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PRP8 intein in dermatophytes: Evolution and species identification

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Abstract

Dermatophytes are keratinophilic fungi belonging to the family *Arthrodermataceae*. Despite having a monophyletic origin, its systematics has always been complex and controversial. Sequencing of nuclear ribosomal ITS and D1/D2 rDNA has been proposed as an efficient tool for identifying species in this group of fungi, while multilocus analyses have been used for phylogenetic species recognition. However, the search for new markers, with sequence and size variation, which enable species identification in only one polymerase chain reaction (PCR) step, is very attractive. Inteins seems to fulfill these characteristics. They are self-splicing genetic elements present within housekeeping coding genes, such as *PRP8,* that codify the most important protein of the spliceosome. The PRP8 intein has been described for *Microsporum canis* in databases but has not been studied in dermatophytes in any other published work. Thus, our aim was to determine the potential of this intervening element for establishing phylogenetic relationships among dermatophytes and for identifying species. It was found that all studied species have a full-length PRP8 intein with a Homing Endonuclease belonging to the family LAGLIDADG. Phylogenetic analyses were consistent with other previous phylogenies, confirming *Epidermophyton floccosum* in the same clade of the *Arthroderma gypseum* complex, *Microsporum audouinii* close to *M. canis*, differentiating *A. gypseum* from *Arthroderma incurvatum*, and in addition, better defining the *Trichophyton interdigitale* and *Trichophyton rubrum* species grouping. Length polymorphism in the HE region enables identification of the most relevant *Microsporum* species by a simple PCR-electrophoresis assay. Intein PRP8 within dermatophytes is a powerful additional tool for identifying and systematizing dermatophytes.

Key words: dermatophytes, PRP8 intein, phylogeny, identification.

Introduction

Dermatophytes are filamentous fungi present in soil, humans, and other animals. This broad capacity for living in different habitats enabled their classification according to their way of life as geophilic, zoophilic, and anthropophilic.[1–3] Three genera are classically established:

Trichophyton, *Microsporum,* and *Epidermophyton*, based on morphological characteristics. They belong to the order Onygenales and possess keratinases for degrading keratinized tissues in animals and humans, causing superficial and cutaneous lesions on skin, hair or nails.[4–8]

The taxonomy of this fungal group has been controversial, and research studies have been published, some of which unify different species into only one and others that propose new cryptic species complexes.[9–12] Besides morphological, physiological, and reproductive studies to classify dermatophytes,[13–17] molecular biology has elucidated some systematic issues by applying the phylogenetic species concept.[3,11,12,18,19]

Some variable genomic markers have been proven useful to identify and classify dermatophytes, such as ITS (internal transcribed spacer) 1 and ITS 2 from the nuclear noncoding ribosomal RNA and D1/D2 regions located in rRNA from the large ribosomal subunit 28S.[3,11,19,20] Other genomic variable regions, such as inteins, that have been applied for identifying fungal species,[21–24] have never been explored in dermatophytes.

Inteins are intervening protein splicing elements firstly identified in 1990 in the *VMA* (ATPase Vacuolar Membrane) gene in *Saccharomyces cerevisiae*.[25,26] Since then, additional studies have applied much effort into understanding the mechanism for intein splicing from the precursor protein, mainly concerning the amino acids (aa) residues, from both inteins and exteins (intein flanking sequences), indispensable for this function.[27–30] Therefore, protein-splicing is defined as the excision of the intein coupled with ligation of N and C-terminal exteins, which leads to an active and functional protein.[31–33] If this mechanism is not performed in a highly efficient manner, it could result in a malfunctional or totally inactive protein (extein). A deficient splicing mechanism may lead to intein loss by selective pressures.[22,34,35]

Inteins are considered ancient elements and can reach new targets by means of a homing endonuclease (HE), generally found within the intein sequences, splitting the splicing domain into N- and C-terminals domains. Inteins are often located in conserved host protein motifs probably by the conservation of the HE specific DNA recognition sites, which possess sufficiently length to promote a DSB (double strand break) in one specific location of the genome, commonly an empty and cognate allele.[36–39] Inteins that contain a HE are denominated full-length inteins and are larger than mini-inteins (those containing only the splicing domain).[22] Several conserved blocks have been characterized for both homing and splicing domains; these blocks represent some residues responsible for a catalytic or structural function. Blocks A, B, F, and G are located in the intein splicing domain, while blocks C, D, E, and H are in the HE.[22,34] It was determined that protein-splicing and endonuclease domains have separate active sites. Mutations of the intein active-site residues do not inhibit endonuclease function and vice versa.[40,41]

