose, 92.5% for mannitol, 92.5% for dextrin, 90% for galactose and 17.5% for xylose) (Fig. 3).

Analysis of the numeric grouping of the biochemical results. Analysis of the dendrogram, using 0.82 of the scale of the program in the Jaccard's coefficient showed the differentiation of the isolates to assimilate different carbon and nitrogen sources the distance displayed among the isolates. This statistical analysis grouped the isolates into 2 main groups **A** and **B**. The group **A** included two subgroups, A1 grouping 2 isolates E8 that cannot assimilate dextrose, dulcitol, lactose and galactose and isolate E37 could not assimilate dulcitol, lactose and galactose; and the subgrouping A2 grouping two isolates E2 that could not assimilate dextrose, dulcitol, lactose, galactose and mannitol and the isolate E35 could not assimilate dextrose, dulcitol, lactose, galactose and manitol. Group **B** which represented 70% of the isolates comprises 3 subgroups B1, B2, B3, B4 and B5. B1 grouping isolate E36 could not assimilate dulcitol, lactose and mannitol the B2 comprised one

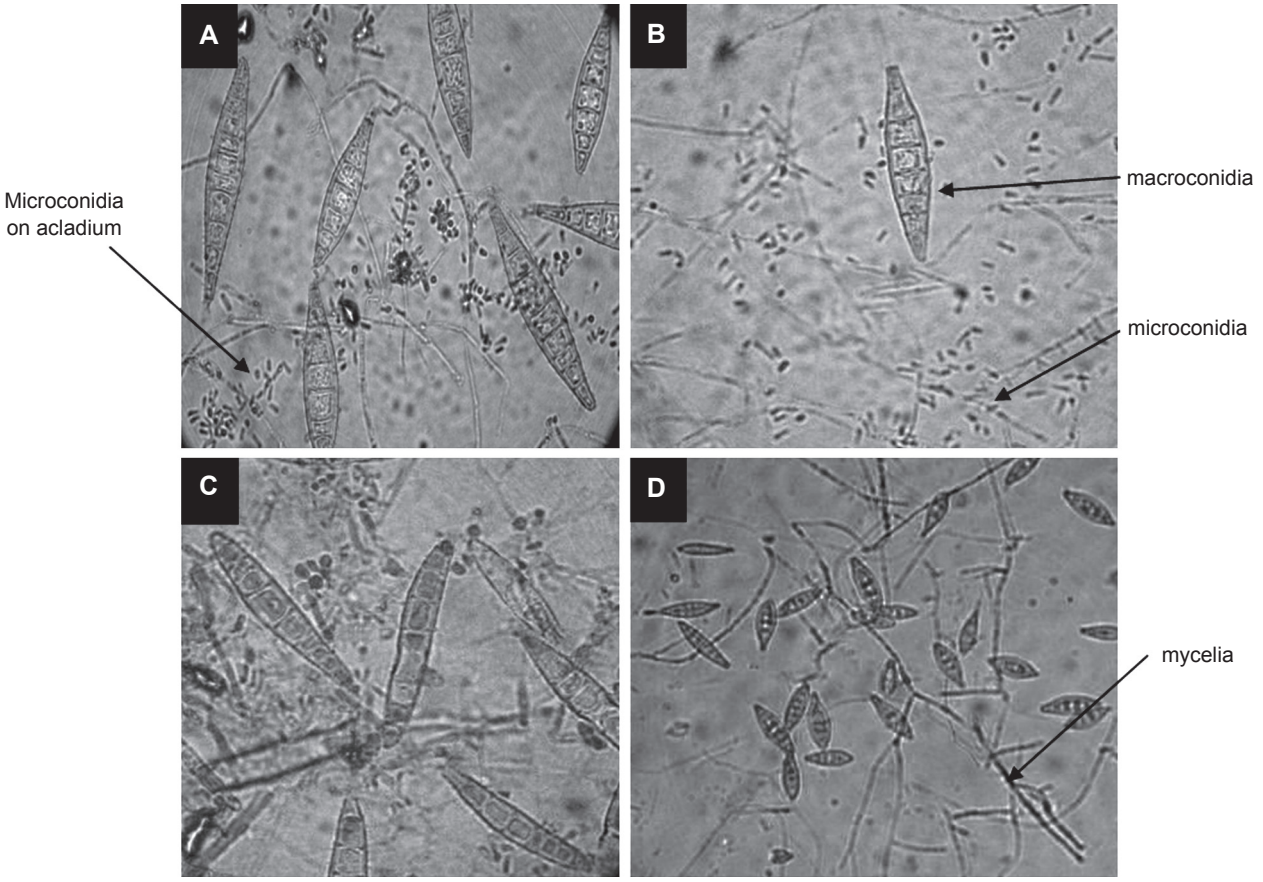

Fig. 2. Microscopic morphology of *M. canis* isolates

(A) Abundant macroconidia 6–10 cells, rough-walled, wiyh thick cell walls and thick septa,microconidia clavate, sessile alongside; (B) Some macroconidia with pyriforme microconidia; (C) Narraw macroconidia and few microconidia.

