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Multiple Rare Opportunistic and Pathogenic Fungi in Persistent Foot Skin Infection

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Abstract: Persistent superficial skin infection caused by multiple fungi is rarely reported. Recently, a number of fungi, both opportunistic and persistent in nature were isolated from the foot skin of a 24-year old male in Malaysia. The fungi were identified as *Candida parapsilosis*, *Rhodotorula mucilaginosa*, *Phoma* spp., *Debaryomyces hansenii*, *Acremonium* spp., *Aureobasidium pullulans* and *Aspergillus* spp., This is the first report on these opportunistic strains were co-isolated from a healthy individual who suffered from persistent foot skin infection which was diagnosed as athlete's foot for more than 12 years. Among the isolated fungi, *C. parapsilosis* has been an increasingly common cause of skin infections. *R. mucilaginosa* and *D. hansenii* were rarely reported in cases of skin infection. *A. pullulans*, an emerging fungal pathogen was also being isolated in this case. Interestingly, it was noted that *C. parapsilosis*, *R. mucilaginosa*, *D. hansenii* and *A. pullulans* are among the common halophiles and this suggests the association of halotolerant fungi in causing persistent superficial skin infection. This discovery will shed light on future research to explore on effective treatment for inhibition of pathogenic halophiles as well as to understand the interaction of multiple fungi in the progress of skin infection.

Key words: *Aureobasidium pullulans*, *Candida parapsilosis*, *Debaryomyces hansenii*, halotolerant fungi, *Rhodotorula mucilaginosa*, skin infection

INTRODUCTION

Skin is a multi-functional organ of human body that forms a barrier for protection against infection. However, the worldwide prevalence of skin and wound infections is high as at least 10,000 death cases were estimated for every million wound patients due to microbial infections (Percival *et al*., 2012). Though bacterial skin infection is frequently encountered, infections due to fungi and yeasts have been significantly recognized (Percival *et al*., 2012). Generally, fungal infection can be classified into six different categories, which are dermatophytosis, mucosal and cutaneous infections, opportunistic fungal infections, systemic mycoses, subcutaneous mycoses and unusual fungal infections (Richardson and Johnson, 2005). Dermatophytosis or superficial fungal infection of skin is believed to affect approximately 20-25% of the world's population and the incidence continues to increase since the 1970s (Ameen, 2010; Carlson, 2012). Superficial fungal infections are affecting the stratum corneum of skin epidermis (Schwartz, 2004). Factors aiding to the widespread of fungal infection include the widespread use of broad-spectrum antibiotics which also eliminates the protective natural microflora, increased number of immune compromised individuals due to HIV infection, cancer treatment and lifestyle disease. These infections can have debilitating effects on a person's quality of life and in some cases it can become persistent and invasive (Garber, 2001). This has resulted in the prevalence and persistence of opportunistic fungi that rarely cause infection in healthy individuals (Carlson, 2012).

Athlete's foot, known as tinea pedis, is a common dermatophytosis that causes the skin itchy, flake and fissure at the dorsal surface of the foot or chronic dryness at soles and heels (Gupta and Cooper, 2008). Although tinea pedis is not as common in many places but untreated infection can facilitate secondary bacterial and fungal infection (Bristow and Mak, 2009). Nearly 10% of

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the world population is affected by tinea pedis (Gupta *et al*., 2003). Dermatophytosis is commonly caused by dermatophytes of *Trichophyton*, *Microsporum* and *Epidermophyton* species (Ho and Cheng, 2010). In a study reported by Ng *et al*. (2001), among the dermatophytes identified were *Epidermophyton floccosum*, *Microsporum audouinii*, *M. canis*, *M. gypseum*, *Trichophyton concentricum*, *T. equinum*, *T. mentagrophytes*, *T. rubrum*, *T. verrucosum* and *T. violaceum*. Besides the dermatophytes, superficial fungal infection could be caused by yeasts such as *Malassezia* and *Candida* species (Gupta and Cooper, 2008; Ho and Cheng, 2010). In addition, other non-dermatophyte fungi which are ubiquitous in the environment have been increasingly common in superficial skin infection and the infection may mimic tinea pedis, tinea manuum and tinea unguium caused by dermatophytes (Ho and Cheng, 2010).

