

Original article

Identification of dermatophytes by arbitrarily primed PCR

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Background: Dermatophytosis is a superficial infection caused by filamentous fungi belonging to the following three genera: *Microsporum*, *Trichophyton* and *Epidermophyton*. Arbitrarily primed PCR (AP-PCR) is a rapid and sensitive procedure for the diagnosis of the fungal species.

Objectives: To identify various dermatophyte species as rapidly and precisely as possible.

Methods: Fifty-two clinical dermatophyte isolates from ten species were recovered from samples obtained from the Department of Medical Mycology and patients in different parts of Iran. All 52 dermatophyte isolates tested belonged to any of *Trichophyton*, *Microsporum*, or *Epidermophyton* genera. Four random primers, OPAA11, OPU15, OPAA17, and OPD18, were used in this study.

Results: The results indicated that all 10 dermatophyte species displayed distinct DNA band patterns after amplification with the random primers OPAA11 and OPU15. Nine species of dermatophytes were distinguished with the random primer OPAA17 using a different DNA band pattern. AP-PCR amplified different PCR products using primer OPD18.

Conclusions: AP-PCR offers a convenient solution to the problems encountered in the recognition of morphological features of dermatophyte species in comparison with conventional morphological methods. This approach represents technological progress in identification of dermatophytosis.

Keywords: AP-PCR, dermatophytosis, dermatophyte, random primer

Dermatophytosis is a superficial infection caused by filamentous fungi belonging to the following three genera: *Microsporum*, *Trichophyton*, and *Epidermophyton*. These fungi cause the most common superficial mycoses worldwide [1]. Dermatophytes are closely related and able to invade the keratinized tissues, including the skin, hair, and nails of humans and animals.

Colonization by dermatophytes is assisted by the release of various proteolytic and other enzymes produced by the fungi, which can cause inflammatory responses [2].

Almost 30 species of dermatophytes are considered involved in the etiology of dermatophytosis. Of dermatophyte species, *Trichophyton rubrum* is the most prevalent. *T. interdigitale*, *T. tonsurans*, *Microsporum canis*, *M. gypseum*, and

Epidermophyton floccosum are also recognized as causative agents of dermatophytosis [3]. While the *Epidermophyton* genus includes only one species, *E. floccosum*, there are many species as members of the genera *Trichophyton* and *Microsporum* that make the detection of these genera more complicated [4]. In addition, different species of these genera display significant variation in their morphological features and in ecological distribution.

Laboratory diagnosis of dermatophyte species is helpful to treat the infection. Routinely, dermatophytes are detected by microscopic examination and by culture, but conventional routine procedures are not specific or sensitive, and the methods are time-consuming.

Several molecular methods have been developed for the rapid identification of various species of dermatophytes [5]. PCR is a very sensitive and precise technique for the diagnosis of microorganisms including fungi. Arbitrarily primed PCR (AP-PCR) is a rapid and sensitive procedure for the diagnosis of

fungal species [6, 7]. Ribosomal and mitochondrial DNA of dermatophytes have shown variation among these agents [8-10].

The identification of dermatophytes has been improved as a result of the aforementioned studies to have more acceptable accuracy and speed. However, the techniques used have required extra manipulation such as restriction enzyme digestion and sequencing phylogenetic relationships between different species. Recent research has focused on developing novel molecular techniques for the rapid detection of dermatophytes from clinical samples at the genus and species level [6, 11, 12]. The identification of dermatophytes with randomly amplified fragments enhanced laboratory discrimination of dermatophytes from other microorganisms [12, 13]. AP-PCR was used in the present study with four random primers for identification of 10 dermatophyte species recovered from patients in Iran. The aim of this study was to identify various dermatophyte species as rapidly and precisely as possible.

Materials and methods

The study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences.

Organisms

Fifty-two clinical dermatophyte isolates of 10 species were recovered from samples obtained from the Department of Medical Mycology and different parts of Iran. All species, except *M. ferrugineum*, are very common in Iran. The isolates were cultured on selective agar (Sabouraud's dextrose agar including cycloheximide and chloramphenicol, Merck, Germany). These isolates were recognized up to the species level with phenotypic techniques including colony morphology, microscopy, physiological and biochemical analysis. After confirming the isolates at a species level, the cultured colonies were saved at room temperature for further molecular diagnosis. All 52 dermatophyte isolates tested belonged to the *Trichophyton*, *Microsporum*, or *Epidermophyton* genera (Table 1).