isolate E38 that could not assimilate dulcitol, lactose, xylose and maltose, the subgroup B3 grouping E15, E13 and E7 these isolates could not assimilate urease, dulcitol, lactose and xylose, B4 subgrouping included 29 isolates unable to assimilate dulcitol, lactose and glucose but were able to assimilate all the others; the subgrouping B5 comprised of E1 and E3 that could not assimilate only dulcitol and lactose (Fig. 3).

PCR. The ITS regions were successfully amplified from all the *Microsporum* isolates by the fungus-specific universal primers ITS1 and ITS4. Polymerase chain reaction electrophoresis showed a typical profile for all the isolates with a single band of 700 bp size.

RFLP. Amplified rDNA from isolates of *M. canis* was digested with *Eco*RI, *Hinf*I and *Dde*I. Each restriction enzyme used showed only one restriction pattern with two, four and five fragments for *Eco*RI, *Dde*I and *Hinf*I, respectively (Table I). Thus, no polymorphism was detected among the isolates. The size of the various bands of digestion is presented in Table I and RFLP bands are shown in Fig. 4, 5 and 6.

Table I

The PCR product patterns of RFLP analysis of rDNA from *M. canis* strains using restriction enzymes *EcoR1*, *Dde1* and *Hinf1*

Enzymes	Restriction site	Fragment size (bp)
***Eco*RI**	present	290, 350
***Dde*I**	present	90, 190, 200, 240
***Hinf*I**	present	140, 200, 240, 260

Sequencing. To confirm the results of RFLP analysis, PCR products were sequenced and the nucleotide sequences were compared with the GenBank Database. All the strains showed a homology from 98% to 100% with *M. canis* (*A. otae*).

Phylogenetic analysis. The ITS phylogenetic trees show that the clinical strains isolated from different patients originating from the North of Tunisia contaminated by animals or human sources are related. They are grouped in the same cluster and genotypically nearly identical to the strains of *M. canis* sequenced by the other authors. The Tunisian clinical strains of *M. canis* are similar to those from other clinical and animal strains (human, rabbit, dog *etc.*) (Fig. 7).

Discussion

M. canis is a frequent cause of *tinea capitis* and *tinea corporis* in Europe and especially in the Mediterranean area (Maraki *et al.*, 2000). Many studies undertaken in

