



Comparative Genomics and Molecular Analysis of *Epidermophyton floccosum*

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Abstract *Epidermophyton floccosum* is one of the most common agents of human superficial fungal infections, compared with genus *Trichophyton* and *Microsporum*, it possesses uniqueness in ecology traits and rarely causing hair infections. *E. floccosum* is so far the only representative species of genera *Epidermophyton*, and it is known as anthropophilic dermatophytes. To further reveal the genome sequences and clues of virulence factors, thus in this study, we sequenced the genome of *E. floccosum* (CGMCC (F) Eld), and performed comparative genomic

analysis with other dermatophytes. It is revealed that *E. floccosum* owns the largest genome size and similar GC content compared with other dermatophytes. A total of 7565 genes are predicted. By comparing with the closest species *N. gypseum*, our study reveals that number and structure of adhesion factors, secreted proteases and LysM domain might contribute to the pathogenic and ecological traits of *E. floccosum*. Mating genes is also detected in genome data. Furthermore, we performed AFLP analysis trying to discuss intraspecific differences of *E. floccosum*, but no significant relationship is found between genotype and geographical distribution. Upon above, our study provides a deeper understanding and strong foundation for future researches about *E. floccosum*.

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Introduction

Dermatophytes cause the most common fungal infections in humans. Among the seven genera (*Trichophyton*, *Microsporum*, *Epidermophyton*, *Lophophyton*, *Paraphyton*, *Nannizzia* and *Arthroderma*) [1], *Trichophyton*, *Microsporum* and *Epidermophyton* species are the three most common causative agents in clinical trials. The genus *Epidermophyton* was first

reported in 1870 and named as *Acrothecium floccosum* [2]. In earlier studies, genus *Epidermophyton* was composed of two species, *E. floccosum* and *E. stockdaleae*, for the latter was geophilic and rarely caused human infections, and they also differed in antifungal susceptibilities and temperature tests [3, 4]. While based on the newly taxonomy of dermatophytes, *E. floccosum* is now the only representative species in the genus *Epidermophyton* [1]. *E. floccosum*, consistent with other dermatophytes, can cause superficial infections like tinea corporis and onychomycosis which is more common in tropical and subtropical areas like Iran [5] and Africa [6]. Severe disseminated infections were also reported as well [7, 8]. But it is widely acknowledged that *E. floccosum* rarely causes hair infections, the reason behind is still unknown.

Recently, with widely applications of next and third generation sequencing platforms, many pathogenic fungi have been sequenced as a consequence. While for dermatophytes, these tools have not been fully applied yet, and might be one of the reason why the pathogenic pattern still not so clear [9]. Whole genome sequences were first obtained in *Arthroderma benhamiae* and *Trichophyton verrucosum* [10], and some clinical important dermatophytes like *Trichophyton rubrum*, *Trichophyton tonsurans*, *Trichophyton equinum*, *Microsporum canis*, *Nannizzia gypseum*, *Arthroderma vanbreuseghemii* [11], *Trichophyton violaceum* [12] were sequenced later. Based on available data, researchers have found that unlike other pathogenic fungi, four gene classes are enriched in dermatophyte genomes, namely proteases secreted to degrade keratin, kinases involved in adaptation to skin, important secondary metabolites for interaction with hosts and LysM domain acting on host immune response. Although gene contents are conserved across dermatophyte genomes, it is widely accepted that dermatophytes differ in morphology, ecology and invasion process [11]. Genome study provides a potential way for exploring reasons behind these divergences. Here we sequenced genome of *E. floccosum* (CGMCC (F) E1d), and did phylogenetic analysis of related dermatophytes. As reported before by molecular method [1], *E. floccosum* shared the closest relationship with *N. gypseum*. However, *E. floccosum* is anthropophilic mainly causing nail and glabrous skin infections, while *N. gypseum* is zoophilic and geophilic causing hair and skin

infections, and the two species also differ in their morphological characters. Therefore, genomic comparison was performed between these two species, trying to reveal reasons accounting for pathogenic and ecology traits of *E. floccosum*.

Sexual reproduction is quite common among eukaryotes. For fungi, sexual reproduction not only is related to host fitness, but also related to influences virulence in some species [13]. Mating type (MAT) locus, harboring two different transcription factors genes (MAT1-1 and MAT1-2). Based on previous studies, geophilic and zoophilic dermatophytes are confirmed to reproduce sexually, while anthropophilic dermatophytes are considered to lose their ability for sexual reproduction when adapting to human host [14]. Genomic comparative analysis of *T. rubrum* and related dermatophytes revealed that genes concerning mating and meiosis are conserved across dermatophytes [11]. *E. floccosum* is known as anthropophilic dermatophyte, thus we suppose that it also tends to adopt asexual way to reproduce like *T. rubrum*, and we try to testify it with both molecular marker and genomic data acquired. Amplified fragment length polymorphism (AFLP) method has been extensively applied in population genetic, epidemiological studies, and it is suited for intraspecific differentiation of closely related groups [15]. So far, there is still limited molecular epidemiology study of *E. floccosum*, hence in this study, we try to discussion genetic variation within *E. floccosum* with AFLP method as well.