Among fungal inteins, the PRP8 intein is the most widespread, despite its sporadic distribution.[22] The *PRP8* gene encodes for the Splicing Factor Prp8, a core protein of the spliceosome complex responsible for removing introns from messenger RNA (mRNA).[42] Many fungal species containing the PRP8 intein are important human pathogens. The PRP8 intein of *C. neoformans* and *C. gattii,* for instance, lacks HE, being a mini-intein, while other species, such as *P. brasiliensis*, *A. fumigatus,* and *H. capsulatum* possess a full-length PRP8 intein, with a HE belonging to LAGLIDADG family.[21–24] The LAGLIDADG protein family was the first intron-encoded proteins to be identified and biochemically characterized. It shows conservation of a ten-residue sequence motif and is the most diverse of the homing endonuclease families.[39]

It has been observed that the differences between the intein sequences reflect the phylogenetic relationship between the host species, being useful for establishing proper systematic classification of complex species groups. [23,24,43–46] The only fully described intein sequence in dermatophytes is the PRP8 intein of *M. canis*[47]; thus, no other intein sequences have been characterized for this group of fungi. Therefore, we aim to characterize the molecular aspects of PRP8 inteins in representative species of dermatophytes in order to better define their phylogenetic relationship and also to evaluate whether this intein might be used as a simple molecular marker, based on polymerase chain reaction (PCR)electrophoresis, for distinguishing species.

Methods

Strains

Forty strains of 11 different dermatophyte species were used for PRP8 intein sequencing. Strains were previously obtained, described, and identified by DNA sequencing of the nuclear ribosome regions ITS1-5.8S-ITS2 and D1/D2 by Garcia Garces et al.[19] Although some current nomenclature changes have been adopted for dermatophytes,[12] the species denomination herein employed was based on Gräser et al.[3] Both denominations might be observed at Table 1 for each strain.

PRP8 intein sequencing

DNA samples previously obtained by Garcia Garces et al. (2016) were used for intein sequencing.[19] Extein regions flanking the intein were determined by aligning

Table 1. Strain identification by using nuclear ribosomal regions (ITS1-5.8S-ITS2 and D1/D2) and PRP8 intein DNA sequences.