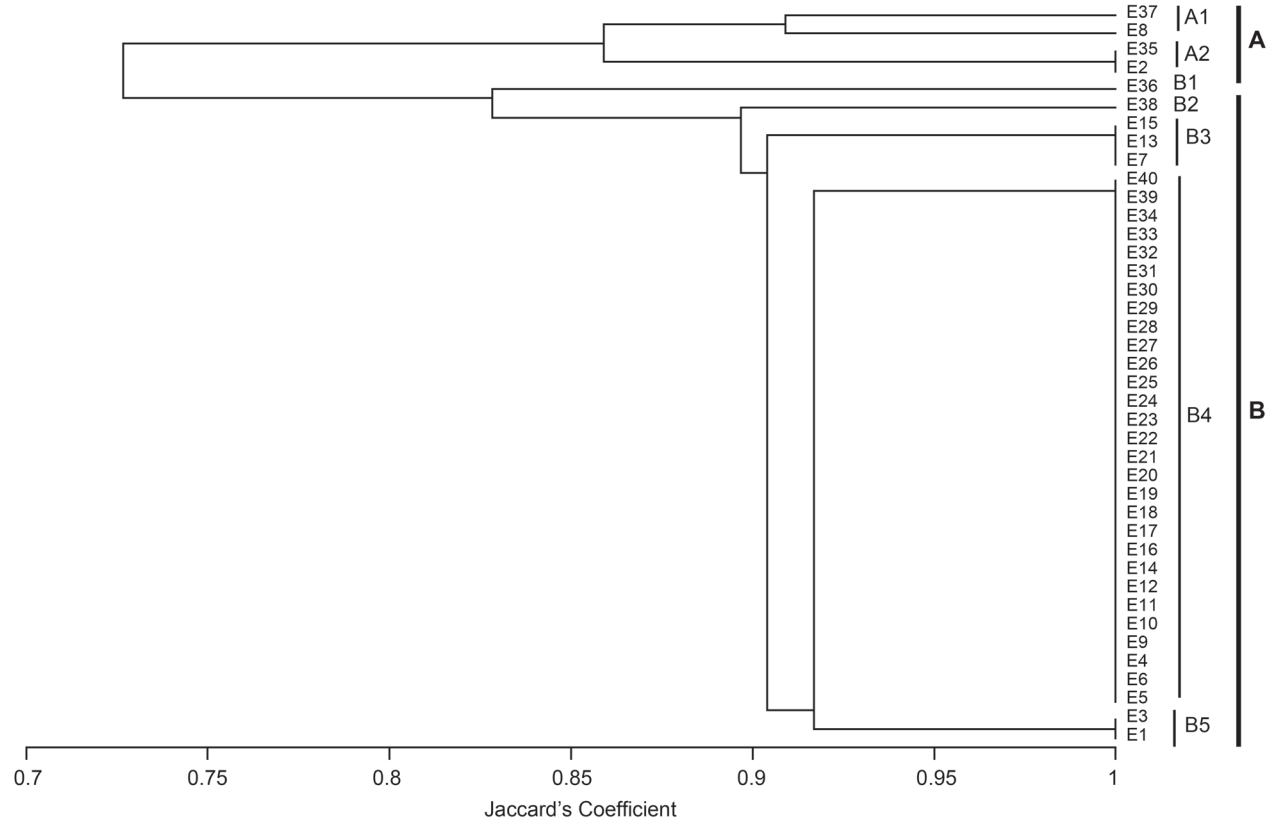

Fig. 3. Dendrogram of the 40 samples isolated from clinical patients. The dendrogram was constructed by UPGMA program from Jaccard's similarity coefficient obtained with assimilation of carbon and nitrogen sources results.

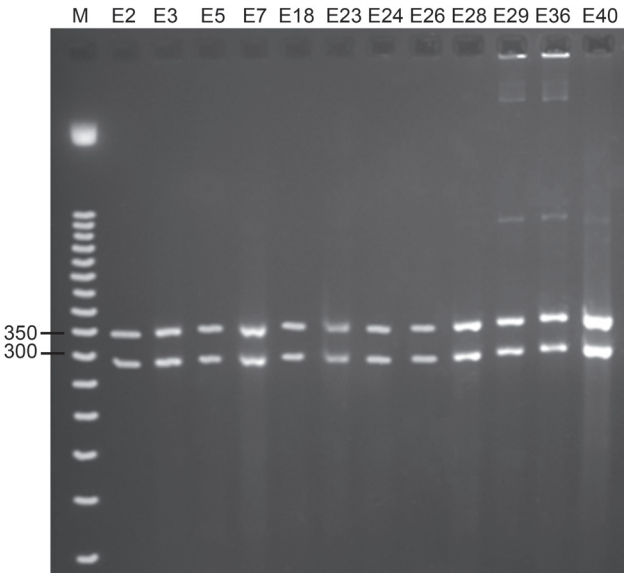

Fig. 4. Polyacrylamide-gel electrophoresis of PCR products digested with *EcoRI* restriction enzyme. The ITS1-ITS4 sets of primers were used to amplify ribosomal DNA including internal transcribed spacers (ITS).

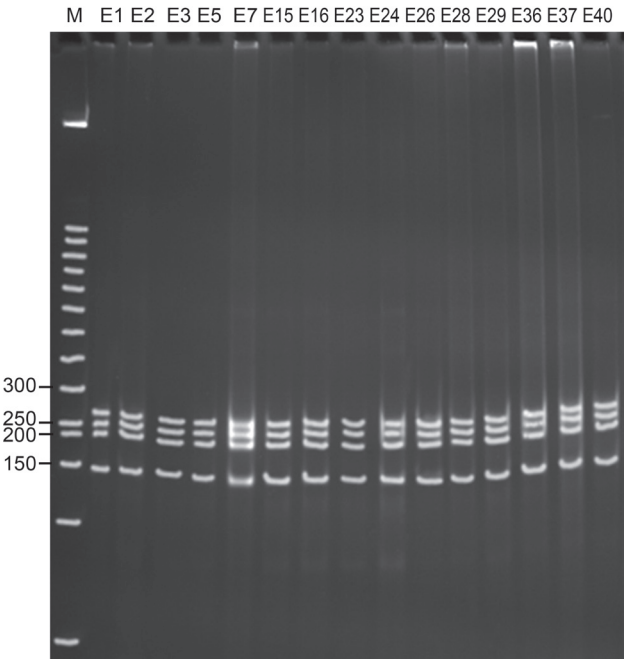

Fig. 5. Polyacrilamide-gel electrophoresis of PCR products digested with *HinfI* restriction enzyme. The ITS1-ITS4 sets of primers were used to amplify ribosomal DNA including internal transcribed spacers(ITS).