Materials and Methods

Fungal Strain and Genomic DNA Extraction

The strain *E. Floccosum* (CGMCC (F) E1d) isolated from a Chinese patient was grown on potato dextrose agar (PDA) medium at 28 °C for 14 days. Genomic DNA extraction was performed by using the Fungi DNA Kit (OMEGA) according to the manufacturer's instructions. The quality of genomic DNA was then quantified by using TBS-380 fluorometer (Turner BioSystems Inc., Sunnyvale, CA). High qualified DNA sample (OD_{260/280} = 1.8–2.0, > 6 µg) was utilized to construct fragment library.

Sequencing, Assembly and Annotation

The sequencing procedure was performed by Illumina HiSeq combined with Pacific Biosciences sequencing platforms. At least 1 µg qualified genomic DNA was used for Illumina sequencing library construction. Paired-end libraries with insert sizes of ~ 400 bp were prepared following Illumina's standard genomic DNA library preparation procedure. The qualified Illumina pair-end library would be used for Illumina HiSeq sequencing at Shanghai Biozeron Biotechnology Co., Ltd (PE150 mode). For Pacific Biosciences sequencing, 20 k insert whole genome shotgun libraries were generated and sequenced on a Pacific Biosciences RS instrument using standard methods. An aliquot of 8 µg DNA was spun in a Covaris g-TUBE (Covaris, MA) at 6000 RPM for 60 s using an Eppendorf 5424 centrifuge (Eppendorf, NY). DNA fragments were then purified, end-repaired and ligated with SMRT bell sequencing adapters following manufacturer's recommendations (Pacific Biosciences, CA). Resulting sequencing libraries were purified three times using 0.45 × volumes of Agencourt AMPure XPbeads (Beckman Coulter Genomics, MA) following the manufacture's recommendations.

We used ab initio prediction method to get gene models for strain (CGMCC (F) E1d). Gene models were identified using Augustus (<http://bioinf.uni-greifswald.de/augustus/binaries/>). Then all gene models were blast against non-redundant (NR in NCBI) database, SwissProt (<http://uniprot.org>), KEGG (<http://www.genome.jp/kegg/>), and COG (<http://www.ncbi.nlm.nih.gov/COG>) to do functional annotation by blastp module. In addition, tRNA were identified using the tRNAscan-SE (v1.23, <http://lowelab.ucsc.edu/tRNAscan-SE>) and rRNA were determined using the RNAmmer (v1.2, <http://www.cbs.dtu.dk/services/RNAmmer/>).

Phylogenetic Analysis

We identified clustering of single-copy genes of acquired dermatophytes genomes with OrthoMCL [16]. Individual amino acid sequences were aligned with MUSCLE v3.8.31 [17]. The phylogenetic tree was inferred by maximum likelihood method with PhyML v3.0 program [18] and 100 bootstraps were used to infer branch support. Beyond *E. floccosum*, genomic sequences of other dermatophytes including

M. canis, *N. gypseum*, *T. benhamiae*, *T. equinum*, *T. interdigitale*, *T. mentagrophytes*, *T. rubrum*, *T. soudanense*, *T. tonsurans*, *T. verrucosum* and *T. violaceum* were downloaded from NCBI genome database (<https://www.ncbi.nlm.nih.gov/genome/>).

Comparative Genomics

Comparative genomic analysis was conducted between *E. floccosum* (CMCC (F) E1d) and *N. gypseum* (MS_CBS118893). Protein sequences were aligned using CD-HIT v4.6.1 [19] (identity > 40%, coverage > 50%).

Adhesion Analysis

For adhesion prediction, three software products were tested, SignalP [20] was used to identify signal peptide, TMHMM [21] was used to predict transmembrane protein domains with default parameters, and Big-PI Predictor [22] was used to identify GPI-anchor sites. Potential adhesions were identified with the following parameters: SignalP positive, TMHMM negative and Big-PI positive.

Proteases Analysis

Local database related to keratin degradation of dermatophytes [23] including endoproteases and exoproteases were downloaded from Uniprot database (<https://www.uniprot.org/>). Orthologs were selected when E values < 1e-3 and similarities > 90%.