Strain	Molecular identification (ITS1-5.8S-ITS2 and D1/D2 region)*	Identification by PRP8 intein (Blasting at whole-genome shotgun contigs database, WGS)			Final identification by Gräser et al. 2008[3]. (By de Hoog et al. 2017[12])
		Base pairs (bp)	% Identity	Identification	
747	*M. canis*/ *A. otae*	1686	100	*A. otae* strain CBS 113480	*M. canis*/ *A. otae*. (*M. canis*)
841	*M. canis*/*A. otae*	1686	100	*A. otae* strain CBS 113480	*M. canis*/ *A. otae*. (*M. canis*)
431	*M. canis*/*A. otae*	1686	100	*A. otae* strain CBS 113480	*M. canis*/ *A. otae*. (*M. canis*)
830	*M. canis*/*A. otae*	1686	100	*A. otae* strain CBS 113480	*M. canis*/ *A. otae*. (*M. canis*)
483	*M. audouinii*	1686	98	*A. otae strain CBS 113480*	*M. audouinii* (*M. audouinii*)
RS3	*T. ajelloi*/*A. uncinatum*	1455	85	*A. otae strain CBS 113480*	*T. ajelloi* / *A. uncinatum* (*A. unicinatum*)
589	*M. gypseum*/*A. incurvatum*	1449	87	*M. gypseum* CBS 118893	*M. gypseum*/ *A. incurvatum* (*Nannizzia incurvata*)
586	*M. gypseum*/*A. incurvatum*	1449	87	*M. gypseum* CBS 118893	*M. gypseum*/ *A. incurvatum (Nannizzia incurvata)*
541	*M. gypseum*/*A. incurvatum*	1449	87	*M. gypseum* CBS 118893	*M. gypseum*/ *A. incurvatum (Nannizzia incurvata)*
B15	*M. gypseum*/*A. incurvatum*	1449	87	*M. gypseum* CBS 118893	*M. gypseum*/ *A. incurvatum* (*Nannizzia incurvata*)
613	*M. gypseum*/*A. gypseum*	1473	100	*M. gypseum* CBS 118893	*M. gypseum*/ *A. gypseum* (*Nannizzia gypsea*)
435	*M. gypseum*/*A. gypseum*	1473	100	*M. gypseum* CBS 118893	*M. gypseum*/ *A. gypseum* (*Nannizzia gypsea*)
546	*M. gypseum*/*A. gypseum*	1473	100	*M. gypseum* CBS 118893	*M. gypseum*/ *A. gypseum* (*Nannizzia gypsea*)
1019	*M. persicolor*/*A. persicolor*	1473	84	*M. gypseum* CBS 118893	*M. persicolor*/ *A. persicolor*(*Nannizzia persicolor*)
1025	*M. fulvum*/*A. fulvum*	1500	89	*M. gypseum* CBS 118893	*M.fulvum*/ *A. fulvum* (*Nannizzia fulva*)
198	*E. floccosum*	1503	85	*M. gypseum* CBS 118893	*E. floccosum* (*E. floccosum*)
84	*E. floccosum*	1503	85	*M. gypseum* CBS 118893	*E. floccosum* (*E. floccosum*)
433	*A. vanbreuseghemii*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale* (*T. mentagrophytes*)
B19	*T. interdigitale (zoophilic)*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale* (*T. mentagrophytes*)
28	*T. interdigitale*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale* (*T. interdigitale*)
B1	*T. interdigitale*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale*(*T. interdigitale*)
B13	*T. interdigitale*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale*(*T. interdigitale*)
B3	*T. interdigitale*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale*(*T. interdigitale*)
B4	*T. interdigitale*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale*(*T. interdigitale*)
B5	*T. interdigitale*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale*(*T. interdigitale*)
B6	*T. interdigitale*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale*(*T. interdigitale*)
B16	*T. interdigitale*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale*(*T. interdigitale*)
B10	*T. interdigitale*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale*(*T. interdigitale*)
B11	*T. interdigitale*	1503	100	*Trichophyton interdigitale* H6	*T. interdigitale*(*T. interdigitale*)
368	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum*(*T. rubrum*)
31	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum*(*T. rubrum*)
1013	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum*(*T. rubrum*)
B7	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum*(*T. rubrum*)
B8	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum*(*T. rubrum*)
B9	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum*(*T. rubrum*)
B12	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum*(*T. rubrum*)
B17	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum*(*T. rubrum*)
1024	*T. rubrum (African population)*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum*(*T. soudanense*)
119	*T. tonsurans*	1503	99	Trichophyton tonsurans CBS 112818	*T. tonsurans*(*T. tonsurans*)
429	*T. tonsurans*	1503	99	Trichophyton tonsurans CBS 112818	*T. tonsurans*(*T. tonsurans*)

*Identification made by Garcia Garces et al. 2016[19].

dermatophyte *PRP8* DNA sequences from the GenBank database of the National Center for Biotechnology Information (NCBI) and Centraal Bureau voor Schimmelcultures (CBS) dermatophyte database for primer design. The primers EXTDF 5′ ATC AAG YTG CAC CTG GAA AC 3′ and EXTDR 5′ GAC AAG TCA RAG ACA TGA TGG 3′ (IDT, Coralville, IA, USA) were designed to anneal at the extein *PRP8* sequences flanking the PRP8 intein. PCR was performed in a Veriti Thermocycler (Applied Biosystems, Foster City, CA, USA) using GoTaq® Green Master Mix (Promega, Madison, WI, USA), PCR kit requirement (25 μl of reaction mixture-containing 3 μl of genomic DNA at 600 ng/μl, 12.5 μl of GoTaq® Green Master Mix 2X, 1.4 μl of each primer at 10 μM and nuclease-free water to complete the reaction volume). Thermal cycling conditions were: 98°C for 2 min followed by 35 cycles at 98°C for 0.5 min, 60°C for 0.5 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

PCR products were detected as a single band of 1400–1700 bp by 1.5% agarose gel electrophoresis. The PCR mixture was purified by using EXOSAP-IT (Affymetrix, Cleveland, OH, USA) and submitted to sequencing of both strands in the 3500 analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

