Molecular Profile of *Trichophyton mentagrophytes* and *Microsporum canis* based on PCR-RFLP of Internal Transcribed Spacer

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**ABSTRACT**

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*Trichophyton mentagrophytes* dan *Microsporum canis* merupakan kapang dermatofit yang biasa menginfeksi hewan maupun manusia. Metode konvensional dan molekuler telah digunakan untuk mengidentifikasi kapang tersebut. Daerah *internal transcribed spacer* (ITS) mempunyai peluang besar untuk digunakan sebagai dasar dalam mengidentifikasi fungi. PCR-RFLP dilaporkan sebagai metode yang berguna untuk membedakan kapang dermatofit. Tujuan dari penelitian ini adalah membandingkan profil molekuler *T. mentagrophytes* dan *M. canis* berdasarkan hasil digesti fragment ITS dengan enzim restriksi Dde I, Hinf I dan Mva I. Kapang diisolasi dari kerokan kulit 18 ekor hewan yang menunjukkan lesi dermatofitosis. Kapang ditumbuhkan pada media agar selama 14 hari pada suhu 37°C kemudian diidentifikasi secara morfologi makro dan mikroskopik. Amplifikasi gen *chitin synthase* digunakan untuk mengkonfirmasi dan memisahkan kapang dermatofit dari kapang-kapang yang lain. Fragmen ITS diamplifikasi dan selanjutnya dipotong menggunakan enzim restriksi Dde I, Hinf I dan Mva I. Hasil menunjukkan bahwa produk digest fragmen ITS dari *T. mentagrophytes* dan *M. canis* berbeda. Fragmen 159 bp dari Dde I, 374 bp dari Hinf I dan 89 bp dari Mva I ada pada *T. mentagrophytes* tetapi tidak ditemukan di *M. canis*. Berdasarkan hasil tersebut, profil spesifik RFLP dari digesti daerah ITS oleh Dde I, Hinf I dan Mva I dapat digunakan sebagai *marker* spesifik untuk spesies dari fungi dermatofit.

**Kata Kunci:** Dermatofit, *Internal transcribed spacer*, PCR-RFLP

**ABSTRACT**

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*Trichophyton mentagrophytes* and *Microsporum canis* are dermatophytes fungi which commonly infect animal and human. Conventional and molecular methods were used for identification of the fungus. The region of internal transcribed spacer (ITS) has a high probability for fungal identification. PCR-RFLP was reported as a useful method to differentiate dermatophytes fungi. The objective of the study was to compare molecular profile of *T. mentagrophytes* and *M. canis* based on the result of ITS fragment digestion using Dde I, Hinf I and Mva I. The molds were isolated from skin scraping of 18 animals which showed dermatophytosis lesion. The isolated molds were grown on agar plate for 14 days of incubation at 37°C and then identified based on macro and microscopic morphologies. Amplification of *chitin synthase* gene was used for confirmation and separation of dermatophytes from other fungi. ITS fragment was amplified and then digested using restriction enzymes Dde I, Hinf I and Mva I. The result showed that digestion products from ITS fragment of *T. mentagrophytes* and *M. canis* were different. The fragment 159 bp from Dde I, 374 bp from Hinf I and 89 bp from Mva I were present in *T. mentagrophytes* but absent in *M. canis*. Based on these results, specific RFLP profile of digestion ITS region by Dde I, Hinf I and Mva I can be used as a specific marker for species of dermatophytes fungi.

**Key Words:** Dermatophytes, *Internal transcribed spacer*, PCR RFLP

**INTRODUCTION**

Dermatophytes are the fungus commonly invading *stratum corneum* of epidermis and keratinized tissues such as skin nails and hair of humans and animals. Cats and dogs are natural hosts which most infected by the fungus (Pasquetti et al. 2017). The fungus is commonly transmitted to human and cause *tinea capitis* and *tinea corporis* (Brillowska-Dabrowska et al. 2013). As dermatophytes transmitted from animal to animal, from animal to human and from human to human, identification and differentiation of the related species is important from an epidemiological point of view (Rezaei-Matehkolaee et al. 2012).

In the conventional identification methods, long incubation (7-14) is needed for characteristic traits to appear making the fungi difficult to be identified. Microscopic examination is limited because of the absence of macro or microconidia and the production of hyphae with prominent cross-walls. Identification was
difficult because of similarities among colonies of variant *Microsporum canis* (Rezaei-Matehkolaee et al. 2012). In addition, clinical isolates with similar geographical conditions of nature may show different phenotypes making identification even more complicated (Brilloska-Dabrowska et al. 2013; Katiae et al. 2016).

A variety of molecular techniques, such as polymerase chain reaction (PCR) need to be considered. Other methods, such as mitochondrial DNA restriction fragment length polymorphism (RFLP) pattern and Chitin synthase 1 nucleotide sequence analysis has reported an as simple, fast and accurate method for identification (Jung et al. 2014). This research used ITS primers (ITS1 and ITS4) because the primers are universal and allow selective amplification of fungal sequences. ITS region is in a ribosomal citron. The nuclear rRNA cistron has been used for fungal diagnostic and phylogenetics for more than 20 years (Begerow et al. 2010). The eukaryotic rDNA cistron consists of the 18S, 5.8S and 28S rRNA genes transcribed as a unit RNA polymerase I. Posttranscriptional processes split the cistron, removing two internal transcribed spacers. These spacers are the 5.8S which is referred as internal transcribed spacer (ITS) and the 18S nuclear ribosomal small subunit rRNA gene (SSU).