Tunisia revealed the re-emergence of *M. canis* (Belhadj *et al*., 2007). The infection can be transmitted by animals, especially dogs and cats or by direct physical contact with an infected person (Gambale *et al*., 1993; Romano *et al*., 1997).

In the present study, 40 isolates of *M. canis* were collected from clinical patients originating from the north of Tunisia, aged between 2 and 19 years affected with

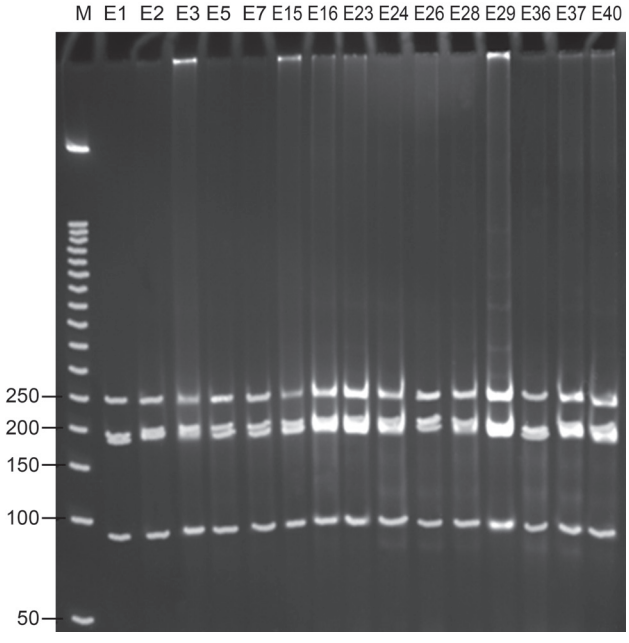

Fig. 6. Polyacrylamide-gel electrophoresis of PCR products digested with *DdeI* restriction enzyme. The ITS1-ITS4 sets of primers were used to amplify ribosomal DNA including internal transcribed spacers (ITS).

tinea capitis, one case with *sycosis pubien* and another case with *onychomycosis*. The majority of them (82.5%) had contact with animals (cat, dog and hamster).

Moreover, from an epidemiological study carried out by Bouden-Mansour (1997), *M. canis* was the most frequent dermatophyte isolated from animals in Tunisia (77.2%) with a high prevalence of isolates from asymptomatic animals. The occurrence of zoonotic transmission from animals to humans has been demonstrated by several studies (Proverbio *et al*., 2014; Drouot *et al*., 2009). For the best therapeutic procedure, identification of this dermatophyte at the species level is very important; the procedure for the identification of *M. canis* is usually based on phenotypical analysis by its macro- and micro-morphological features.

In mycological laboratories in Tunisia, especially in the Mycology Unit of the Hospital La Rabta (Tunisia), the identification of dermatophytes was just established by macroscopic and microscopic methods. *M. canis* colonies are, in general, apiculated with low cottony texture and present pigment varying from canary yellow pigment to ochre (Evans and Richardson, 1989; De Hoog *et al*., 2000). According to Brilhante *et al*. (2005), the morphology of *M. canis* colonies analyzed in different media showed a large diversity which could be attributed to an adaptation of the strain to the variation of environmental conditions. On Sabouraud agar, the isolates grew in 10 days and produce colonies that spread flat. Fluffiness was the majority. The color of the colonies varied between white-yellowish to orange-brown to brownish yellow front and upside down, this was also described by Evans and Richardson (1989). The microscopic appearance showed the presence of hyphal cells; macroconidia in time with the distaff side with an elongated shape containing 5 to 10 stalls, pointed ends, a thick wall and verrucae surface. The microconidia were unicellular pyriform and of variable number. The abundance of fruiting bodies varied from one isolate to another and seemed to be influenced by the nature of the culture medium; for isolates E17 and E30 the absence of fructification was observed on Sabouraud agar but reappeared on the fruiting medium PDA. Marvroudeas *et al*. (1996) suggested that exogenous factors such as the concentration of glucose or thiamine in the culture medium could affect the structure of macroconidia. Several hypotheses have been advanced to explain the phenotypic variation observed in dermatophytes. According to several studies (Makimura *et al*., 1999; Faggi *et al*., 2001; Brilhante *et al*., 2003; Sidrim *et al*., 2004), changing environmental factors such as medium composition, incubation time and temperature could be responsible for the phenotypic variation observed. Besides morphological characteristics, dermatophytes of the genus *Microsporum* could also be typed by means of biochemical tests such as secretion of enzymes (keratinase, collagenase, elastase, urease), assimilation of carbon and nitrogen sources and susceptibility to yeast killer toxins and antifungals (Brilhante *et al*., 2005). There were few variations in the assimilation of nitrogen sources, all the isolates assimilated nitrate and asparagine sources except for three (E7, E13 and E15) which were not able to assimilate urease. For the carbon sources, all the isolates were able to assimilate glucose, mannose and sorbitol but not dulcitol and lactose. Moreover, the assimilation of dextrose, xylose, galactose, mannitol and mannose showed variations between isolates in the collection. In contrast, Mai *et al*., 2001, showed that all the strains of *Microsporum canis* isolated from dogs and cats in Brazil could assimilate nitrogen sources as well as carbon sources, mannitol, maltose, mannose, sorbitol, and dextrin and that not all the samples assimilated galactose and none of them were capable of growing with dulcitol, lactose, or xylose as the only carbon source. This show that biochemical characteristics did not reflect the morphological ones for classification, which may explain the inadequacy of the use of different carbon sources in the typing of *M. canis*. The identification of *M. canis* was based primarily on morphological and biochemical tools (Ginnis, 1980) but the observed change requires the development of other procedures (Maia *et al*., 2001). The success in typing dermatophytes according to phenotyping criteria such as colony morphology, microscopy or biochemical reaction has been limited. Over the past years genotypic approaches have proven useful in solving the problems of identification