In this study, we aimed to isolate and identify fungal pathogens from a persistent case of superficial fungal infection of a 24-year old healthy male from Malaysia. The case was diagnosed as tinea pedis (athlete's foot) infection and had been treated with various types of antifungal creams, either prescribed or non-prescribed from various medical practitioners and clinics. The persistent infection affected the patient for more than 12 years and had resulted in the skin at the infected area of the feet to dry, scaly and crack (Fig. 1). Fungal identification was based on the 18S rDNA sequence variation and the variation was addressed based on the phylogenetic relationships among the fungal species. This fungal identification study has revealed the cause of rare incidence of persistent foot skin infection. An interesting characteristic of the fungal isolates to be halotolerant was also noted and this is for the first time that halophilic fungi are associated with persistent fungal infection.

MATERIALS AND METHODS

Fungal isolation: The infected area was swabbed with alcohol and the skin of feet was scrapped onto Sabouraud Dextrose Agar (SDA). The plates were incubated for 2 day at 30°C. The isolates designated as ZH5, ZH9, ZHA, ZH12, ZH11, ZH1 and ZH14 were maintained at 4°C on SDA plates.

Genomic DNA Isolation, PCR Amplification and Identification: The fungal cells were lysed by liquid nitrogen and genomic DNA isolation was done using Wizard Genomic DNA Purification Kit (Promega, USA). The isolated genomic DNA was viewed under UV

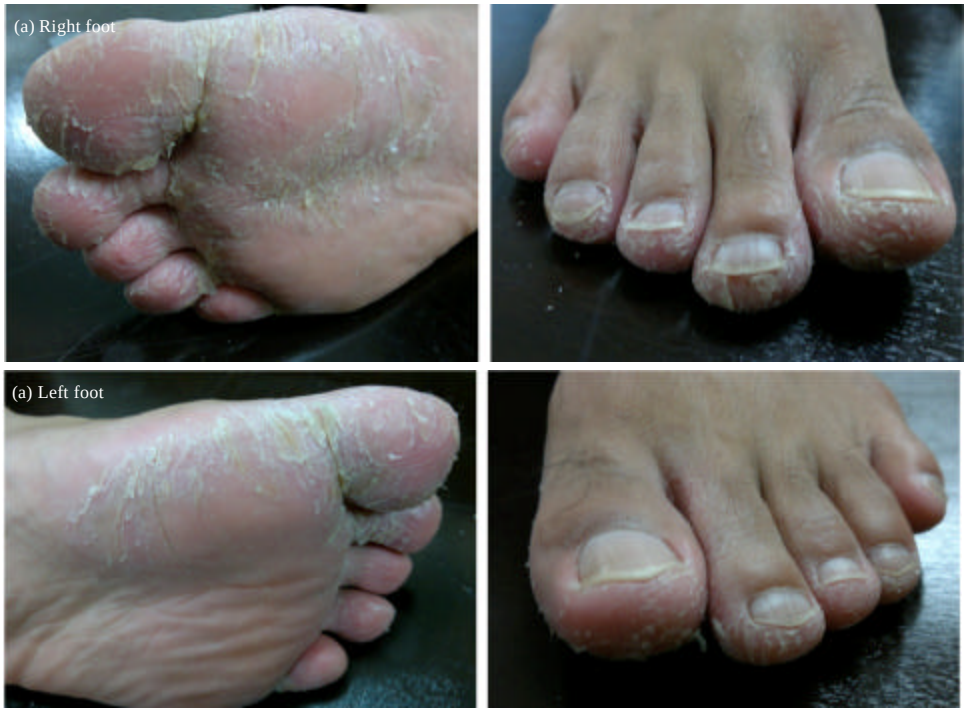


Fig. 1: Skin infection on both feet of a healthy 24-year old male patient

transillumination in 1% agarose. PCR of 18S rRNA gene was performed using NS1 (5'-GTAGTCATATGCTTGTCTC) and NS8 (5' TCCGCAGGTTCACCTACGGA) universal primers (White *et al*., 1990), following standard amplification procedures (25 cycles of 30 sec at 94°C, 30 sec at 50°C and 1.5 min at 72°C) using Go*Taq* Flexi DNA polymerase (Promega, USA). The amplified DNA fragment was purified using QIAquick PCR Purification Kit (QIAGEN, USA). Sequencing of the 18S rRNA genes was done using NS1 and NS8 primers. The sequences were assembled and submitted to online BLASTn analysis. The sequences were aligned with sequences from related fungi available from GenBank database using ClustalW. MEGA version 5 was used for construction of neighbor-joining phylogenetic trees with bootstrap values calculated based on 1000 replicates (Tamura *et al*., 2011).