The ITS region has the highest probability of accurate identification for fungi. ITS was referred to a candidate of fungal barcode (Schoch et al. 2012). However, amplification of the internal transcribed spacer (ITS) region representing organism diversity was still unsatisfactory as the sequences of *T. mentagrophytes*, *T. tonsurans*, *T. rubrum* and *Microsporum gypseum* are very similar (Jung et al. 2014). PCR-RFLP of ITS fragment is a method that combines PCR and enzymatic digestion of the PCR products. The method was reported to be a rapid and accurate technique for fungal identification by generating band patterns on agarose gel electrophoresis, which takes only 5 hours to be carried out (Mohammadi et al. 2015). ITS PCR and RFLP have also been used for differentiation of brewing yeast and brewery wild yeast contaminant (Pham et al. 2011). Mirzahoseini et al. (2009) reported that PCR-RFLP was a reliable tool to identify dermatophytes from a clinical specimen.

Application of the Mva I and the Dde I restriction enzyme to the ITS amplicon resulting good, stable and reproducible in the identification of the dermatophytes species (Elavarashi et al. 2013). Previously, it used one or two restriction enzymes to compare molecular profile of dermatophytes fungi. This research used three enzymes to produce fragments of profile from digestion products. It was hoped that application of more enzymes produces more specific molecular profile. In addition, the data would provide information about the most suitable enzyme which used for species identification. Therefore, differentiation among dermatophytes species are more accurate. As dermatophytes fungi infect human and animals such as pets, wild and livestock, the samples were taken from cat and dog which represent pet animals and cattle which represent livestock. This research was conducted to compare molecular profile of *Microsporum canis* and *Trichophyton mentagrophytes* based on the result of ITS fragment digestion using Dde I, Hinf I and Mva I. *T. mentagrophytes* and *M. canis* produced different digestion product which can be used to distinguished both species.

**MATERIALS AND METHODS**

**Clinical isolate**

Scraping skin sample was collected from infected cat and dog patients which came to animal hospital around Bogor, Jakarta and Sukabumi, Indonesia. The scrap was inoculated in Sabouraud dextrose agar (SDA) with chloramphenicol 0.05 mg/mL and cycloheximide 0.5 mg/mL (Pal & Dave 2013), to inhibit bacteria and spreading mold. The plates were incubated at 37°C for 7-14 days. Dermatophytes fungi were purified by picking selected single colony and inoculated in new agar plate.

**Conventional identification**

The fungus was identified by colonies observation and microscopic direct examination using KOH 10%. The scraping skin was put in object-glass, KOH dropped in surface, press using cover glass. Fixation was done by trough the glass up the flame. Microscopic morphology was examined under microscope. Identification was performed based on mycelia and conidia form.

**DNA extraction**

DNA extraction was conducted according to White et al. (1990) with some modification. Mycelium of dermatophyte fungi was placed into microtube 1.5 mL. Two grams of mycelia were ground using micro pestle to form small particles. Amount of 500 µL sodium deodecyl sulphate (SDS) was added, then incubated at 65°C for 30 minutes. The mixture was let until cold, added with 500 µl chloroform isoamyl (CI 24:1) and centrifuged at 10,000 x g for 20 minutes. Supernatant was placed into a new tube and 500 µL phenol-chloroform isoamyl (PCI 25:24:1) was added and centrifuged at 10,000 x g for 10 minutes. The supernatant was placed into new tube and 100 µl Na acetate 2 M (pH 5.2) and 500 µl ethanol 100% were
Polymerase chain reaction and electrophoresis

Primer Chitin Synthase (CHSI: forward 5'-GAA GAA GAT TGT CGT TTG CAT CGT CTC-3' dan reverse 5'-CTC GAG GTG AAA AGC ACG CCA GAG-3') (Putty et al. 2018) were used to amplify dermatophytes specific sequence gene from mold. Primer ITS 1 (forward: 5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (reverse: 5'-TCC TGC TAT TGA TAT GC-3') were used to amplify ribosomal DNA (rDNA) and produced 600–700 bp (Zhang et al. 2011; Tartor et al. 2016). Amplification was performed using HotStarTaq@ Master Mix Kit (Invitrogen). Total volume was 50 µl (25 µl HotStarTaq@ Master Mix, 1 µl for each primer (10 µM), 10 µl platinum® GC enhancer, 10 µl DNA template (10 ng) and Nuclease free water until 50 µl). Polymerase chain reaction (PCR) process was conducted in pre denaturation at 95°C, 5 minutes, denaturation 95°C for 3 seconds, annealing 56°C for 60 seconds, extension 3 seconds, followed by final extension 72°C for 5 minutes. The PCR product was kept at -20°C until used. Electrophoresis for PCR product was performed using agarose 1.5 % and SYBR® safe staining, run at voltage 100 Volt. The bands were visualized using UV transilluminator.