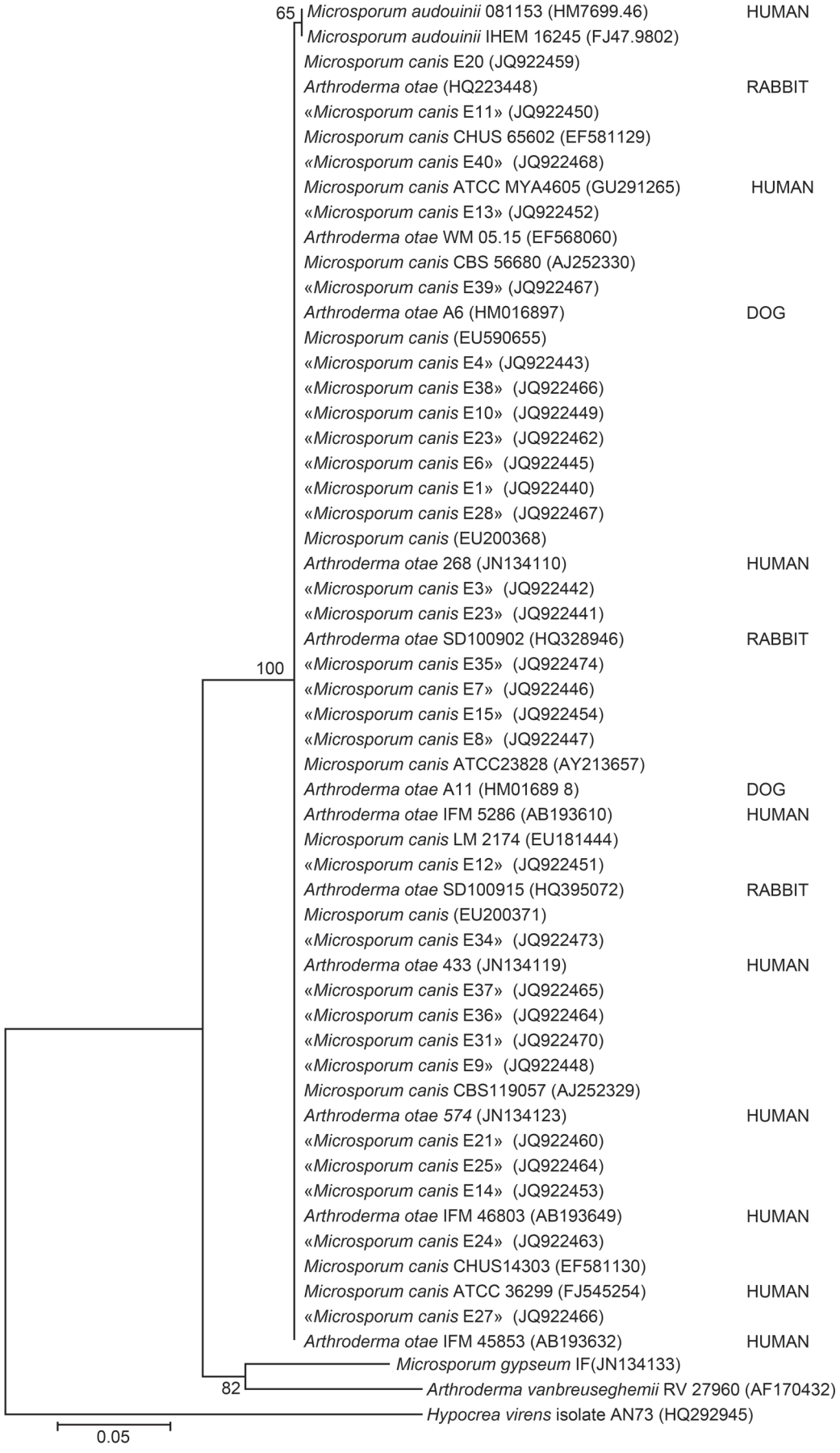

Fig. 7. Consensus tree of all the species listed in the Tbale1 obtained by Mega4 analysis using sequences of the ITS1, 5.8S and ITS2 rDNA regions.

of dermatophytes. Indeed, the genotypic characteristics are more stable and accurate in relation to phenotypic characteristics (Mochizuki *et al*., 2003; Liu 2000). Thus, molecular techniques have been used to better identify the isolates. Several molecular studies have used PCR-RFLP to identify strains of *M. canis*, this technique provides a rapid and practical tool for the identification of dermatophytes (Mochizuki *et al.*, 2003; Kanbe *et al.*, 2003). In this study, the ITS region was subjected to enzymatic digestion by the enzymes *Eco*RI, *Dde*I and *Hinf*I. Endonuclease analysis provides a rapid and reliable technique for molecular differentiation of the collection of the isolates. The *Eco*RI digestion revealed the presence of two bands, while four bands were obtained by *Dde*I digestion for all the samples analyzed in the study. These results are in agreement with those of Brilhante *et al.* (2005) who have identified a collection of *M. canis* from veterinary and clinical origins using *Eco*RI, *Dde*I and *Rsa*I and showed the same profiles for all the strains. Moreover, the identification of an atypical isolate of *Microsporum* with six other strains of *M. canis* by submitting the ITS region to the digestion with *Eco*RI and *Mva*I confirmed the membership of this isolate in *M. canis* species (Leon Mateos *et al.* 2006). The work of Mirzahoseini *et al.* (2009) showed that the *Hinf*I enzyme cannot digest the ITS region of *M. canis,* but in our study we have revealed the presence of 4 bands within this enzyme. Based on the RFLP results, we conclude that the collection of *M. canis* used in this study is genetically identical; such data are in agreement with Brilhante *et al.* (2005), and Faggi *et al.* (2001).

The utilization of direct sequence analysis was an important technical development in the field of amplification-based genotypic pathogen identification (Relman *et al*., 1993) The sequence homology within the rDNA genes of fungi (18S, 5.8S and 28S genes) and differences within the spacer regions (ITS1 and ITS2) are a genetic basis for the organization of the fungi into taxonomic