Analysis of the PRP8 intein sequences

After sequencing, sense and antisense sequences (approximately 600 to 700 bp) were aligned and a second set of internal primers was designed in order to complete the whole intein sequence. Primers INTDF 5′TCC TKG GRC TYT GGC TTG G 3′ and INTDR 5′CGG ACR GYR CGC GAA TTG 3′ (IDT, Coralville, IA, USA) were used for amplifying the internal regions of the PRP8 intein. PCR cycling conditions, detection by electrophoresis, purification, and sequencing were the same but using 3.0 μl of previous PCR reaction, instead of genomic DNA, for amplification and 58°C as the annealing temperature.

The entire PRP8 intein DNA sequence was constructed by aligning the four previously obtained sequences (two from primers EXTD and two from primers INTD) for each strain. Sequences edition, alignment with Clustal W, conversion to protein sequence and further phylogenetic analysis were executed by using the software MEGA v 6.0.[48]

Additionally, all PRP8 DNA sequences were submitted for molecular identification by Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nlm.nih.gov/BLAST/) from the NCBI site to confirm the previous ITS1-5.8s-ITS2 and D1/D2 identification[19] and to validate the sequencing edition. Each species was identified from the best-scoring reference sequence of the blast output with an identity >98% compared to the query sequence (only for previously deposited species). The Whole-Genome Shotgun contigs database (WGS) was useful for that purpose. BLASTp was also used in order to confirm identification by protein sequences.

Comparative studies between obtained sequences and other PRP8 intein sequences[22] allowed us to locate both the splicing and homing-endonuclease conserved blocks. The PRP8 intein nucleotide sequences reported in this paper were submitted to the GenBank database under accession numbers: KX463279-81, KX497134-59, and KX510280-90.

The protein sequences for each species were exported to a CLC sequencer viewer v7.7.1 available at www.clcbio.com (QIAGEN Aarhus A/S, Aarhus, Denmark); alignment was carried out and exported to PDF (Supplementary Material 1).

The dS/dN rates were calculated for both splicing and HE conserved block domains by using synonymous–nonsynonymous mutation rates between sequences containing ambiguous nucleotides (Syn-SCAN)[49] available at https://hivdb.stanford.edu/pages/synscan.html, in order to infer the conservation and possible function of both domains.

Experimental identification of putative introns in the PRP8 inteins from *M. canis* and *M. audouinii*.

Strains 747 and 830 of *M. canis* and *483* of *M. audouinii* were used for RNA extraction by using TRIZOL® reagent (Invitrogen, Cincinnati, OH, USA) and treated with DNase I (Thermo Scientific, Carlsbad, CA, USA). The mRNA was converted to complementary DNA (cDNA) by using the IScript™cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA), according to the manufacturer's instructions. Primers McaP241F 5′CGC TGT CTT CTT GAA CAA TTC G 3′ and McaP242R 5′AGC TGA TGT CCA TCA ACT CGG 3′ (IDT, Coralville, IA, USA) were designed from intein regions surrounding the supposed intron. Three μl of cDNA samples was amplified by PCR, visualized in gel electrophoresis, purified and sequenced following the same requirements for the intein sequencing but with an annealing temperature of 55°C.

Phylogenetic analysis

Two phylogenetic analyses were performed. Alignment was accomplished through Clustal W alignment method for DNA sequences[50] and a Point Accepted Mutation (PAM) method for protein alignment.[51] The maximum likelihood (ML) method[52] was performed by applying the Kimura Parameter[53] for intein DNA sequences with bootstrap of 1000 replicates with random additions.[54] The same method, but with Jones-Taylor-Thornton Parameter,[55] was applied for

protein sequences using the same bootstrap values. For both constructions, *Chrysosporium queenslandicum* (accession number: LJPI00000000.1) and *Uncinocarpus reesii* (accession number: CH476615.1) were used as out-groups because applying phylogenetic analysis of the PRP8 gene (data not shown) it can be seen that *Uncinocarpus reesii* is the closest species out of dermatophyte containing a PRP8 intein. *Chrysosporium queenslandicum* is the only *Chrysosporium* species available in NCBI databases with a whole genome deposited (intein never previously described), whereas a close relationship has been proven between *Chrysosporium* species and dermatophytes.[3]

The data and phylogenetic constructions were deposited at treeBASE (www.Treebase.org). Access: http://purl.org/phylo/treebase/phylows/study/TB2:S20861 and http://purl.org/phylo/treebase/phylows/study/TB2:S20862 for analyses related to DNA sequences and protein sequences, respectively.