DNA sequencing and analysis

PCR products from amplification of ITS region were sequenced and identified. The PCR product was sent to First Base Laboratories Sdn Bhd All Right Reserved, Selangor, Malaysia for sequencing. DNA sequences were analyzed using Bioedit and Mega-X and aligned with Gene Bank database using BLAST program (www.ncbi.nlm.nih.gov) and clustalw2 (https://www.ebi.ac.uk/Tools/msa/clustalo/). Open Reading Frame was determined using https://www.ncbi.nlm.nih.gov/orffinder/

Polymerase Chain Reaction-Restriction fragment length polymorphism (PCR-RFLP)

Restriction fragment length polymorphism (RFLPs) for PCR products were performed following Mohammadi et al. (2015) using enzymes Dde I, Hinfl I and Mva I (Thermo Fisher Scientific Inc). The procedure for enzymes treatment was conducted according to the protocol of each enzyme from the company. Ten µl of ITS PCR product were mixed with 18 µl nuclease-free water (NFW), 2 µl 10x Tango buffer (composed by 33 mM Tris-acetate pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA) and 1 µl Dde I (10U/µL) (Thermo Fisher Scientific Inc). The mixture was incubated at 37°C for 1 hours. The reaction was stopped by incubation in 65°C water bath for 20 minutes. For Hinfl I, ITS PCR product 10 µl, was mixed with 17 µl NFW, 2 µl 10x green buffer and 1 µl Hinfl I (Thermo Fisher Scientific Inc) then incubated at 37°C for 5 minutes. The reaction was stopped by incubation in 65°C water bath for 20 minutes. All digestion products were stored at -20°C until used. Electrophoresis for PCR digested product was performed using agarose 1.5 % and SYBR® safe staining, run at voltage 100 Volt. The bands were visualized using UV transilluminator.

RESULTS AND DISCUSSION

Isolation of dermatophytes fungi

Dermatophytes fungi were isolated from cat, dog and cattle which came to animal hospital, pet clinics and animal husbandry around Jakarta, Bogor and Sukabumi city. There was a total of 18 patients which showed clinical signs of dermatophytosis such as itchy, red, scaly, circular rush and some hair loss as showed in Figure 1. The fungi infect certain organs or even around the body.

Table 1 provides information regarding the animal and the organ which had suspected dermatophytosis in this study. The patients were dominated by cats. It may due to less dog population compare to cat. Besides cats and dogs, dermatophytes such as Microsporum canis and Trichophyton mentagrophytes were also infected calves (Pal & Dave 2013). In this research, only one from 100 examined cattle were infected. Intensive husbandry with good sanitation reduced the possibility to be infected by the dermatophytes fungi. As shown in table 1, there was no organ or breeds preference. Age ≤ 12 months more frequently infected by the fungi. Aneke et al. (2018) reported that in dogs and cats, male and young individuals develop more frequently clinical lesions. Ihan et al. (2016) found no significant association between genders in cats. The most likely risk factor for dermatophytes infection were seasons and age of animals. Winter and spring are the
Figure 1. Dermatophytosis lesion detected in cat (a) and dog (b)

Table 1. Frequency of clinical sign of dermatophytosis based on the animal, age, infected organ and breeds

| No | Animal | Breeds | Age (month) | Infected organ       | Origin of sample         |
|----|--------|--------|-------------|----------------------|--------------------------|
| 1  | Cat    | Local  | 36          | back                 | Sindangbarang, Bogor     |
| 2  | Cat    | Local  | 24          | Back, neck           | Sindangbarang, Bogor     |
| 3  | Cat    | Local  | 24          | almost all body      | Loji, Bogor              |
| 4  | Cat    | Persia | 24          | tail, neck           | Darmaga, Bogor           |
| 5  | Cat    | Local  | 4           | tail                 | Sindangbarang, Bogor     |
| 6  | Cat    | Local  | 18          | tail base            | Darmaga, Bogor           |
| 7  | Cat    | Persia | 9           | abdomen, elbow, tail | Pasirkuda, Bogor         |
| 8  | Dog    | Golden retriever | >24 | back, neck | Pasirkuda, Bogor         |
| 9  | Cat    | Local  | 12          | neck, tail           | Gunungbatu, Bogor        |
| 10 | Dog    | Local  | 8           | tail                 | Gunungbatu, Bogor        |
| 11 | Cat    | Local  | 12          | tail                 | Ciomas, Bogor            |
| 12 | Cat    | Local  | 12          | abdomen              | Darmaga, Bogor           |
| 13 | Cat    | Local  | 12          | neck                 | Sindangbarang, Bogor     |
| 14 | Cat    | Local  | 12          | tail base            | Darmaga, Bogor           |
| 15 | Cat    | Local  | 6           | neck, head           | Sindangbarang, Bogor     |
| 16 | Cattle | FH     | ±15         | Face, neck, leg      | Sukabumi                 |
| 17 | Cat    | Local  | 18          | Tail                 | Animal hospital, Jakarta |
| 18 | Cat    | Local  | 24          | Head                 | Animal hospital, Jakarta |

Seasons when cases of dermatophytes were higher. *Microsporum canis* is the most common dermatophyte in cat (90-100%) globally (Torres-Guerrero et al. 2016).