Nucleotide sequence accession numbers: The sequences of the 18S rRNA genes were deposited to GenBank database and are available under accession numbers JN903910, JQ838010, JQ838011, JQ838012, JX303662, JX303663 and JX303664 for isolates ZH5, ZH9, ZHA, ZH12, ZH11, ZH1and ZH14, respectively.

RESULTS

Fungal cultures: All the fungal isolates of ZH5, ZH9, ZHA, ZH12, ZH11, ZH1 and ZH14 could grow on Sabouraud dextrose agar at 30°C. The colony morphology of the fungal isolates is shown in Fig. 2. As shown in Fig. 2a, the colonies of isolate ZH5 were small, cream-colored and most of the colonies are smooth at the surface. Colonies of isolate ZH9 were glistening, mucoid and pigmented in red (Fig. 2b). As shown in Fig. 2c, large

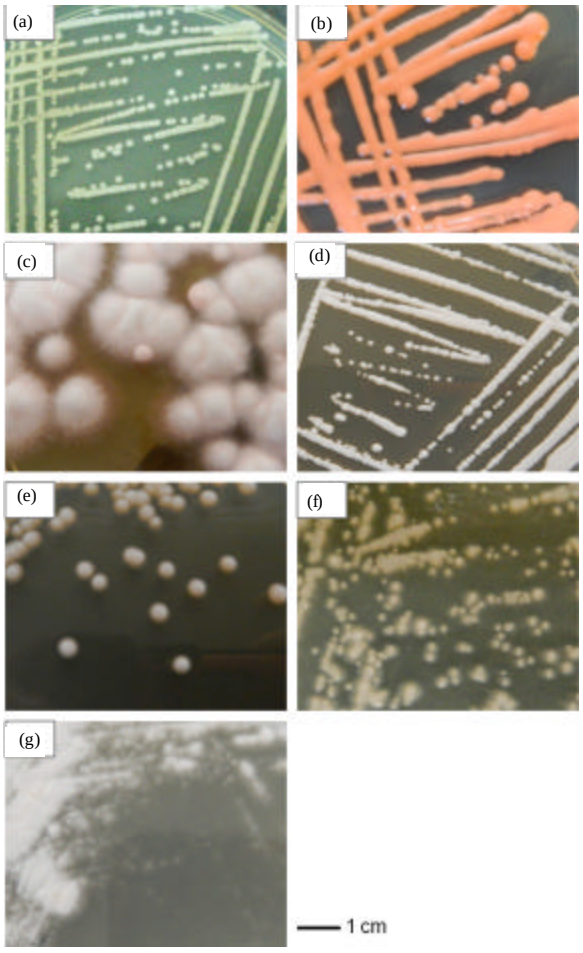


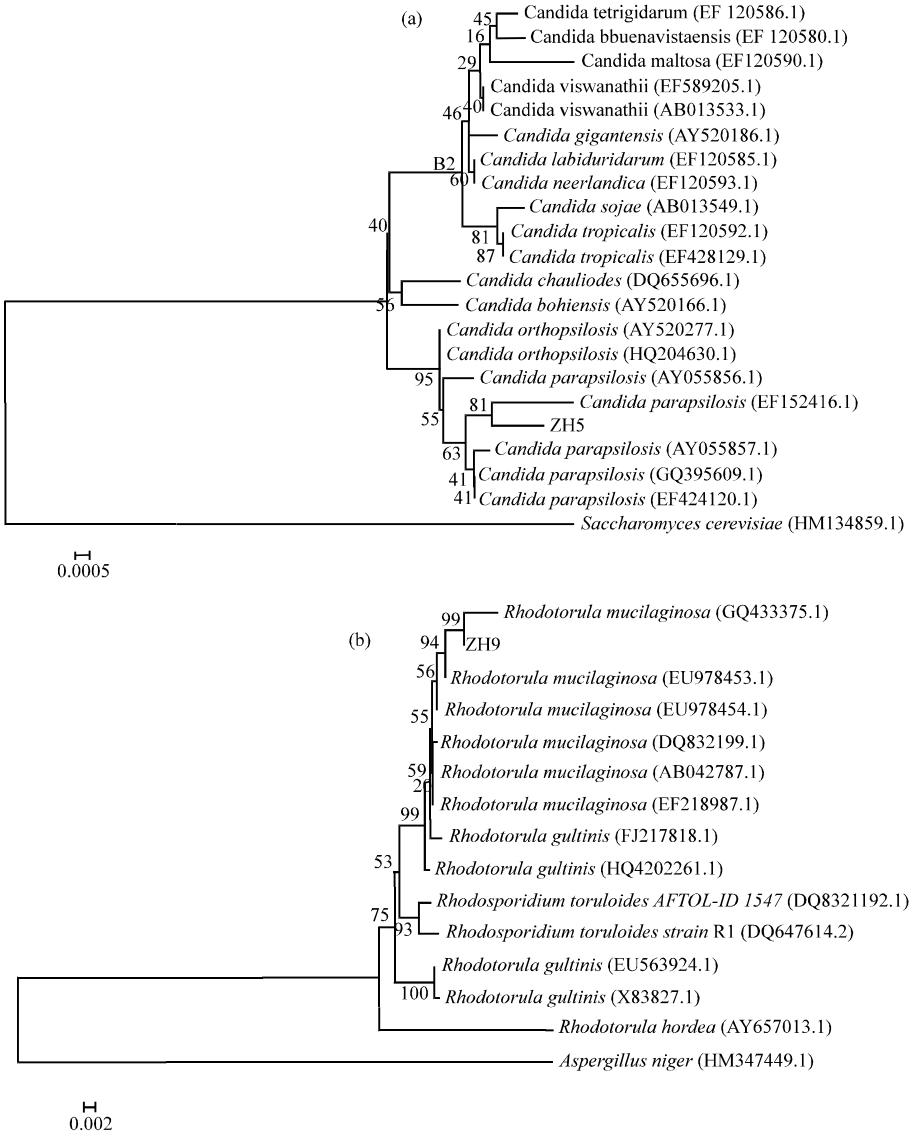
Fig. 2(a-g): Colony morphology of fungal isolates grown on Sabouraud dextrose agar. (a) ZH5, (b) ZH9, (c) ZHA, (d) ZH12, (e) ZH11, (f) ZH1 and (g) ZH14