LysM Domain Analysis

Proteins with LysM domains were identified with Pfam database (<http://pfam.xfam.org/>) based on multiple sequence alignments and hidden Markov models (HMMs) as previously reported [11].

AFLP Analysis

A total of 19 *E. floccosum* strains were collected from the Institute of Dermatology, Chinese Academy of Medical Sciences and CBS (Table 1). All strains were identified by both morphological methods and sequencing of internal transcribed spacer (ITS) regions [24]. AFLP genotyping was performed according to previously described methods [25, 26].

Table 1 *E. floccosum* information in study

Number	Current taxon name	New taxon name	Resources/lesion site	Country
CBS 100148	<i>A. uncinatum</i> / <i>E. stockdaleae</i>	<i>E. floccosum</i>	Human/groin	India
CBS 108.67	<i>E. floccosum</i> var. <i>floccosum</i>	<i>E. floccosum</i>	Human	Netherlands
CBS 230.76	<i>E. floccosum</i> var. <i>floccosum</i>	<i>E. floccosum</i>	Human	Unknown
CBS 240.67	<i>E. floccosum</i> var. <i>floccosum</i>	<i>E. floccosum</i>	Human	Netherlands
CBS 457.65	<i>E. floccosum</i> var. <i>nigricans</i> Frágnér	<i>E. floccosum</i>	Unknown	Netherlands
CBS 130793	<i>E. floccosum</i>	<i>E. floccosum</i>	Human	Iran
CBS 130802	<i>E. floccosum</i>	<i>E. floccosum</i>	Human	Iran
CBS 214.63	<i>E. floccosum</i> var. <i>floccosum</i>	<i>E. floccosum</i>	Human	Germany
CBS 236.90	<i>E. floccosum</i> var. <i>floccosum</i>	<i>E. floccosum</i>	Human/foot	Pakistan
CBS 358.93	<i>E. floccosum</i> var. <i>floccosum</i>	<i>E. floccosum</i>	Human	Netherlands
CBS 566.94	<i>E. floccosum</i> var. <i>floccosum</i>	<i>E. floccosum</i>	Human/ear	Unknown
CBS 767.73	<i>E. floccosum</i> var. <i>nigricans</i> Frágnér	<i>E. floccosum</i>	Human/groin	Germany
CBS 970.95	<i>E. floccosum</i> var. <i>floccosum</i>	<i>E. floccosum</i>	Human/nail	Unknown
CMCC(F) E1c	<i>E. floccosum</i>	<i>E. floccosum</i>	Human	China
CMCC(F) E1d	<i>E. floccosum</i>	<i>E. floccosum</i>	Human	China
CMCC(F) E1e	<i>E. floccosum</i>	<i>E. floccosum</i>	Human	China
CMCC(F) E1f	<i>E. floccosum</i>	<i>E. floccosum</i>	Human	Netherlands
CMCC(F) E1g	<i>E. floccosum</i>	<i>E. floccosum</i>	Human	China
CMCC(F) E1h	<i>E. floccosum</i>	<i>E. floccosum</i>	Human/hand	China

Briefly, first step was usage of two restriction endonucleases (HpyCH4 IV and MseI) to obtain restriction fragments. HpyCH4 IV adapter, MseI adapter and T4 DNA ligase were adopted to perform a combined restriction-ligation procedure. Fluorescently labeled primer (HpyCH4IV) was used for sample PCR reaction. The products were 10 × diluted and combined with GeneScan LIZ500 internal size standard (Applied Biosystems, Foster City, CA, USA). After denaturation and cool down process, the samples were subjected to 96 capillary 3730xl DNA Analyzer platform (Applied Biosystem). Raw data were imported into Genemapper v4.1 software (Applied Biosystem) and analyzed by using UPGMA clustering and Sorensen's Coefficient. DNA fragments in the range of 50–500 bp were submitted for analysis.

Mating Genes

Mating type analysis was performed for 19 isolates with primers TR- α and TR-HMG separately as described previously [27]. PCR amplification was carried out at the following condition: initial

denaturation at 94 °C for 5 min, 35 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 90 min, and extension at 72 °C for 60 s, with final extension at 72 °C for 10 min. Amplified products were analyzed visually by 1% (w/v) agarose gel electrophoresis in TBE buffer at 90 V for 30 min. The products of TR- α primers are indicative of the MAT1-1 mating type, or the products of TR-HMG primers are indicative of the MAT1-2 mating type. Since mating genes of MAT1-1 and MAT1-2 had been revealed in genome data of related dermatophytes, blast was performed of genome sequences of CGMCC (F) E1d accordingly.