groups and they provide the most relevant information, which can also be easily exchanged between laboratories. The results of the present study show that the ITS, 5.8S and ITS2 sequences of *M. canis* isolates from different patients, thus supporting the hypothesis that the infections are caused by the same strain. The DNA sequences of the ITS regions of nuclear ribosome in the dermatophytes have been proven to be useful for species identification and for resolving phylogenetic relationships between close taxonomic species. Gräser *et al*., (2000) reported that 40 *M. canis* isolates displaying different colonies morphologies did not reveal any DNA polymorphism when analyzed using molecular techniques, suggesting a strictly clonal mode of reproduction and a strong adaptation to human skin. The present results show that the *Microsporum* group is heterogeneous and that new isolates and other genome regions need to be analyzed to elucidate the taxonomic relationships of a group that shows differences. According to Yu *et al.,* (2004), the ITS region was always thought to have interspecies polymorphism and little variance at the intra-species level. Thus, our results suggest that strains from different origins which present the same DNA sequence in the ITS region may result from small variances in this region for intra-species isolates from patient with *tinea capitis* or from the environment. The accessibility of the sequencing of the amplification products, combined with the continued enrichment of databases, allows introducing the sequencing method in a rational approach to compare dermatophytes around the word so DNA sequencing is thought to be the most direct method for species discrimination. In our study of isolate differentiation, we tried to examine all the isolates of *M. canis* using the method of DNA sequencing. The ITS region of rDNA was chosen as the target region of the study. The results showed that the strains present some biochemical diversity to assimilate carbon and nitrogen sources, but no genetic differences were detected between isolates from different patients. It resulted in the inference that all the isolates were a single strain which had spread among different sites and to susceptible children. *M. canis* strains from Tunisian patients are very clonal genetically.