and filamentous colonies were observed for isolate ZHA. The colonies of isolate ZH12 were small, smooth and cream in color (Fig. 2d). On the other hand, the whitish colonies of ZH11 were cottony (Fig. 2e). Colonies of ZH1 appeared to be yellowish, filamentous (Fig. 2f) and the colonies could change color to black upon exposure to light. As shown in Fig. 2g, the colonies of isolate ZH14 were downy in texture and whitish in color.

18s rRna identification of fungal isolates: The 1.8 kb genes of 18S rRNA were amplified from the fungal isolates of ZH5, ZH9, ZHA, ZH12, ZH11, ZH1 and ZH14 by using universal primers of NS1 and NS8. The gene sequences were aligned with related sequences obtained from GenBank database. The rooted and scaled phylogenetic trees for the fungal isolates are shown in Fig. 3. From these phylogenetic trees, ZH5, ZH9, ZHA, ZH12, ZH11,ZH1 and ZH14 are closely related to *Candida parapsilosis*, *Rhodotorula mucilaginosa*, *Phoma* sp., *Debaryomyces hansenii*, *Acremonium* sp., *Aureobasidium pullulans* and *Aspergillus* sp., respectively.

DISCUSSION

Skin infection is commonly diagnosed using microscopy and culture from skin scrapping. This report highlights the advantage of molecular identification in