Table 1 was only performed on the animal which showed the clinical sign of dermatophytoses, but the fungi had not been yet identified. In some cases, the sign leads to dermatophytoses, but the dermatophytes fungi failed to be isolated in culture and not detected in native preparation or molecular identification. The scrapping of infected skins was then observed under a microscope using KOH 10% and some were inoculated in agar medium. Colonies and microscopic of dermatophytes fungi are shown in Fig 2. Identification was performed based on the macro and microscopic morphology and confirmed by molecular identification.

As shown in Figure 2, colony of *Microsporum canis* is coarsely fluffy, furrier on top and darker in the underside of the growth medium than that of
Trichophyton mentagrophytes. The dark yellow pigment was absent in some strains of M. canis due to failure to develop macroconidia and retardation of colony growth. Macroconidia divided into compartments which are separated by coss-wall. Microconidia M. canis also resemble other dermatophytes therefore it is not useful for diagnostic or identification. Spora of Trichophyton mentagrophytes was more abundant therefore easily recognized. On the contrary for Microsporum canis, even with prolonged 14 days incubation, the conidia were still hardly present. As consequence, molecular identification is a necessity.

In this research, internal transcribed spacer and chitin synthase were amplified for fungal identification and characterization. According to Cafarchia et al. (2013), the first and second internal transcribed spacers (ITS1 and ITS2) of nuclear ribosomal DNA and the part the chitin synthase gene (pchs1) have shown promise as markers for specific identification of dermatophytes.

Internal Transcribed Spacer (ITS)

ITS primers were used for amplification DNA region specific for fungi. The ITS bands were detected in fungi such as Candida sp., Fusarium sp. and dermatophytes but absent in bacteria (Elavarashi et al. 2013). Therefore, the ITS amplification products can be used as a fungal marker. This research use primers ITS 1 and ITS 4 to amplify both of marker specific and conserved sequence. These primer pairs are universal primers and are commonly used for fungal molecular diagnostic and identification (Ferrer et al. 2001; Aala 2012). The length sizes were various among genus and species. Amplification of the region using primer ITS 1 and ITS 4 in some dermatophytes from the previous research indicated that the region was conserved among dermatophytes fungi. PCR product using ITS1 and ITS4 primers is shown in Figure 3. PCR products were then sequenced for identification.

As shown in Figure 3 some of the fungi sequenced identified as dermatophytes fungi. One isolate was identified as Trichophyton mentagrophytes (Tm) and 7 isolates were Microsporum canis (Mc). However, not all fungi isolated from skin sent for sequencing. Mostly the fungi which had been confirmed as dermatophytes as the chitin synthase amplicon was detected (Figure 3 and 4), or the fungi which genus identified from macro and microscopic morphology.

PCR product using ITS 1 and ITS 4 primer was reported producing 690-720 bp for T. mentagrophytes and M. canis (Abdel-Fatah et al. 2013). ITS amplicon of Microsporum genus was also reported varied in size from ~851 bp in Microsporum gypseum to ~922 bp in Microsporum canis and ITS region of M. canis being ~50 bp longer than that of other dermatophytes (Cafarchia et al. 2013).

In this research, both T. mentagrophytes and M. canis produced 686-739 bp. Using the same primers pairs, the M. canis amplicon shorter than that reported by Zhang et al. (2019) (760 bp). Confirmable result was reported by Dhieb et al. (2014), 700bp. Elavarashi et al. (2013) reported that T. mentagrophytes ATCC 9533

![Figure 2](image-url)

Figure 2. Colonies and microscopic morphologies of Microsporum canis (A, B) and Trichophyton mentagrophytes (C, D) at 37°C for 14 days incubation.
produced almost similar amplicon, 690 bp. ITS fragments, produced by non-dermatophytes fungi (Table 2) such as *Aspergillus niger* (600bp), *Aspergillus bridgeri* (600 bp), yeast (around 500bp) and *Chaetomium pachypodiodes* (around 500 bp), were shorter than that of dermatophytes fungi. A similar result was reported by (Elavarashi et al. 2013) who revealed that ITS 1 and ITS 4 pairs primer produced around 550-600 bp PCR products in *Candida sp* and *Fusarium sp*. This result showed that PCR products of ITS 1 and ITS 4 pair primers can be used to distinguish dermatophytes and non-dermatophytes fungi.

**Chitin synthase**

Amplification of chitin synthase region was aimed to confirm that the isolate was dermatophytes fungi. The existence of chitin synthase band indicated a dermatophytes fungi. Saprophytic fungi isolated from skin scrapping did not produce this band. PCR for chitin synthase 1 gene was powerful to identify the presence of dermatophytes fungi from clinical isolate such as skin scraping and hair. Sharma et al. (2017) found 10 samples that were negative on the fungal culture but were positive for dermatophytes by PCR of chitin synthase indicating that PCR was more sensitive than culture.