Results and Discussion

Genome Sequencing and Annotation

The genome of *E. floccosum* (CGMCC (F) E1d) was successfully sequenced using Illumina HiSeq combined with Pacific Biosciences platforms and the sequence was submitted to NCBI (ID:

PRINA528555). The estimated genome size of 24.4 Mb. Compared with genome size of other clinical important dermatophytes [11], *T. rubrum* (22.5 Mb), *T. interdigitale* (23.0 Mb), *M. canis* (23.1 Mb) and *N. gypseum* (23.2 Mb), *E. floccosum* owns the largest genome size. The GC content is 48.5%, which is in similar with other dermatophytes. The number of assembled scaffolds is 46 with N50 of 1.44 Mb, and the length of largest one is 4.72 Mb. 7565 protein coding genes are predicted accounting for 49.39% of genome. When refers to the genome contents, they are relatively conservative within dermatophytes, which further confirms the conclusions of previous study [11].

Phylogeny Analysis

A phylogenetic tree was done between 12 dermatophytes (Fig. 1). Genomic data of genus *Trichophyton* are more sufficient than that of *Microsporium*. Considering the similarity of gene sequences, we can conclude that genomes are highly similar between the dermatophytes. *N. gypseum* contains the largest number of unique genes, while *T. violaceum* contains the least ones. Among all selected dermatophytes, *E.*

floccosum shares the closest relationship with *N. gypseum* which consistent with previous study with gene markers [1]. It is widely known that *E. floccosum* is anthropophilic mainly causing infections of glabrous skin and nails. While for *N. gypseum*, which belongs to geophilic species, it is a pathogen of hair and skin of human and animals. Thus comparative genomic analysis was performed between these two species trying to reveal the reason of the specificity in ecology and pathogenicity of *E. floccosum*.

Comparative Genomic Analysis

Adhesion Analysis

Adherence to host surface is first and indispensable step for invasion. Table 2 shows probable adhesions of *E. floccosum* and *N. gypseum* (19 and 22 separately), most of the genes (29/41) are annotated as hypothetical protein for limited database. Several genes have definite function which are common within these two species like cu-Zn superoxide dismutase (E1dA0486 and MGYG-04560), carboxypeptidase S1 (E1dA4066 and MGYG-03675), UTR2 protein (E1dA6473 and MGYG-06064). E1dA2196 of *E. floccosum* is 1,

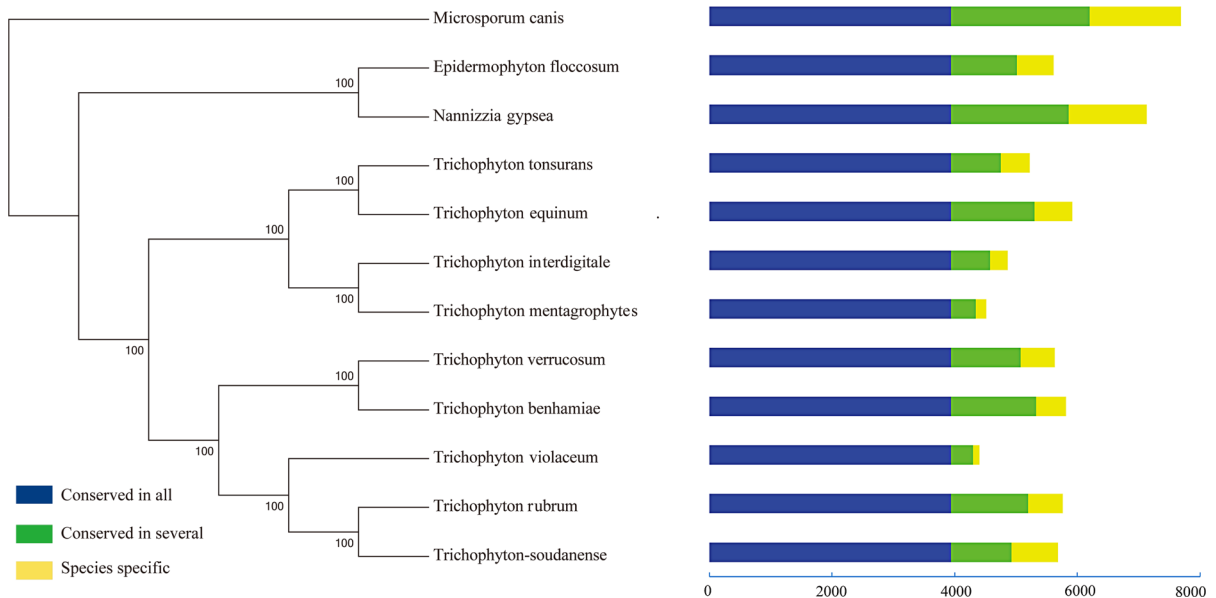


Fig. 1 Phylogenetic relationship and gene conservation of dermatophytes, blue color means genes conserved in all included dermatophytes, green color means genes conserved

in more than one dermatophytes, and yellow color means those are species specific genes