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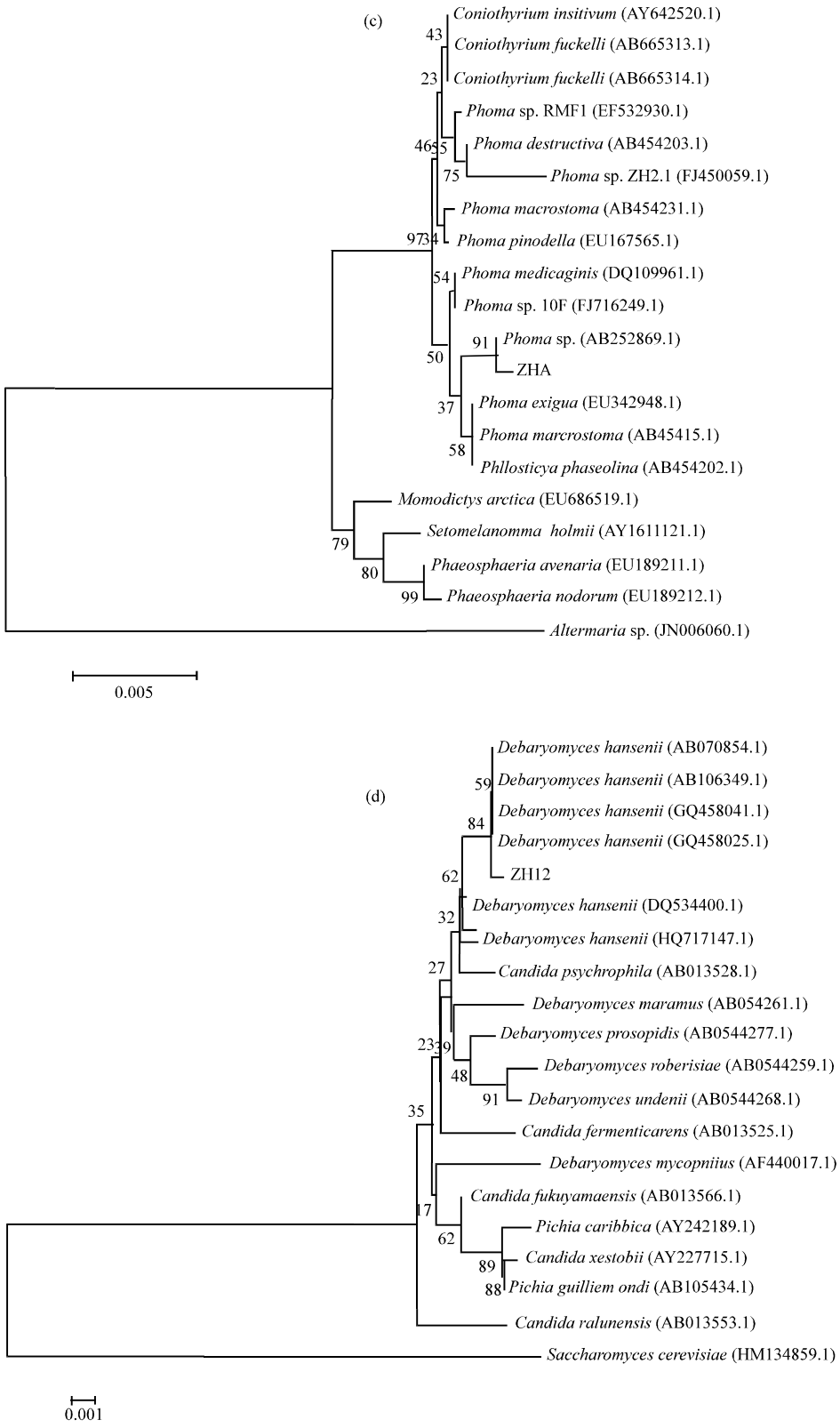