Identifying *Microsporum* species by PRP8 intein

Two PCR electrophoreses were designed for differentiating clinical important *Microsporum* species. Figure 1 summarizes the proposed diagram for this experiment. The first assay was performed by using the primers HE1F 5′TTC CTK GGR CTY TGG CTT GG 3′ and HE2R 5′ADY AAA CCD GCR AGG ACG G3′ (IDT, Coralville, IA, USA) to distinguish *M. canis* and M. *audouinii* from the rest of the other clinically important dermatophytes, including the genus *Trichophyton*. PCR was performed following the same conditions employed for amplifications of inteins, with an annealing temperature of 58°C. The electrophoresis assay was carried out in a 2.5% agarose gel for 2.5 h at 120 V.

The second assay was designed for differentiating within the *A. gypseum* species complex herein studied. A third reverse primer HE3R 5′GTC CAG ATC RYC CWC TTT SG 3′ (IDT, Coralville, IA, USA) was designed, using as forward the same primer used for *M. canis*/ *M. audouinii* identification (HE1F). The annealing temperature was 57°C, and the bands were visualized by electrophoresis in a 2.5% agarose gel after 2 h at 120 V.

Results

Dermatophytes might be identified using the PRP8 intein sequences.

The PRP8 intein was efficiently amplified for all species and individuals; all of them are full-length inteins. Among the samples herein evaluated, *M. canis* and its closest species

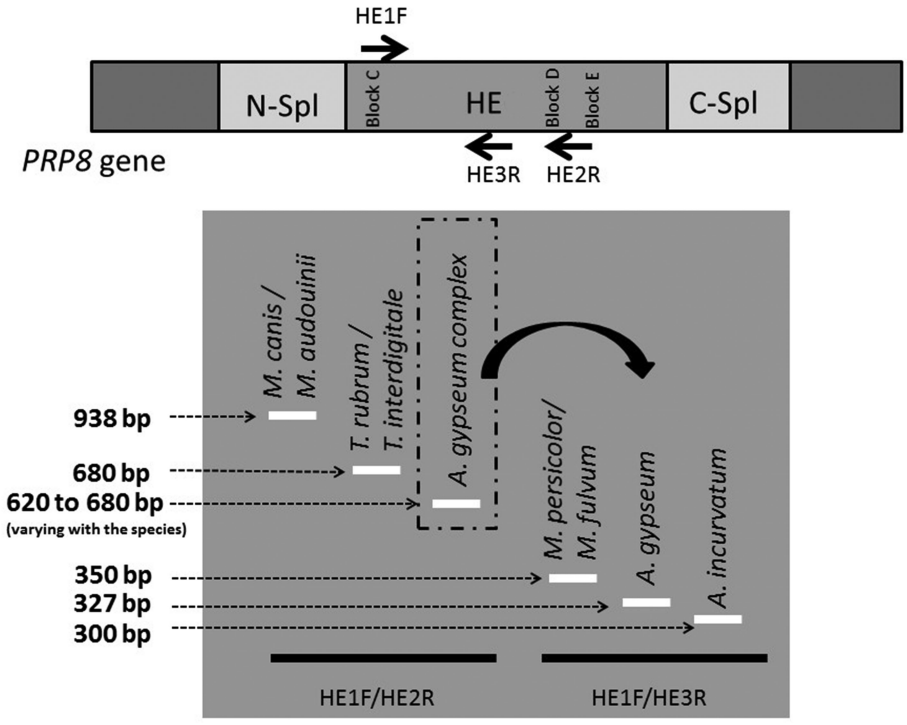

Figure 1. Proposed diagram to differentiate clinically important dermatophytes (*Microsporum* species), according to the PRP8 intein (HE domain) length polymorphism. Primers HE1F and HE2R anneal into Block C and Blocks D / E of the HE region, respectively. The first assay, using HE1F and HE2R primers, differentiates *M. canis* / *M. audouinii* (938 bp) from the other dermatophyte species. The second, using HE1F and HE3R primers, differentiates within the *A. gypseum* species complex.