Putty et al. (2018) reported that amplification of chitin synthase 1 gene resulting in 288 bp product size. They added that amplification of the gene was able to be considered as a rapid test for dermatophytosis to decided appropriate antifungal therapy. In this research, the same primer pairs produced longer PCR products, around 400 bp. According to Emam & Abd El-salam (2016) PCR products may be varied among the dermatophyte genus and amplicon size 288 bp was

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**Figure 3.** Internal transcribed spacer (ITS) gene of fungi isolated from skin scrapping. Dermatophytes fungi M: molecular mass DNA marker, 1, 4, 6, 9, 11, 17, 18: *Microporum canis* (650 bp) and 16: *Trichophyton mentagrophytes* (650bp). 2, 3, 5, 7, 8, 10, 12, 13, 14, 15: other fungi which was isolated from skin scrapping samples.

**Figure 4.** PCR products of chitin synthase 1 gene of fungi isolated from skin scrapping. Dermatophytes fungi produced chitin synthase I band (350-400 bp) for *Microporum canis* [6 (400bp), 7 (400 bp), 10 (350bp), 11 (350 bp), 12 (350bp), 17 (400 bp), 18 (400bp)] and *Trichophyton mentagrophytes* [1, 16 (400bp)]. M: marker. The bands were absent in non-dermatophytes fungi.
found mostly in genus *Trichopyton*. However, as shown in Figure 4, both *Trichophyton mentagrophytes* and *Microsporum canis* produced amplicon around 400 bp. Hryncewicz-Gwóźdź et al. (2011) use the same primer to amplify chitin synthase gene of dermatophytes fungi. The result showed that both *Trichophyton tonsurans* and *T. mentagrophytes* produced 366 bp, almost similar to the PCR product in this research. Based on the result, amplification of the chitin synthase using primer CHS 1 was powerfull to differenciate dermatophytes and non dermatophytes but did not able to distinguish among genera within dermatophytes. This result also indicates that primer CHS 1 can be used for determination of dermatophytes fungi from clinical samples such as skin scraping from the animals suspected suffer from dermatophytosis.

### Identification of dermatophytes fungi

Sequence analysis of PCR product of ITS genes showed that they were confirmed as *Trichophyton mentagrophytes* and *Microsporum canis*. The similarity percentage of both fungi with sequence database in GeneBank is more than 99% (Table 2).

Seven dermatophytes fungi were identified based on the characteristic colonies, microscopic morphologies and their nucleotide sequences of ITS PCR product. The fungi were identified as *M. canis* and *T. mentagrophytes*. Sequencing result of ITS 1 to ITS 4 regions of Mc1-7 showed that they had similarity almost 100% with ITS regions of *M. canis* from GeneBank. Conventional identification using macro dan microscopic morphology of *T. mentagrophytes* was also confirmed by sequencing result of the ITS region. Identification of dermatophytes and non-dermatophytes fungi isolated from cats, dogs and cattle suspected dermatophytosis as displayed in Table 3.

### Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

For further molecular profile, PCR products of ITS fragment were then digested using restriction enzymes Dde I, Hinf I and Mva I. The enzymes cut the ITS sequence in their cleavage site. ITS sequence affects the cleavage site position resulting difference in fragment size of digestion products. The digestion product was presented at Figure 5. *T. mentagrophytes* (Tm) and *M. canis* (Mc) were digested by Dde I produced different fragments. Fragment 159 bp in Tm and 201 bp in Mc was able to differentiate both genera. *Microsporum canis* (Mc) 1-5 isolated from Bogor has a different pattern with Mc 6-7 which was isolated from Jakarta. This different pattern may represent different strains circulating between both regions, although it needs further examination to prove it.

Digestion ITS sequence using Hinf I showed that there was almost no difference pattern among *M. canis*. *T. mentagrophytes* revealed 374 bp at Hinf I and 89 bp at Mva I digestion products which were absent in

### Table 2. Similarity of dermatophytes in this research with database in GeneBank

| Dermatophyte fungi in this research | Percent similarity | Accession number          |
|------------------------------------|--------------------|----------------------------|
| *Microsporum canis* (Mc-1)         | 99.54%             | *Microsporum canis* MT487816.1 |
| 100%                               | *Microsporum canis* MT423728.1 |
| *Microsporum canis* (Mc-2)         | 99.35%             | *Microsporum canis* MT487816.1 |
| 100%                               | *Microsporum canis* MT423728.1 |
| *Microsporum canis* (Mc-3)         | 99.40%             | *Microsporum canis* MT487816.1 |
| 100%                               | *Microsporum canis* MT423731.1 |
| *Microsporum canis* (Mc-4)         | 100%               | *Microsporum canis* MT633048.1 |
| 100%                               | *Microsporum canis* MT632638.1 |
| *Microsporum canis* (Mc-5)         | 99.68%             | *Microsporum canis* MT487816.1 |
| 100%                               | *Microsporum canis* MT423728.1 |
| *Microsporum canis* (Mc-6)         | 99.86%             | *Microsporum canis* MT361863.1 |
| 99.29%                             | *Microsporum canis* KF733019.1 |
| *Microsporum canis* (Mc-7)         | 99.59%             | *Microsporum canis* MT423731.1 |
| 99.59%                             | *Microsporum canis* MT423730.1 |
| *Trichophyton mentagrophytes* (Tm) | 99.56%             | *Trichophyton mentagrophytes* LC317435.1 |
| 99.56%                             | *Trichophyton mentagrophytes* LC317440.1 |
Table 3. Isolated fungi from skin scrapping of animal suspected dermatophytosis