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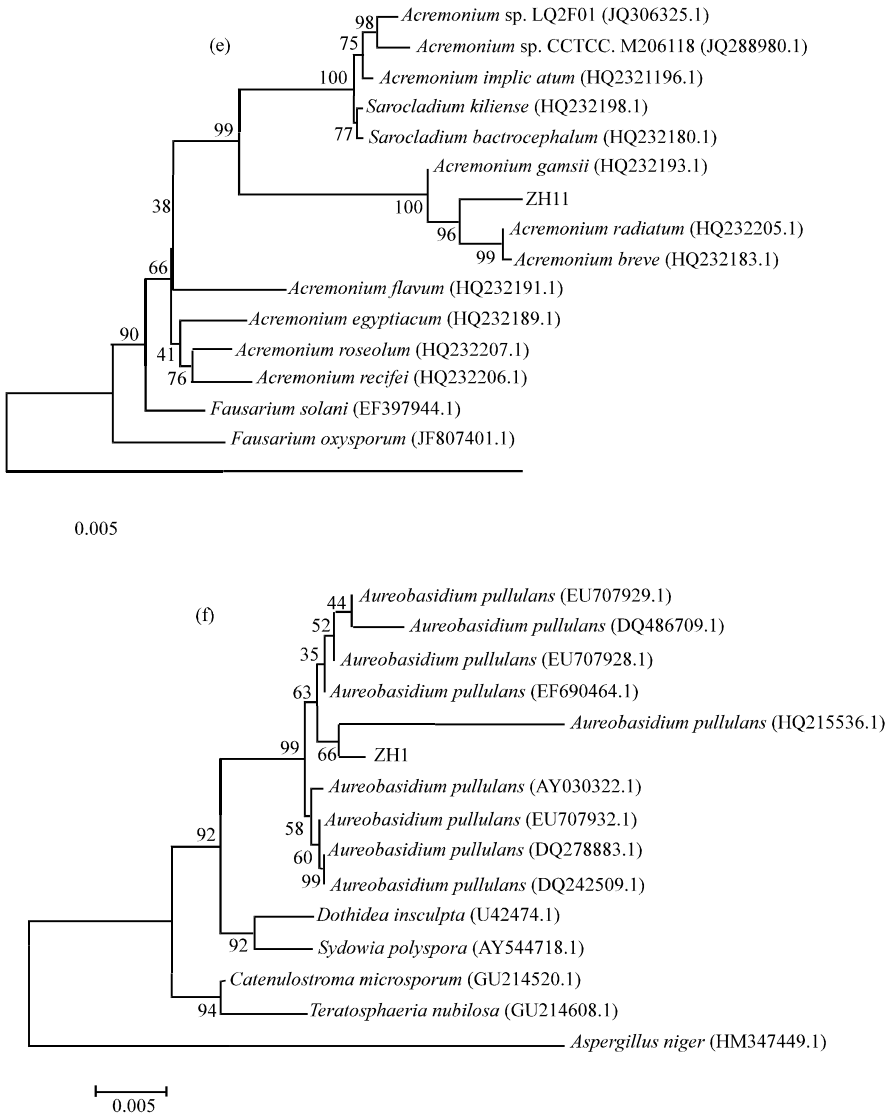

Fig. 3(a-g): Continue

characterization of fungi that caused skin infection which resulted in an accurate and thorough characterization of the fungal isolates. The 18S rRNA gene codes for the nuclear small subunit ribosomal DNA and this was selected for identification of fungi at the genus and species levels due to several reasons. The reasons include the presence of several established universal fungal primers based on the conserved regions of 18S rRNA gene that makes it possible to obtain the PCR products from most of the fungi for sequencing (White *et al*., 1990), the deposit of a large number of 18S rRNA gene sequences in GenBank database that makes similarity searches convenient and the repetitive nature of 18S rRNA which is over 100 copies per fungal genome that makes the rRNA-based amplification easier (Wu *et al*., 2003). Thus, this gene is suitable to find consensus conserved and varied regions within a group of fungi to establish the phylogenetic relationships among closely related fungi.

In this study, the case was initially diagnosed as athlete's foot and the infection is medically referred to as tinea pedis. Although rarely life-threatening, the infection affects the patient's quality of life and such persistent infection may spread or become invasive. Tinea pedis is commonly caused by dermatophytes from the genera of *Trichophyton*, *Microsporum* and *Epidermophyton*, though the inter- and intra-continental variability of species in causing global incidence was observed