Table 2 Adhesions predicted of (a) *E. floccosum*, (b) *N. gypseum*

<i>E. floccosum</i>	Length	Annotation
(a)		
E1dA0486	265	cu-Zn superoxide dismutase
E1dA1029	892	Hypothetical protein
E1dA1910	321	Hypothetical protein
E1dA2196	508	1,3-Beta-glucanosyltransferase gel3
E1dA2258	643	Hypothetical protein
E1dA2325	95	Hypothetical protein
E1dA2581	258	Hypothetical protein
E1dA3102	289	Hypothetical protein
E1dA3222	321	Hypothetical protein
E1dA3319	237	Hypothetical protein
E1dA3518	192	Uncharacterized protein
E1dA3615	187	Hypothetical protein
E1dA4066	651	Carboxypeptidase S1
E1dA4664	172	Hypothetical protein
E1dA4905	275	GPI anchored serine-rich protein
E1dA5120	454	Hypothetical protein
E1dA6364	233	Hypothetical protein
E1dA6473	448	UTR2 protein
E1dA7405	386	Hypothetical protein
(b)		
E1dA0486	265	cu-Zn superoxide dismutase
E1dA1029	892	Hypothetical protein
E1dA1910	321	Hypothetical protein
E1dA2196	508	1,3-Beta-glucanosyltransferase gel3
E1dA2258	643	Hypothetical protein
E1dA2325	95	Hypothetical protein
E1dA2581	258	Hypothetical protein
E1dA3102	289	Hypothetical protein
E1dA3222	321	Hypothetical protein
E1dA3319	237	Hypothetical protein
E1dA3518	192	Uncharacterized protein
E1dA3615	187	Hypothetical protein
E1dA4066	651	Carboxypeptidase S1
E1dA4664	172	Hypothetical protein
E1dA4905	275	GPI anchored serine-rich protein
E1dA5120	454	Hypothetical protein
E1dA6364	233	Hypothetical protein
E1dA6473	448	UTR2 protein
E1dA7405	386	Hypothetical protein

3-beta-glucanosyltransferase gel3 which is specific to *E. floccosum*. The 1, 3-beta-glucanosyltransferase GEL family remains in *Aspergillus fumigatus* and *Neurospora crassa*, and it is reported that gel3 is an important factor for mycelial growth [28, 29]. The importance of gel3 in *E. floccosum* needs more fundamental studies.

Protease Analysis

Secretion of various proteases plays a vital role for keratin degradation and is considered to be important virulence factors. Enrichment of proteases of dermatophytes has been validated with previous genomic analysis [11, 30]. Among all secreted proteases, subtilisin (S8A family) and fungalsin (M36) of extracellular endoproteases are prevalent in dermatophytes [12]. Our study (Fig 2) shows that highly similarity is found between *E. floccosum* and *N. gypseum* in M36 (Mep3-Mep5). For S8A family, which has been proven involving in mutualisms with hosts of many fungi [31]. In *T. rubrum*, the most prevalent dermatophytes in China, sub7 expresses highest on nail-keratin [32]. In our study, 5 genes encode sub7 for *E. floccosum* while 1 for *N. gypseum*, which may be a partial reason for more nail infections for *E. floccosum*. In addition, it has been proved that sub1 and sub3 expression, sub3 especially, are required for adherence in guinea pig model for *M. canis* [33, 34]. In our analysis, no gene encodes sub1 or sub3 annotated with local database in *E. floccosum*, thus we hypothesized that sub1 and sub3 may partly explain the anthropophilic character of *E. floccosum*. However, gene expression analysis is needed to testify this hypothesis.

LysM domain analysis

The LysM was originally considered as an enzyme degrading bacterial cell walls and first reported in fungus *Cladosporium fulvum*, acting as a virulence factor [35]. Hitherto, LysM is found among fungi with various lifestyles [36]. It is supposed that LysM effectors in dermatophytes may take part in breaking down certain products of fungal cell walls which can serve as triggers of host immunity during invasion process, resulting in chitin mask, therefore avoiding host immunostimulation [11, 36]. LysM domain can also facilitate adhesion of pathogens to human skin