DNA extraction

The genomic DNA from all 52 dermatophytes was extracted and applied as templates for PCR amplifications. Dermatophyte isolates were grown in selective agar. A small portion of fresh culture was transferred into a 1.5 microtube containing 400 µL of

lysis buffer (100 mM Tris-HCl, pH 8.0, 30 mM EDTA, pH 8.0, SDS 5% w/v) using a sterile tooth pick. The microtubes were boiled for 20 min, and then 150 µL of 3 M potassium acetate was added. The suspension was kept at -20°C for 10 min, and centrifuged at 12000 rpm at 4°C for 10 min. After transferring the supernatant to a new 1.5 mL microtube, 250 µL phenol-chloroform-isoamyl alcohol (25:24:1, v/v) was added. The tube was briefly vortexed and centrifuged at 12000 rpm for 10 min. The supernatant was transferred to a new 1.5 mL microtube and 250 µL chloroform-isoamyl alcohol was added. Then, it was kept at -20°C for 10 min and centrifuged at 12000 rpm for 10 min. After transfer of the supernatant to a new 1.5 mL tube, and the addition of an equal volume of iced-cold 2-propanol, the microtube was kept at -20°C for 10 min, then centrifuged at 12000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 300 µL 70% ethanol. After that, the supernatant was discarded by centrifuging at 12000 rpm for 10 minutes. The DNA pellet was air dried and resuspended in 50 µL of distilled H₂O.

AP-PCR

AP-PCR was conducted for all isolates with each random primer separately. Amplification reactions were performed with volumes of 50 µL containing reaction buffer, 2.2 mM MgCl₂, 200 µM each dNTP (dATP, dCTP, dGTP, and dTTP), 1.5 U of Taq DNA polymerase (Roche, Germany), 50 ng template DNA, 15 pmol of each random primer [6] OPAA11 (5'-ACCCGACCTG-3'), OPU15 (5'-ACGGGCCAGT-3'), OPAA17 (5'-GAGCCCGACT-3'), and OPD18 (5'-GAGAGCCAAC-3'). The PCR cycling conditions were 3 cycles of 94°C for 60 s, 36°C for 45 s, and 72°C for 90 s, followed by 32 cycles 94°C for 30 s, 36°C for 45 s, and 72°C for 90 s. The PCR products were electrophoresed in 1.5% agarose gel in 1× TAE buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA) and stained with ethidium bromide.

Results

A total of 52 isolates belonging to any of 3 genera, *Microsporum*, *Trichophyton*, and *Epidermophyton* containing 10 species were analyzed with AP-PCR.

The genomic DNA of 10 dermatophytes was amplified with random primers OPAA11, OPU15, and OPD18 separately. However, primer OPAA17 was used to amplify the genomic DNA of 9 dermatophytes (Table 1, Figures 1-4). No band was generated

from the negative control without a DNA template. For consistency, the experiences were repeated and the same results were obtained.

Using primer OPAA11, the PCR exclusively amplified different products for dermatophytes between 300 bp to 1800 bp. The results indicated that all 10 dermatophyte species displayed a distinct DNA band pattern after amplification with random primer OPAA11 (**Figure 1**). This primer amplified the genomic DNA of *E. floccosum* and *M. canis* with a similar band pattern 350, 450, 650, and 750 bp. Therefore, *E. floccosum* and *M. canis* must be recognized with another random primer, for example, OPU15. Primer OPAA11 generated only 2 bands, 350 and 750 bp from *T. mentagrophytes*. AP-PCR with primer OPU15 amplified various products for dermatophytes between 250 bp to 1800 bp. All 10 species of dermatophytes could be identified with random primer OPU15 by their distinctive DNA band pattern (**Figure 2**). The fewest distinctive bands belonged to *T. verrucosum*, with 3 bands 250, 350, and 800 bp for primer OPU15.

As shown in **Figure 1**, the PCR with the primer OPAA17 generated different products, from 300 bp

to 1400 bp, for 9 species of dermatophytes. No PCR product was obtained for *T. schoenleinii* with the primer OPAA17 (**Figure 3**). Nine species of dermatophytes could be distinguished with the random primer OPAA17 by different DNA band pattern. The fewest distinctive bands belonged to *M. ferrugineum*, with 3 bands 300, 500, and 700 bp, and *T. verrucosum*, with 300, 500, and 850 bp for primer OPAA17.