| No | Animal | Fungi                           |
|----|--------|---------------------------------|
| 1  | Cat    | Yeast                           |
| 2  | Cat    | White colony mold               |
| 3  | Cat    | Yeast, Aspergillus sp           |
| 4  | Cat    | Aspergillus niger               |
| 5  | Cat    | Yeast                           |
| 6  | Cat    | Yeast, Microsporum canis (Mc-1) |
| 7  | Cat    | White colony mold               |
| 8  | Dog    | White colony mold, Microsporum canis (Mc-2) |
| 9  | Cat    | Yeast                           |
| 10 | Dog    | Aspergillus bridgeri, Microsporum canis (Mc-3) |
| 11 | Cat    | White colony mold               |
| 12 | Cat    | Yeast, Microsporum canis (Mc-4) |
| 13 | Cat    | Microsporum (Mc-5)              |
| 14 | Cat    | Yeast                           |
| 15 | Cat    | White colony mold               |
| 16 | Cattle | Trichophyton mentagrophytes     |
| 17 | Cat    | Chaetomium pachypodiodes, Microsporum canis (Mc-6) |
| 18 | Cat    | Aspergillus bridgeri, Microsporum canis (Mc-7) |

Figure 5. ITS fragment PCR product after digestion with Dde I, Hinf I and Mva I. M: molecular marker, Mc: Microsporum canis, Tm: Trichophyton mentagrophytes. Specific fragments for Tm are 159 bp at Dde I, 374 bp at Hinf I and 89 bp at Mva I digestions.
Table 4. In silico analysis of ITS sequence digested using Dde I, Hinf I and Mva I restriction enzymes

| Dermatophytes species | ITS size (bp) | Dde I (CTNAG) (bp) | Hinf I (GANTC) (bp) | Mva I (CCWGG) (bp) |
|-----------------------|--------------|--------------------|---------------------|-------------------|
|                       | Cut site     | In silico of RFLP fragments | Cut site | In silico of RFLP fragments | Cut site | In silico of RFLP fragments |
| *T. mentagrophytes*    | 686          | 159/260, 260/261 | 7, 145.157, 165,303,311, 374,382,540 | 407/409, 531/533, 581/583, 595/597 | 12, 48, 62, 89, 103, 122, 153, 172, 186, 277, 407, 531, 581, 595 |
| *M. canis* (Mc-1)     | 738          | 205/206, 296/297, 42/43 | 7, 142, 179, 187,193,201,213, 322,330,381,407,541,524,595 | 440/442, 605/607, 633/635 | 26, 103, 131, 163, 191, 296, 440, 605, 633 |
| *M. canis* (Mc-2)     | 738          | 207/208, 296/297, 544/545 | 7, 142, 179, 187,193,201,213, 322,330,381,407,541,524,595 | 442/444, 607/609, 635/637 | 26, 101, 129, 163, 191, 294, 442, 607, 635 |
| *M. canis* (Mc-3)     | 738          | 205/206, 296/297, 542/543 | 7, 144, 179, 187,193,201,213, 323,331,381,407,541,524,595 | 440/442, 605/607, 633/635 | 26, 103, 131, 163, 191, 296, 440, 605, 633 |
| *M. canis* (Mc-4)     | 739          | 205/206, 296/297, 542/543 | 7, 143, 179, 187,193,201,213, 323,331,381,407,541,524,595 | 440/442, 605/607, 633/635 | 26, 104, 132, 163, 191, 294, 440, 605, 633 |
| *M. canis* (Mc-5)     | 739          | 207/208, 296/297, 543/545 | 7, 69, 77, 122,142,179,187, 192,200,215,25,7,322,330,388,800,408,416,523,596 | 440/444, 606/608, 634/636 | 26, 103, 131, 163, 191, 296, 441, 606, 634 |
| *M. canis* (Mc-6)     | 738          | 205/206, 296/297, 542/543 | 7, 143, 178, 186,193,201,213, 323,330,380,407,541,524,594 | 440/442, 604/606, 632/634 | 26, 104, 132, 162, 190, 294, 440, 604, 632 |
| *M. canis* (Mc-7)     | 738          | 206/207, 297/298, 543/544 | 7, 141, 179, 187,201,214,193, 321,329,381,408,416,523,596 | 441/443, 606/608, 634/636 | 26, 102, 130, 163, 191, 295, 441, 606, 634 |

ITS size was determined from sequence between 5' forward and 3' reverse primer annealing position in this study. Bold: the fragments present in electrophoresis gel