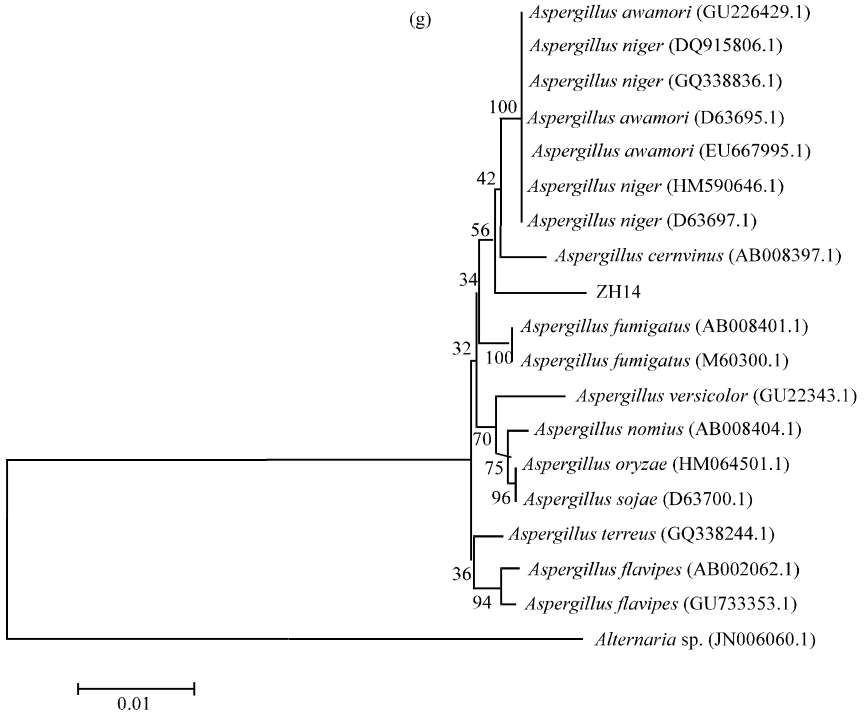

Fig. 3(a-g): Neighbor-joining tree based on 18S rRNA gene sequence alignments showing the relationship between fungi of different genus/species and fungal isolates. (a) ZH5, (b) ZH9, (c) ZHA, (d) ZH12, (e) ZH11, (f) ZH1 and (g) ZH14. Numbers in bracket indicate the GenBank accession numbers. Bootstrap values of 1,000 replicates are shown on each of the branches

(Havlickova *et al*., 2008). However, the identified fungal isolates from the patient's skin scrapping were none of those commonly reported fungi that cause tinea pedis. The isolated fungal strains presented here are closely related to *Candida parapsilosis*, Rhodotorula *mucilaginosa*, *Phoma* sp., *Debaryomyses hansenii*, *Acremonium* sp., *Aureobasidium pullulans* and *Aspergillus* sp. These multiple co-isolates are proposed to be unique in causing the infection to be persistent. To the best of our knowledge, foot skin infection caused by multiple of the rare fungal strains is reported here for the first time. On the other hand, polymicrobial infection due to Gram-positive and Gram-negative bacteria has been commonly associated with diabetic foot infections (Dryden, 2010).

Among the fungi identified in this study, *Candida parapsilosis* is one of the commonly reported yeasts isolated from human skin and nails (Jautova *et al*., 2001). Formerly, *C. parapsilosis* strains was divided into groups I, II and III on the basis of molecular printing. In 2005, groups II and III were proven to be a new species and renamed as *C. orthopsilosis* and *C. metapsilosis*, respectively (Tavanti *et al*., 2005). Strains from Group I remained as *C. parapsilosis* and these were predominantly observed among clinical isolates (Tay *et al*., 2009). The virulence of *C. parapsilosis* on reconstituted human tissue caused severe attenuation, morphological changes and cellular damage. The damage of upper tissue layer and epithelium was worsened by pseudohyphae production (Gacser *et al*., 2007). In addition, *C. parapsilosis* was reported to form biofilm, a cause for persistence in the hospital environment (Trofa *et al*., 2008). The presence of polymicrobial biofilm with sessile microbial community attached to a surface or to each other which is embedded in Extracellular Polymeric Substances (EPS) has been recognized as one of the major barriers to healing of skin and wound infection (Percival *et al*. 2012).

In this study, *Rhodotorula mucilaginosa* was also among the fungi isolated. *R. mucilaginosa* has many synonyms, including strains of *Sporobolomyces alborubescens, R. grinbergsii, R. rubra, R. pilimanae, R. matritensis* and *R. mucilaginosa* that demonstrated identical LSU rRNA sequence alignments (Fell and Statzell-Tallman, 1998). Species of *Rhodotorula* have been isolated from feces, nails, skin, sputum, digestive tract and

adenoids and since 1999, the yeast species have been reported to cause both local and systemic infections (Galan-Sanchez *et al*., 1999). A systematic review of 128 cases by Tuon and Costa (2008) revealed that *Rhodotorula* infection caused 79% fungemia (103 cases), 7% eye infections (nine cases) and 5% (six cases) peritonitis associated with continuous ambulatory peritoneal dialysis. *R. mucilaginosa* was shown as the most common species of fungemia found in 74% of the cases (Tuon and Costa, 2008). However, the yeast was a rare source of skin infection though being reported as a source of dermatitis affecting the skin of the back and thighs of chickens and cause of skin lesions in a Southern sea lion (*Otaria flavescens*) (Beemer *et al*., 1970; Alvarez-Perez *et al*., 2010).

Additionally, *Debaryomyces hansenii* was also identified from the isolates. This is an opportunistic and hemiascomycetous yeast fungal pathogen, which is able to cause fungal skin infection. The yeast was detected as one of the species that cause infection on the feet and toe webs (Mlinaric-Missoni *et al*., 2005). In addition, *D. hansenii* has also been implicated as a potential emerging pathogen under its anamorphic form, *Candida famata*. *C. famata* was isolated from skin and mucosal surfaces and considered part of the growing list of opportunistic fungi frequently found among immuno-compromised patients (Nishikawa *et al*., 1997; Beyda *et al*., 2013).