With the use of primer OPD18, AP-PCR amplified different PCR products with distinct DNA band patterns between 250 bp to 1600 bp for 10 dermatophyte species (**Figure 4**). Similar band patterns were obtained for *M. gypseum* and *T. verrucosum* with the OPD18 primer. Therefore, these 2 dermatophytes must be identified with another random primer, for example OPAA17. The fewest distinctive bands belonged to *T. mentagrophytes* with 4 bands 350, 400, 750, and 1000 bp for primer OPD18. Amplification success rate for primers OPAA11, OPU15, and OPD18 was 100%, and for primer OPAA17 was 90%.

Table 1. PCR product patterns (bp) from dermatophyte species

Dermatophyte species	Number tested	OPAA11	OPU15	OPAA17	OPD18
<i>Epidermophyton floccosum</i>	8	350-450-650-750	250-400-600-650-750-1000	300-500-750-850	250-350-400-600-750-900-1100-1500
<i>Microsporium canis</i>	4	350-450-650-750	250-350-450-600	200-300-450-700-900-1400	250-350-400-450-700-750-800-1000-1500
<i>M. gypseum</i>	8	300-450-600-700-800-1000-1200-1800	300-400-550-650-700-800-1000-1200-1500	300-450-500-600-800-900	350-450-600-700-800-900-1100-1600
<i>M. ferrugineum</i>	2	350-450-700	250-350-400-600-700-900-1300-1800	300-500-700	300-400-500-700-800-1000-1400
<i>Trichophyton mentagrophytes</i>	10	350-750	250-400-450-600-1000	300-450-500-700-900	350-400-750-1000
<i>T. rubrum</i>	5	350-450-650	250-400-550-750-800-1000	300-500-700-800-1200-1400	350-400-650-800-900-1300-1500
<i>T. verrucosum</i>	9	300-400-600	250-350-800	300-500-850	350-450-600-700-800-900-1100-1600
<i>T. tonsurans</i>	3	350-450-600-750-850	250-300-400-500-600-800-900-1400	300-400-500-700-900-1200	300-600-800-1000-1400-1500
<i>T. violaceum</i>	2	350-450-650	250-350-400-500-600-750-1000-1200	300-500-700-900-1000	250-300-500-700-800-1000-1500
<i>T. schoenleinii</i>	1	350-450-550-650	250-350-400-500-550-650-800-900-1000	*****	250-350-400-450-500-600-700-800-1000-1300-1500
Total	52				