Acknowledgements

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Literature

Belhadj S., H. Jeguirim, S. Anane, E. Kaouech, K. Kallel and E. Chaker. 2007. Évolution des teignes du cuir chevelu à *Microsporum canis* et à *Trichophyton violaceum* à Tunis. *J. Med. Mycol.* 17: 54–57.

Bouden-Mansour R., S. Belhadj, L. Idir, A. Bouattour, M. Kilani and E. Chaker. 1997. Prevalence and etiology agents of animal ringworm in the region of Tunis. *Mycol. Med.* 7: 145–148.

Brilhante R.S., C.S. Cavalcante, F.A. Soares-Junior, R.A. Cordeiro, J.J. Sidrim and M.F. Rocha. 2003. High rate of *Microsporum canis* feline and canine dermatophytoses in North east Brazil: epidemiological and diagnostic features. *Mycopathologia* 156(4): 303–308.

Brilhante R.S., M.F. Rocha, R.A. Cordeiro, S.H. Rabenhorst, T.B. Granjeiro, A.J. Monteiro and J.J. Sirdim. 2005. Phenotypical and molecular characterization of *Microsporum canis* strains in northeast Brazil. *J. App. Microbiol.* 99(4): 776–782.

De Hoog G.S., P. Mayser, G. Haase, R. Horré and A.M. Horrevorts. 2000. A new species, *Phialophora europaea*, causing superficial infection in humans. *Mycoses* 43(11–12): 409–416.

Doyle J.J and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11–15.

Drouot S., B. Mignon, M. Fratti, P. Roosje and M. Monod. 2009. Pets as the main source of two zoonotic species of the *Trichophyton mentagrophytes* complex in Switzerland, *Arthroderma vanbreuseghemiiand, Arthroderma benhamiae*. *Vet. Dermatol.* 20(1): 13–18.

Evans E.G.V and M.D. Richardson (eds). 1989. Medical mycology – a practical approach. Oxford University Press.

Faggi E., G. Pini., E. Campisi, C. Bertellini, E. Difonzo and F. Mancianti. 2001. Application of PCR to distinguish common species of dermatophytes. *J. Clin. Microbiol.* 39(9): 3382–3385.

Gambale W., G.E. Larsson, M.M. Moritami, B. Correa, C.R. Paula and V.M. Framil. 1993. Dermatophytes and other fungi of the haircoat of cats without dermatophytosis in the city of Sao Paula, Brazil. *Feline Prac.* 21(3): 29–33.

Jung H.J., S.Y. Kim, J.W. Jung, H.J. Park, Y.W. Lee, Y.B. Choe and K.J. Ahn. 2014. Identification of dermatophytes by polymerase chain reaction-restriction fragment length polymorphism analysis of metalloproteinase-1. *Ann Dermatol*. 26(3):338–42.

Ginnis M.R. 1980. Laboratory handbook of Medical Mycology. Academic Press, Inc. New York.

Graser Y., M. El Fari, W. Presber, W. Sterry and H.J. Tietz. 1998. Identification of common dermatophytes (*Trichophyton*, *Microsporum*, *Epidermophyton*) using polymerase chain reactions. *Br. J. Dermatol.* 138(4): 576–582.

Graser Y., A.F. Kuijpers, M. El Fari, W. Presber and G.S. De Hoog. 2000. Molecular and conventional taxonomy of the *Microsporum canis* complex. *Med. Mycol.* 38: 143–153.

Kanbe T., Y. Suzuki, A. Kamiya., T. Mochizuki, M. Fujihiro and A. Kikuchi. 2003. PCR- based identification of common dermatophyte species using primer sets specific for the DNA topoisomerase II genes. *J. Dermatol Sci.* 32(2): 151–61.

Kano R., K. Okabayashi, Y. Nakamura, S. Ooka, M. Kashima and M. Mizoguchi. 2000. Differences among chitin synthase 1 gene sequences in *Trichophyton rubrum* and *T. violaceum*. *Med. Mycol.* 38(1): 47–50.

Kawasaki M., M. Aoki, H. Ishizaki, K. Nishimura and M. Miyaji. 1996. Phylogeny of *Epidermophyton floccosum* and other dermatophytes. *Mycopathologia* 134(3): 121–128.

Leon-Mateos A., C. Pares-Suarez, M.J.R. Pereiro and J. Toribio. 2006. Study of the ITS region in an atypical isolate and comparison with six species of *Microsporum*. *Mycoses* 49(6): 452–456.

Liu D., S. Coloe, R. Baird and J. Pedersen. 1997. PCR identification of *Trichophyton mentagrophytes* var. *interdigitale* and *T. mentagrophytes* var. *mentagrophytes* dermatophytes with a random primer. *J. Med. Microbiol* 46(12): 1043–1046.