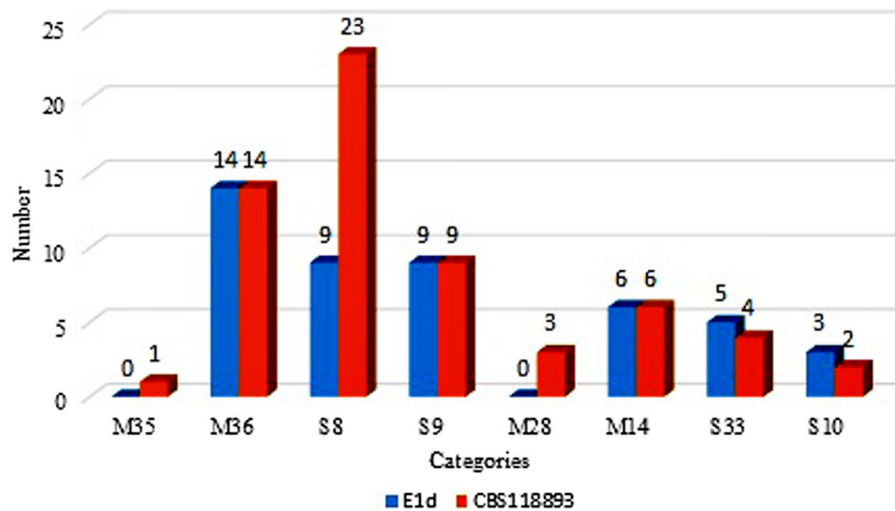


Fig. 2 Proteases genes predicted of *E. floccosum* and *N. gypseum*. Including endoproteases (M35, M36 and S8) and exoproteases (S9, M28, M24, S33 and S10)

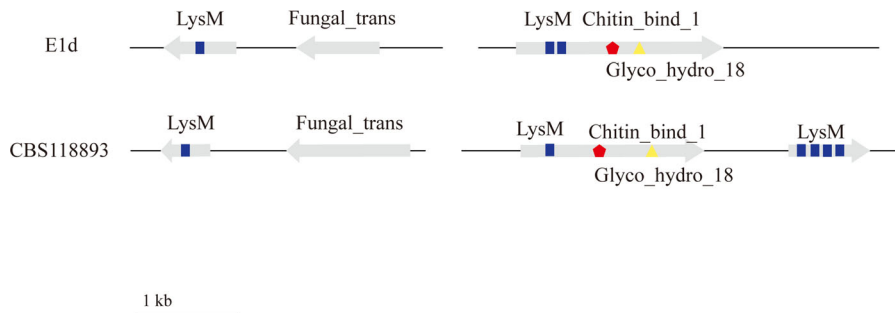


Fig. 3 Structure of LysM gene cluster of *E. floccosum* (E1d) and *N. gypseum* (CBS118893)

[37]. Figure 3 shows the LysM structure of *E. floccosum* and *N. gypseum*. The number of LysM genes within dermatophytes varies (10–31) [38]. *E. floccosum*, in this study, only possesses 7 related genes. The number of LysM genes varies greatly within fungi, the small number of LysM genes can serve as proof that chitin protection is not a necessary method for this fungus. Secretion signals, similar with *N. gypseum*, are also detected in *E. floccosum*. Based on the structure of LysM proteins, they can be classified into 5 types (A–E), type A and type B constitute structure of both *E. floccosum* and *N. gypseum*, with number varies. Type A is considered to play a role in invasion process, and type B is thought to take part in hydrolytic activity [36]. The number of LysM genes and type A, as we suggest, might play a role in their different reaction to host immunity thus resulting in different pathogenic process. What's

more, opposite direction in LysM domain is revealed between the two species, while the reason and meaning of this need further researches.

Mating Genes

The results of gel electrophoresis are shown in Figs. 4 and 5. All isolates show positive amplification result with TR-HMG primers (MAT1-2) and the fragments are about 700 bp. In genome sequence, *N. gypseum* (CBS118893) was reported to be type MAT1-1, comparing with it, *E. floccosum* (CGMCC (F) E1d) is revealed to be type MAT1-2. Reproduction is known as a powerful way adapting to environment alteration, *E. floccosum*, anthropophilic fungus, also lose its ability to reproduce sexually when colonize or infect humans as predicted. What's more, opposite