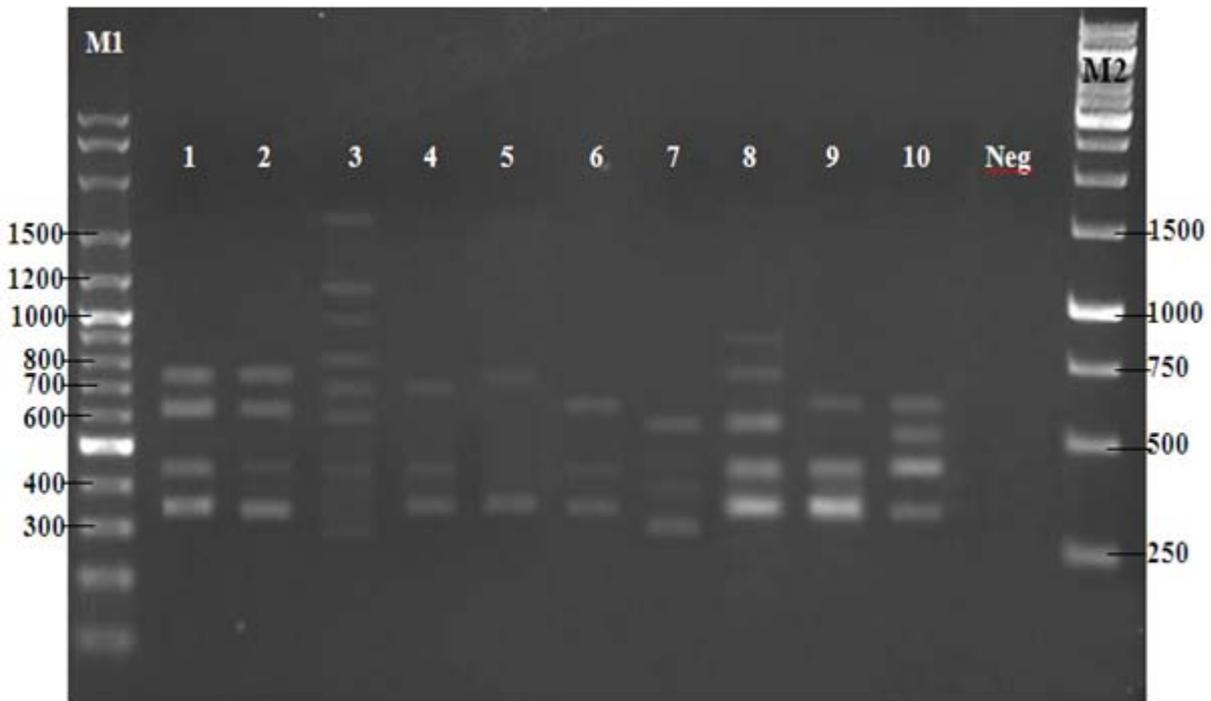


Figure 1. PCR products of dermatophyte species with random primer OPAA11
 Lanes: M1, 100 bp ladder; 1, *Epidermophyton floccosum*; 2, *Microsporium canis*; 3, *M. gypseum*; 4, *M. ferrugineum*;
 5, *Trichophyton mentagrophytes*; 6, *T. rubrum*; 7, *T. verrucosum*; 8, *T. tonsurans*; 9, *T. violaceum*; 10, *T. schoenleinii*;
 Neg, no template DNA; M2, 1 kb ladder

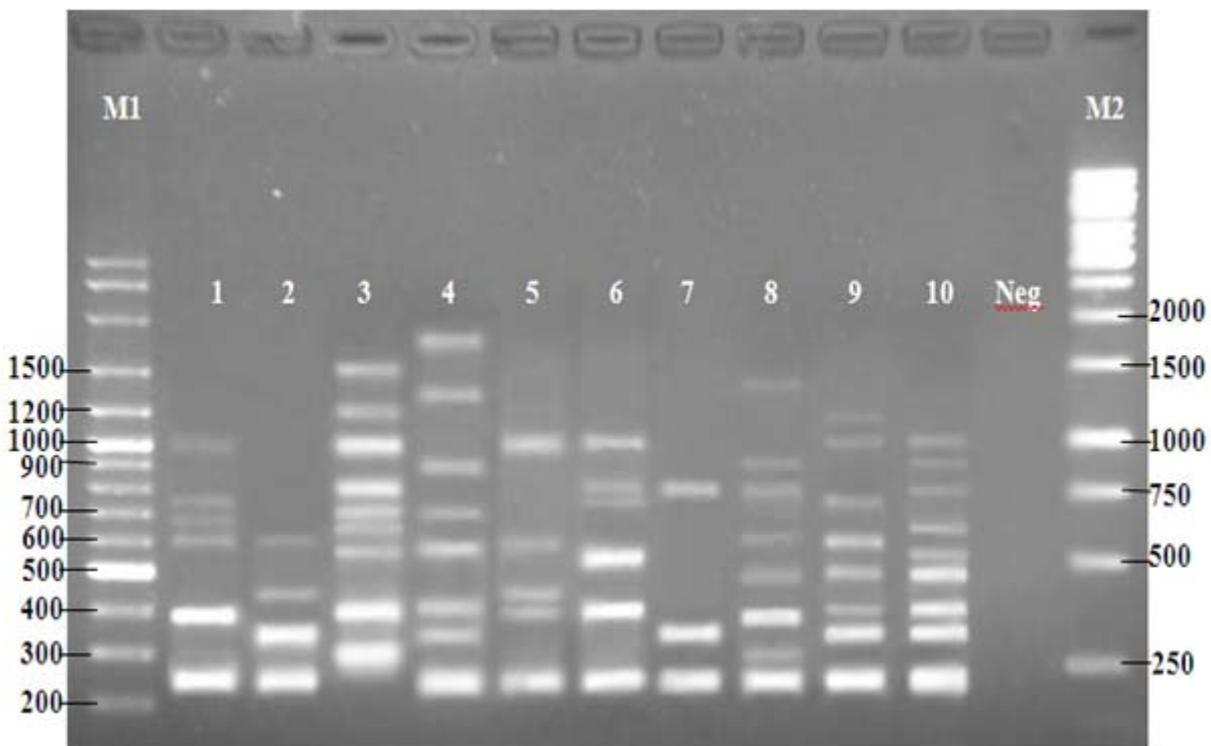


Figure 2. PCR products of dermatophyte species with random primer OPU15. Lanes: M1, 100 bp ladder; 1, *Epidermophyton floccosum*; 2, *Microsporium canis*; 3, *M. gypseum*; 4, *M. ferrugineum*; 5, *Trichophyton mentagrophytes*; 6, *T. rubrum*; 7, *T. verrucosum*; 8, *T. tonsurans*; 9, *T. violaceum*; 10, *T. schoenleinii*; Neg, no template DNA; M2, 1 kb ladder