Liu D., S. Coloe, R. Baird and J. Pedersen. 2000. Application of PCR to the identification of dermatophyte fungi. *J. Med. Microbiol.* 49(6): 493–497.

Maia L.S., J.I. Dos Santos, F.C. Viani, C.E. Larsson, C.R. Paula and W. Gambale. 2001. Phenotypic characterisaton of *Microsporum canis* isolated from cats and dogs. *Mycoses* 44(11–12): 480–486.

Makimura K., Y. Tamura, T. Mocgizuki, A. Hasegawa, Y. Tajri, R. Hanazawa, K. Uchida, H. Saito and H. Yamaguchi. 1999. Phylogenetic classification and species identification of dermatophyte strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. *J. Clin. Microbiol.* 37(4): 920–924.

Maraki S and Y. Tselentis. 2000. Survey on the epidemiology of *Microsporum canis* infections in Crete, Greece over a 5-year period. *Int. J. Dermatol.* 39(1): 21–24.

Mavroudeas D., A. Velegraki, J. Leonardopoulos and U. Marcelou. 1996. Effect of glucose and thiamine concentrations on the formation of macroconidia in dermatophytes. *Mycoses* 39 (1–2): 61–66.

Meziou T.J., A. Dammak, T. Zaz, M. Mseddi, S. Boudaya, L. Bouzid, F. Akrout, S. Maalej, A. Ayadi and H. Turki. 2011. Scalp ringworm *tinea capitis* in Tunisian infants. *Med. Mal. Infect.* 41 (9): 486–488.

Mirzahoseini H., E. Omidiana, S. Ghahfarokhi, G. Sadeghi and R. Abyanehni. 2009. Application of PCR-RFLP to Rapid Identification of the Main Pathogenic Dermatophytes from Clinical Specimens. *Iran. J. Publ. Health* 38(1): 18–24.

Mochizuki T., N. Sugie and M. Uehara. 1997. Random amplification of polymorphic DNA is useful for the differentiation of several anthropophilic dermatophytes. *Mycoses* 40(11–12): 405–409.

Mochizuki T., H. Tanabe, M. Kawasaki, H. Ishizaki and C.J. Jackson. 2003. Rapid identification of *Trichophyton tonsurans* by PCR-RFLP analysis of ribosomal DNA regions. *J. Dermatol. Sci.* 32(1): 25–32.

Nei M. and W.H. Li.1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci.* 76(10): 5269–5273.

Proverbio D., R. Perego, E. Spada, G. Bagnagatti de Giorgi, A. Della Pepa and E. Ferro. 2014. Survey of Dermatophytes in Stray Cats with and without Skin Lesions in Northern Italy. *Vet. Med. Int*. doi: 10.1155/2014/565470.

Relman D.A. 1993. The identification of uncultured microbial pathogens. *J. Infect. Dis*. 168(1): 1–8.

Romano C., L. Valenti and R. Barbara. 1997. Dermatophytes isolated from asymptomatic stray cats. *Mycoses* 40(11–12): 471–472.

Sadfi-Zouaoui N., I. Hannachi, M. Rouaissi, M.R. Hajlaoui, M.B. Rubio, E. Monte, A. Boudabous and M.R. Hermosa. 2009. Biodiversity of *Trichoderma* strains in Tunisia. *Can. J. Microbiol.* 55(2): 154–162.

Sharma R., R.C. Rajak, A.K. Pandey and Y. Graser. 2006. Internal Transcribed Spacer (ITS) of rDNA of appendaged and non-appendaged strains of *Microsporum gypseum* revals *Microsporum appendiculatum* as its synonym. *Antonie Van Leeuwenhoek* 89(1): 197–202.

Sidrim J.C., T.E.F. Meireles, L.M.P. Oliveira and M.J.N. Diogenes. 2004. Aspect Clinique des dermatophytoses. *Mycol. Med*. 135–161.

Kanbe T. 2008. Molecular Approaches in the Diagnosis of Dermatophytosis. *Mycopathologia* 166: 307–317.

Turin L., F. Riva, G. Galbiati and T. Cainelli. 2000. Fast simple and highly sensitive double-rounded polymerase chain reaction assay to detect medically relevant fungi in dermatological specimens. *Eur. J. Clin. Invest.* 30(6): 511–518.

White T.J., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. A Guide to Methods and Applications, Academic Press In.

Yu J., Z. Wan, W. Chen, W. Wang and R. Li. 2004. Molecular typing study of the *Microsporum canis* strains isolated from an outbreak of *tinea capitis* in a school. *Mycopathologia* 157(1): 37–41.