*M. canis*. Therefore, 374 bp Hinf I and 89 bp Mva I digestion product of ITS were as a marker for *T. mentagrophytes* which can be used to distinguish it from *M. canis*. The different pattern between *M. canis* from Bogor and Jakarta was also showed by digestion using Mva I. *M. canis* isolated from Jakarta (Mc 6.7) produce more bands compared to that from Bogor. The pattern of *M. canis* isolated from Jakarta was almost similar to what was reported by Rezaei-Matehkolaei et al. (2012) (Table 5). As shown in Table 4 and 5, not all fragments in silico analysis present on an electrophoresis gel. The absent fragments concentration might be very low, and they were not detected in electrophoresis gel. It was also still leaving uncut fragments 686–738 bp in Dde I digestion which may be caused by the condition or
Table 5. Size of PCR and RFLP products of dermatophytes fungi present on electrophoresis gel

| Dermatophytes species | PCR product ITS fragments after digested by enzyme (bp) |
|-----------------------|--------------------------------------------------------|
|                       | ITS | Dde I | Hinf I | Mva I |
| T. mentagrophytes     | 686 | 159, 260, 425, 526, 686 | 157, 374 | 89, 122, 186, 407, 595 |
| M. canis (Mc-1)       | 738 | 195, 336, 441, 542, 738 | 201 | 163, 440 |
| M. canis (Mc-2)       | 738 | 193, 336, 440, 544, 738 | 201 | 162, 442 |
| M. canis (Mc-3)       | 738 | 195, 336, 441, 542, 738 | 201 | 163, 440 |
| M. canis (Mc-4)       | 739 | 196, 336, 442, 542, 739 | 201 | 163, 440 |
| M. canis (Mc-5)       | 739 | 195, 335, 440, 543, 739 | 215 | 103, 441 |
| M. canis (Mc-6)       | 738 | 205, 245, 296, 441, 532, 738 | 143, 201 | 104, 162, 440, 604 |
| M. canis (Mc-7)       | 738 | 206, 245, 297, 440, 531, 738 | 141, 201 | 102, 163, 441 |
| T. mentagrophytes (Abdel-Fatah et al 2013) | 680 | 240, 200, 190, 90 | 375, 158, 81, 65, 8 | - |
| M. canis (Dhielb et al 2014) | 700 | - | 140, 200, 240, 260 | - |
| M. canis (Rezaei-Matehkolaei et al 2011) | 737 | - | - | 441, 165, 103, 28 |
| M. canis (Didehdar et al 2016) | 737 | - | - | 441, 165, 103 |
| M. canis (Abdel-Fatah et al 2013) | 720 | No cutting pattern | - | - |

digestion time was not optimum. Ratio enzyme and DNA might not suitable as the concentration of PCR product was not measured. As in enzyme protocol mentioned the reaction is placed 1-16 hours. It is possible that the digestion processes needed further optimization. In another case, Abdel-Fatah B, et al. (2013) also reported that no cutting pattern in ITS fragments digested by Dde I.

As shown in Table 3, 4, 5 and Figure 5, digestion products from Dde I, Hinf I and Mva I has similar RFLP profile among M. canis from cats and dogs. The digestion profile from the three enzymes was able to differentiate between T. mentagrophytes and M. canis and also M. canis from Jakarta and Bogor. Based on these results, it is possible that specific RFLP profile of digestion ITS region using Dde I, Hinf I and Mva I are used as a specific marker, especially to distinguish among species and strain. However, it still needs further research to compare more samples isolated from more region. Recently, PCR RFLP is commonly used to generate species-specific DNA which used for dermatophytes identification (Mohammadi et al. 2015). Amplification of ITS region and digestion using Mva I and Dde I was also reported equally good for RFLP analysis and identification of dermatophytes directly from clinical material (Elavarashi et al. 2013).

CONCLUSION

Molecular profil from PCR_RFLP using Dde I, Hinf I and Mva I was different between Trichophyton mentagrophytes and Microsporum canis. Digestion product 159 bp from Dde I, 374 bp from Hinf I and 89 bp from Mva I were present in T. mentagrophytes but absent in M. canis. Based on these differences, it is possible that specific RFLP profile of digestion ITS region using Dde I, Hinf I and Mva I are used as a specific marker to differentiate among species, especially between T. mentagrophytes and M. canis local isolate from Indonesia.

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AUTHOR CONTRIBUTIONS

Endrawati D and Kusumaningtyas E had full access to all data in this study and contributed equally to this work.

REFERENCES

Aala F. 2012. Conventional and molecular characterization of *Trichophyton rubrum*. African J Microbiol Res. 6:6502–6516.

Abdel-Fatah B, Ahmad M, Moharam M, El-Din A, Moubasher AH A-RM. 2013. Genetic relationships and isozyme profile of dermatophytes and Candida strain from Egypt and Libya. Am J Biochem Mol Biol. 3:271–292.

Aneke CI, Otranto D, Cafarchia C. 2018. Therapy and antifungal susceptibility profile of *Microsporum canis*. J Fungi. 4:1–14.

Begerow D,Nilsson H,Unterseher M MW. 2010. Current state and perspectives of fungal DNA barcoding and rapid identification procedures. Appl Microbiol Biotechnol. 87:99–108.

Brillowska-Dabrowska A,MichaLek E, Saunte DML, Sogaard Nielsen S, Arendrup MC. 2013. PCR test for *Microsporum canis* identification. Med Mycol. 51:576–579.

Cafarchia C, Gasser RB, Figueredo LA, Weigl S, Danesi P, Capelli G, Otranto D. 2013. An improved molecular diagnostic assay for canine and feline dermatophytosis. Med Mycol. 51:136–143.

Dhib C,Esgghaier B,El Euch D, Sadfi-Zouaoui N. 2014. Phenotypical and molecular characterization of *Microsporum canis* strains in North-Tunisia. Polish J Microbiol. 63:307–315.