Figure 3. PCR products of dermatophyte species with random primer OPAA17. Lanes: M1, 100 bp ladder; 1, *Epidermophyton floccosum*; 2, *Microsporum canis*; 3, *M. gypseum*; 4, *M. ferrugineum*; 5, *Trichophyton mentagrophytes*; 6, *T. rubrum*; 7, *T. verrucosum*; 8, *T. tonsurans*; 9, *T. violaceum*; 10, *T. schoenleinii*; Neg, no template DNA; M2, 1 kb Ladder

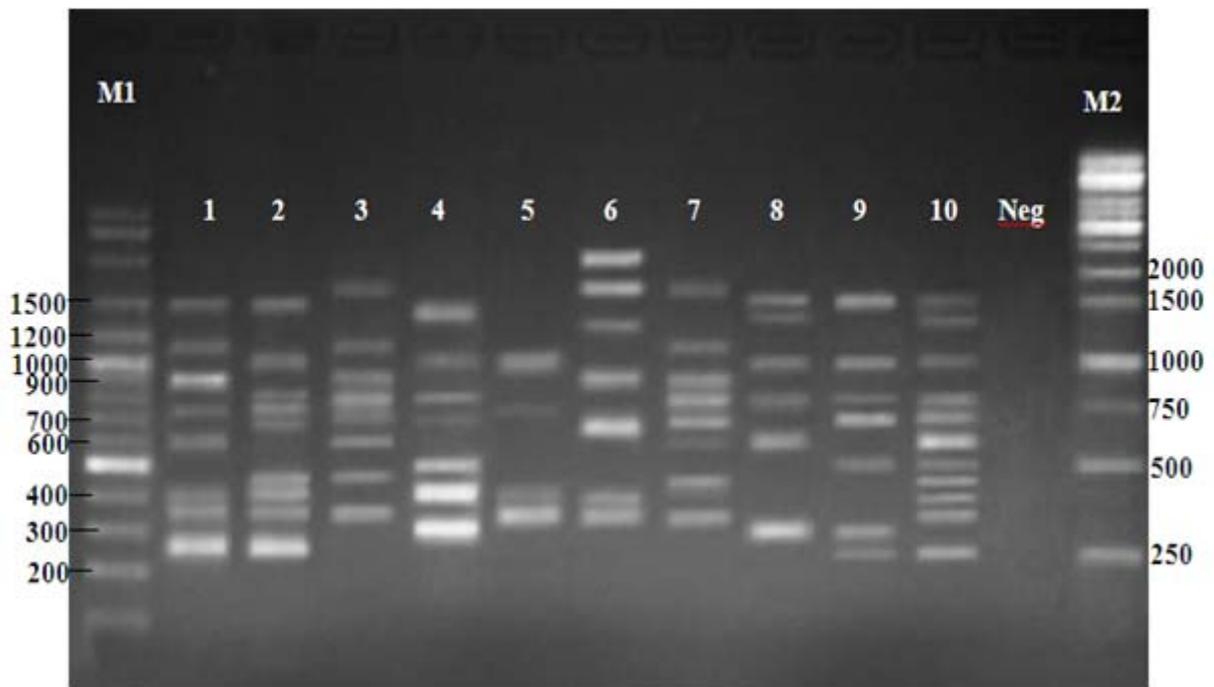


Figure 4. PCR products of dermatophyte species with random primer OPD18. Lanes: M1, 100 bp ladder; 1, *Epidermophyton floccosum*; 2, *Microsporum canis*; 3, *M. gypseum*; 4, *M. ferrugineum*; 5, *Trichophyton mentagrophytes*; 6, *T. rubrum*; 7, *T. verrucosum*; 8, *T. tonsurans*; 9, *T. violaceum*, 10, *T. schoenleinii*; Neg, no template DNA; M2, 1 kb ladder

Discussion

Identification of dermatophytes is not straightforward because of the existence of various species. They grow slowly and the morphology of the isolates is different. The conventional diagnosis of dermatophytosis is founded on microscopic detection of fungal hyphae directly from lesion followed by culture. Although this technique is rapid and economical, but it is not species specific, and is relatively insensitive, presenting false-negative results of up to 15% [2]. Molecular methods are more helpful for the diagnosis of dermatophytes because these procedures are more sensitive and rapid. Furthermore, these methods rely on genetic structures, which are more constant than phenotypic features, and they are able to recognize atypical dermatophytes that cannot be distinguished with morphological approaches [14-16]. The molecular approaches can recognize the dermatophyte species or the strain levels [8, 17]. The use of molecular techniques has facilitated great progress in identification and phylogenetic analysis of dermatophytes [18, 19].