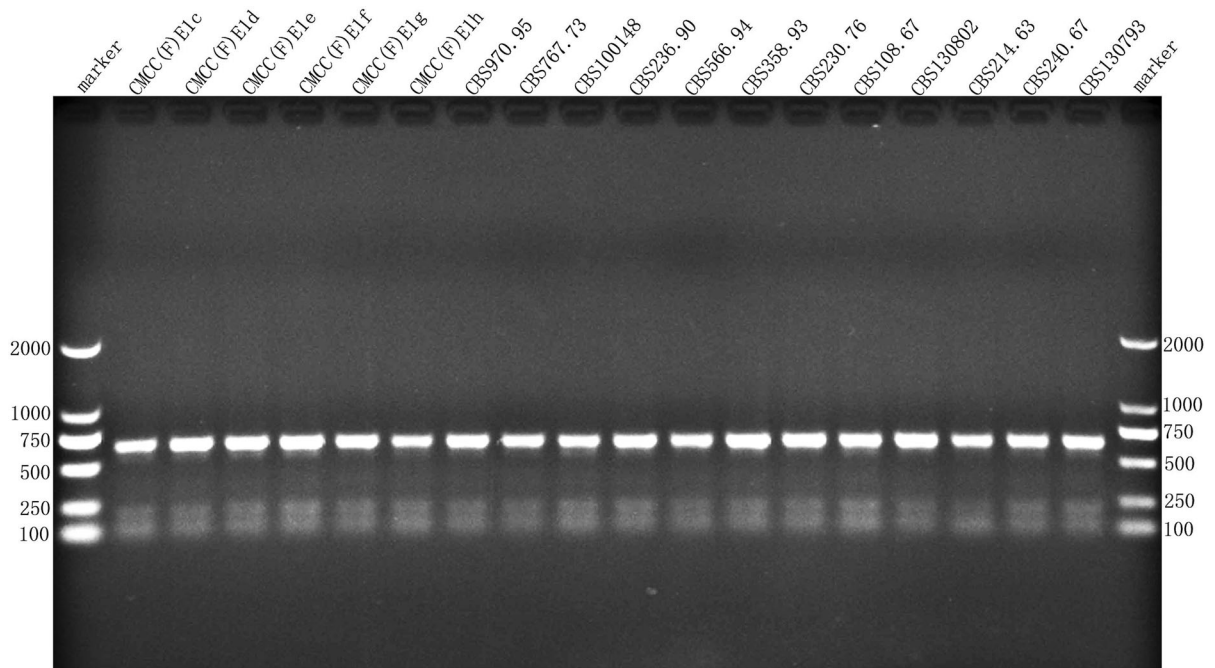


Fig. 4 Gel electrophoresis result with TR-HMG primers of *E. floccosum* which is indicative of MAT1-2, fragments about 700 bp

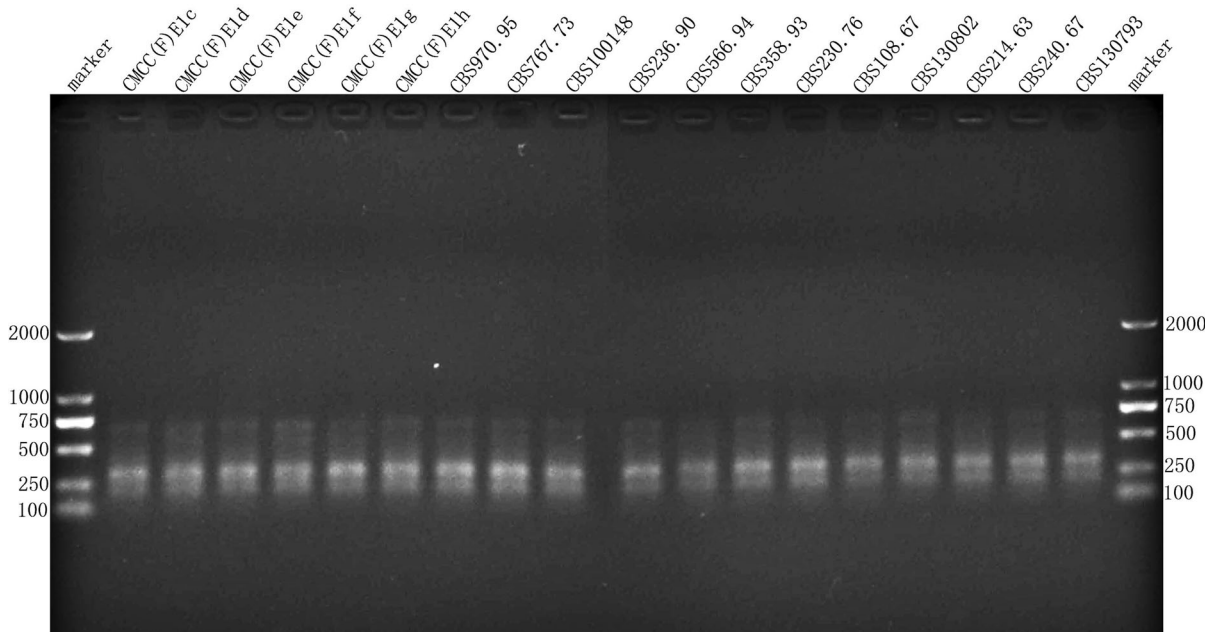


Fig. 5 Gel electrophoresis result with TR- α primers of *E. floccosum* which is indicative of MAT1-1

direction is also found between the two species, further studies needed as well.