Elavarashi E,Kindo AJ,Kalyani J. 2013. Optimization of PCR-RFLP directly from the skin and nails in cases of dermatophytosis, targeting the ITS and the 18s ribosomal DNA regions. J Clin Diagnostic Res. 7:646–651.

Emam SM, Abd El-salam OH. 2016. Real-time PCR: A rapid and sensitive method for diagnosis of dermatophyte induced onychomycosis, a comparative study. Alexandria J Med. 52:83–90.

Ferrer C, Colom F, Frasés S, Mulet E, Abad JL, Alió JL. 2001. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. J Clin Microbiol. 39:2873–2879.

Hryniewicz-Gwóźdź A, Beck-Jendroscheck V, Brash J, Kalinowska K, Jagielski T. 2011. *Tinea capitis* and *Tinea corporis* with a severe inflammatory response due to *Trichophyton tonsurans*. Acta Derm Venereol. 91:708–710.

Ilhan Z, Karaca M, Ekin IH, Solmaz H, Akkan HA, Tutuncu M. 2016. Detection of seasonal asymptomatic dermatophytes in Van cats. Brazilian J Microbiol. 47:225–230.

Jung HJ, Kim SY, Jung JW, Park HJ, Lee YW, Choe YB, Ahn KJ. 2014. Identification of dermatophytes by polymerase chain reaction-restriction fragment length polymorphism analysis of metalloproteinase-1. Ann Dermatol. 26:338–342.

Katifrae F, Asharafi Helan J, Teifoori F. 2016. Multiple Cases of Feline Dermatophytosis Due to *Microsporum anis* Transmitted to Their Owners. J Zoonotic Disease. 1:24–27.

Mirzahoseini H, Omidinia E, Shams-Ghalharokhi M, Sadeghi G, Razzaghi-Abyaneh M. 2009. Application of PCR-RFLP to rapid identification of the main pathogenic dermatophytes from clinical specimens. Iran J Public Health. 38:18–24.

Mohammadi R, Abastabar M, Mirhendi H, Badali H, Shadzi S, Chadeganipour M, Pourfathi P, Jalalizand N, Haghani I. 2015. Use of restriction fragment length polymorphism to rapidly identify dermatophyte species related to dermatophytosis. Jundishapur J Microbiol. 8:4–9.

Pal M, Dave P. 2013. Ringworm in cattle and man caused by *Microsporum canis*: Transmission from dog. Int J Livest Res. 3:100.

Pasquetti M, Min ARM, Scacchetti S, Dogliero A, Peano A. 2017. Infection by *Microsporum canis* in paediatic patients: A veterinary perspective. Vet Sci. 4:2–7.

Pham T, Wimalasena T, Box WG, Koivuranta K, Storgårds E, Smart KA, Gibson BR. 2011. Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery “wild” yeast contaminants. J Inst Brew. 117:556–568.

Putty K, Shiva Jyothi J, Sharanya M, Srikanth Reddy M, Sai Ram Sandeep G, Abhilash M, Venkatesh Yadav J, Purushotham P, Kesavulu Naidu I, Uma Chowdhary A, et al. 2018. PCR as a rapid diagnostic tool for detection of dermatophytes. Int J Curr Microbiol Appl Sci. 7:2021–2025.

Rezaei-Matehkolaei A, Makimura K, Sybren de Hoog G, Shidfar MR, Satoh K, Najafzadeh MJ, Mirhendi H. 2012. Multilocus differentiation of the related dermatophytes *Microsporum canis*, *Microsporum ferrugineum* and *Microsporum audouinii*. J Med Microbiol. 61:57–63.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Bolchacova E, Voigt K, Croux PW, et al. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci U S A. 109:6241–6246.

Sharma R, Gupta S, Asati DP, Karuna T, Purwar S BD. 2017. A pilot study for the evaluation of PCR as a diagnostic tool in patients with suspected dermatophytosis. Indian Dermatol Online J. 8:176–180.

Tartor YH, El Damaty HM MY. 2016. Diagnostic performance of molecular and conventional methods for
identification of dermatophyte species from clinically infected Arabian horse in Egypt. Vet Dermatol. 27:401-e102.

Torres-Guerrero E, González de Cossío AC, Segundo ZC, Cervantes ORA, Ruiz-Esmenjaud J, Arenas R. 2016.Microsporum canis and other dermatophytes isolated from humans, dogs and cats in Mexico city. Glob Dermatology. 3:275–278.

White TJ, Bruns TD, Lee SB, Taylor JW. White TJ, Bruns TD, Lee SB TJ. 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: PCR Protoc A Guid to Methods Appl. New York (US): Academic Press; p. 315-322.

Zhang F, Tan C, Xu Y, Yang G. 2019. FSH1 regulates the phenotype and pathogenicity of the pathogenic dermatophyte Microsporum canis. Int J Mol Med. 44:2047–2056.

Zhang R, Ran Y, Day Y, Zhang H L Y. 2011. A case of kerion celsi caused by Microsporum gypseum in a boy following dermoplasty for a scalp wound from a road accident. Med Myco. 49:90–93.