In this study, the AP-PCR with four random primers OPAA11, OPU15, OPAA17, and OPD18 was used for identification of ten species of dermatophytes. After examining 52 isolates from 10 species of dermatophytes, it was revealed that these primers are able to identify DNA from tested dermatophytes, generating bands between 250 bp to 1800 bp in PCR. However, OPAA17 could not identify the DNA of *T. schoenleinii*.

Three random primers, OPAA11, OPAA17, and OPU15 are not able to differentiate among three dermatophytes *T. rubrum*, *T. soudanense*, and *T. gourvilii* with AP-PCR, but random primer OPD18 generates slight differences among these species of dermatophytes [6, 20].

Neji et al. identified 3 dermatophytes *T. mentagrophytes*, *T. rubrum*, and *M. ferrugineum* with a 450 bp fragment obtained from CHS1 gene. Their method requires sequencing and only can identify 3 dermatophytes compared with 10 dermatophytes in our AP-PCR [21].

DNA based techniques rely on the recognition of genotypic variations in pathogenic microorganisms [22]. The genotypic features are less possibly affected by external influences, for example temperature differences and chemotherapy. The PCR methods such as arbitrarily primed PCR (AP-PCR) or random amplified polymorphic DNA (RAPD) [6, 23-25] have

improved the sensitivity of the diagnostic procedures. A number of laboratories have successfully applied the PCR method to the diagnosis and phylogeny of fungal pathogen in the small (18S) [26] and large (25S) [27] ribosomal DNA regions, and the gene for chitin synthase 1 [28]. These approaches resulted in a more precise assessment of phylogenetic associations among dermatophytes and other fungi [29], and improved the identification of several frequent dermatophytes [30]. However, because of the techniques commonly required to the additional treatments such as restriction enzyme digestion, sequencing or hybridization after amplification, they are not simply accepted for regular use in clinical laboratories. Furthermore, usually these methods are able to distinguishing only a few dermatophyte species and, therefore, they are more suitable for the genus-specific rather than species-specific recognition.

Our results showed that AP-PCR analysis of the internal transcribed spacer (ITS) region is a rapid, reliable and reproducible method, which allows the identification of major dermatophytes at a species level.

There are several reports from prevalence of dermatophytosis in Iran. An epidemiological study of dermatophytosis in Tehran, Iran, showed that the most frequent dermatophytes isolated were *E. floccosum* (32%), *T. rubrum* (26%), and *T. mentagrophytes* (19.9%). Identification of dermatophyte isolates was on the basis of morphological characteristics and conventional methods [31]. Mirzahoseini et al. among 160 skin scrapings examined in Teheran (Iran) identified 6 species of dermatophyte including *T. mentagrophytes*, *T. rubrum*, *T. verrucosum*, *T. tonsurans*, *M. canis*, and *E. floccosum* were based on morphology characteristics and molecular methods. Their study showed that specific PCR products from the ITS region and the use of restriction fragment length polymorphism (RFLP) contributed to the differentiation of isolated dermatophytes at the genus or species level [32].

An epidemiological aspect of dermatophytosis in Southwest Iran demonstrated that tinea cruris and tinea corporis were the most frequent clinical appearance in both men and women. *E. floccosum* was the most commonly isolated dermatophyte (39.25%), followed by *T. verrucosum* (27.33%), and *T. rubrum* (8.41%) [33].

By comparison with other studies of dermatophytes in Iran, our study demonstrated

the rapid and reliable identification of common dermatophytes at a species level. Regarding the detail of the different fragments of DNA amplified from dermatophytes with one of the four random oligonucleotides, gene regions were distinguished, and these oligonucleotides could be evolutionarily variable. Nucleotide sequence analysis of amplified bands is required for clarification of the accurate structures and functions of the gene regions.

In conclusion, AP-PCR offers a convenient solution to the problems encountered in the recognition of morphological features of dermatophyte species in comparison to conventional morphological methods. This approach shows a technological progress in identification of dermatophytosis.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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