The presence of *Aureobasidium pullulans* among the fungal isolates may also contribute to the persistent of the infection. In a different case, *A. pullulans* was coisolated with *Candida orthopsilosis* from the skin with persistent fungal infection of an immuno-compromised patient (Chan *et al*., 2011). As being probed from its genome, the fungus possesses genes coding for virulent factors such as lipases, phospholipases, proteases and beta-lactamases (Chan *et al*., 2012). Most of the isolates of *A. pullulans* from the skin scrapping of healthy individuals were low in virulence and unlikely to be pathogenic (Pritchard and Muir, 1987). Furthermore, cutaneous infections caused by *A. pullulans* were formerly reported by Pikazis *et al*. (2009) and Joshi *et al*. (2010).

Though being regarded as a plant pathogen and rare cause of infection in human, *Phoma* species has been reported for causing subcutaneous infection of immuno-compromised patient with renal transplant (Everett *et al*., 2003). *Phoma minutella* was identified as the cause of subcutaneous phaeohyphomycosis on the foot, which was characterized by hyphae and pigmented fungal cells in the infected tissue (Baker *et al*., 1987).

Among the fungal isolates, *Acremonium* species could cause invasive disease in humans. The fungi of the *Acremonium* genus are among the saprophytic fungi commonly isolated in clinical laboratories. Besides onychomyosis, it has been known as a cause of chronic granulomatous infections of the skin and hypoderma, which are called eumycotic mycomas (Asadi *et al*., 2009; Kouvousis *et al*., 2003).

The association of *Aspergillus* sp. with skin infection is possible. Fungi of the genus *Aspergillus* are opportunistic pathogens which are commonly transmitted by air. Patients with large areas of wounds or burns on the skin may acquire the infection by airborne contamination as *Aspergillus* species produces minute, invisible spores (Rath *et al*., 2002). Infections caused by *Aspergillus* are known as aspergillosis (Stevens *et al*., 2000). The most frequently isolated pathogenic *Aspergillus* were *Aspergillus fumigates* and *Aspergillus flavus* (Van Burik *et al*., 1998). Outbreaks of aspergillosis involving the skin, oral mucosa or subcutaneous tissues are more often associated with *A. flavus* than other species (Hedayati *et al*., 2007).

Interestingly, most of the isolated fungi from this case of persistent infection including *C. parapsilosis*, *R. mucilaginosa*, *D. hansenii* and *A. pullulans* are known halotolerant fungi. *C. parapsilosis* and *D. hansenii* were reported to be cultivated in media containing up to 17 and 25% NaCl, respectively (Butinar *et al*., 2005). Black yeast such as *A. pullulans* was rare extremophilic eukaryote, which was able to grow at salinities up to 17% (Kogej *et al*., 2005). *R. mucilaginosa* could grow at NaCl concentrations ranging up to 2.5 M (Lahav *et al*., 2002). Furthermore, three other isolates identified in this study, namely *Phoma* sp., *Acremonium* sp. and *Aspergillus* sp. are probably halophilic and halotolerant fungi, as these species were frequently isolated from the marine environments (Koh *et al*., 2000; Raghukumar, 2004; Wiese *et al*., 2011). To our knowledge, there is no report on the cause of persistent skin infection by halotolerant fungi. Infection caused by these halophilic and halotolerant fungi could be due to their capability to survive on the skin surface that accumulates high concentration of NaCl from perspiration. Due to their halotolerant nature, the opportunistic fungi tend to survive and remain persistent. This observation will aid in the future endeavor to explore on effective treatment for inhibition of pathogenic halophiles that cause skin infection, as well as to understand the interactions among rare opportunistic and pathogenic fungi in the progress of skin infection.

CONCLUSION

From this study, different fungi were identified as the source of persistent foot skin infection. The multiple co-isolates, closely related to *Candida parapsilosis*, *Rhodotorula mucilaginosa*, *Phoma* sp., *Debaryomyses hansenii*, *Acremonium* sp., *Aureobasidium pullulans* and *Aspergillus* sp. were noted for their halophilic and halotolerant nature, which may result in the persistence of the 12-year infection.

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