AFLP Analysis

AFLP method has been successfully done in several *Trichophyton* and *Microsporum* species [15] but not in

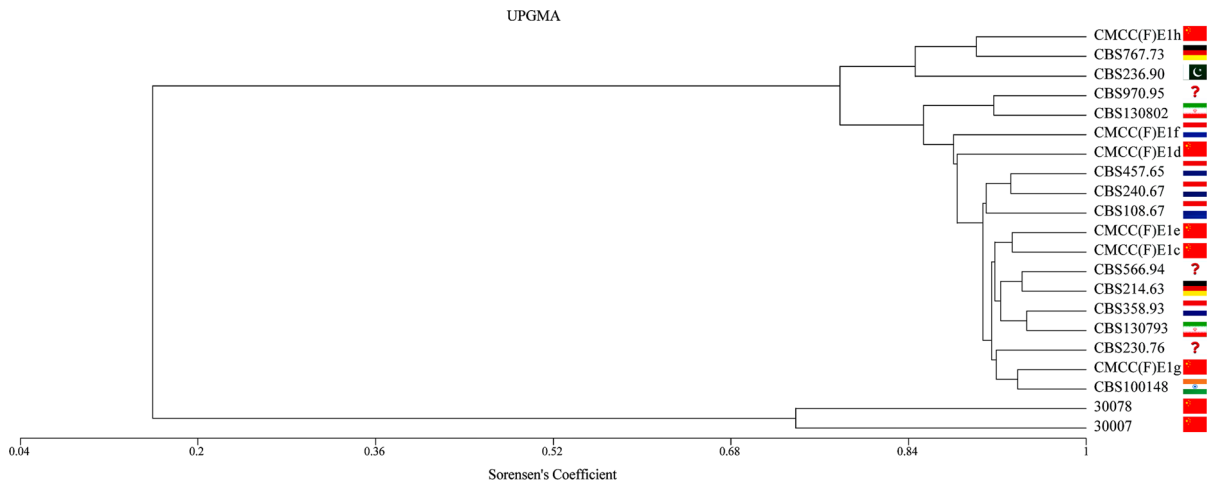


Fig. 6 AFLP analysis of 19 *E. floccosum* and 2 outgroups. The scar bar indicates the percentage similarity

E. floccosum. In this study, The AFLP analysis was efficiently performed in *E. floccosum* and two outgroup strains (30,078 and 30,007), the phylogenetic analysis obtained with UPGMA is shown in Fig. 6. Four clusters (group1-4) could be distinguished at about a cutoff value of 90% similarity. Group 2 contain only one isolate from Pakistan. Group 4 is the most common group contained isolates from different countries (China, the Netherlands, Germany, Iran and India). Isolates from the Netherlands ($n = 5$) were all in group 4. Consistent with previous studies [39–41], existence of genetic diversity in *E. floccosum* also validated with AFLP method. Unlike random amplified polymorphic DNA PCR (RAPD) method [40], all isolates can be divided into four rather than three main gene types. In geographical distributions, isolates from the Netherlands (5/16) are located in the main gene type, while isolates from China, Germany and Iran spread scattered otherwise. Limited by number and distribution of tested strains, the significance of this genotype needs further studies. In earlier studies, *E. floccosum* and *E. stockdaleae* were two distinct species of the genus *Epidermophyton*, for the latter behaves differently in antifungal susceptibilities, temperature tests and seldom causes human infections [21, 22]. *E. floccosum* is now recognized to be the only representative species by new taxonomy [1]. In this study, CBS100148 isolated from India (formerly classified as *E. stockdaleae*), it belongs to the main gene type and shares the closest relationship with CGMCC (F) E1g (*E. floccosum*) from China, it does

not form a distinct gene type, this phenomenon further confirms the reliability of newly taxonomy. In addition, considering the classification varies with methods alteration, we propose that standard criteria need to be made in the near future.

Conclusion

To our best knowledge, this is the first report of the genome sequences of *E. floccosum*, and we did phylogenetic analysis based on genome data of other clinical important dermatophytes. It is verified that dermatophytes share similar gene content. The number and structure of protease like sub3 and sub7, adhesion factors and LysM genes may contribute to the specificity of *E. floccosum*. In addition, *E. floccosum* also lose its ability to reproduce sexually for anthropophilic feature. Four genotypes acquired with AFLP method, seems not correlated with geographical distribution, the significance needs larger samples and further study.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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