M. audoinii present the largest intein with 1686 bp, while *A. incurvatum* has the smallest with 1449 bp (Table 1). All species with previously deposited sequences of the PRP8 gene (complete genomes at WGS database) showed an identity of 100% or 99% with the query sequence and E value of 0.0, corroborating the previous identifications using nuclear ribosomal regions for the same strains.[19]

The PRP8 intein protein sequences, which obviously are more conserved than their corresponding DNA sequences, showed 99% or 100% similarity to those previously deposited identified species (data not shown). Interestingly, this was not observed for *M. canis,* whose PRP8 intein protein sequence (converted from the DNA sequence herein obtained) showed 98% similarity to the deposited PRP8 intein region of this species. Analyzing this result, we found a sequence of 11 aa corresponding to 33 nucleotides (just beside the block E of the HE) within the intein that was not translated at the GenBank database for *Arthroderma otae* CBS 113480 (accession number XP_002846024). Therefore, we hypothesized that these 33 nucleotides might constitute an intron. In fact, all characteristics that define an intron were found in this region.[56] Considering that translation for this protein sequence is made by conceptual translation (theoretically) and that similar aligned sequences for this region are present in other remaining dermatophytes species with 100% identity when compared to query sequences, we carried out a reverse transcription polymerase chain reaction assay in *M. canis* and *M. audouinii* for determining whether this putative intron really occurs, and may be spliced and, therefore, not expressed. Since the 33 nucleotide sequences were found in cDNA from the *PRP8* gene, for strains 747, 830, and 483, we concluded that this is not a functional intron and in this case is normally translated. Thus, the whole DNA sequence for *M. canis* and *M. audouinii* was translated and deposited in GenBank including this region.

Dermatophytes present a full-length PRP8 intein with an apparently active LAGLIDADG HE.

For all species, a full PRP8 intein with a HE belonging to LAGLIDADG family was found. The splicing domain showed no length polymorphism among the different dermatophytes. However, a significant length polymorphism was noticed between blocks C and D of the HE domain for different species. For dermatophytes herein studied, the length from block C to block D was 279 aa for *M. canis* and *M. audouinii*, 195 aa for *T. interdigitale*, *Trichophyton tonsurans* and *T. rubrum*, 193 aa for *E. floccosum* and *Microsporum fulvum*, 189 aa for *A. gypseum*, 188 aa for *Trichophyton* ajelloi, 183 aa for *Microsporum persicolor,* and 177 aa for *A. incurvatum* (Supplementary Material 1)

The analysis of dS/dN rates of conserved block of PRP8 intein provided systematically high values both for splicing and HE domains in all species herein evaluated (Supplementary Material 2 and 3).[49] The dS/dN rates for HE conserved-block domains are nearly identical to the splicing conserved-block domains for all compared species, indicating that besides splicing activity, the HE might also be active in this fungal group. Higher rates were particularly observed for the geophilic species *T. ajelloi* and species of *A. gypseum* complex (*A. gypseum*, *A. incurvatum*, *M. fulvum,* and *M. persicolor*) and for the anthropophilic species *E. floccosum,* both for splicing and HE domains. The zoophilic dermatophyte *M. canis* seems to have a lower dS/dN rate but still strongly positive, suggesting HE activity.

The presence of two essential residues of aspartic acid was also determined within blocks C and E related to HE functioning.[22,57] It was found that these two essential residues are present but a substitution of Asp by glutamic acid (Glu) is present in all *A. gypseum* complex species and *E. floccosum* into Block E (Supplementary Material 1).

Phylogenetical analyses of PRP8 intein sequences reflect evolution of dermatophytes

Phylogenetic constructions employing DNA and protein sequences of PRP8 intein, carried out in a representative group of dermatophytes (40 strains,11 species), generated similar topologies, with high bootstrap values, confirming the robustness of the analyses (Figs. 2 and 3). The following clades and/or species complex were well defined, as proposed by Gräser et al. (2008): i) *T. interdigitale* close to *T. tonsurans*, which belongs to the *Arthroderma vanbreugseghemii* complex; ii) *T. rubrum* as an isolated group; iii) *M. canis* close to *M. audouinii*, which belongs to the *A. otae* complex; iv) the *A. gypseum* complex, which groups *Microsporum gypseum* / *A. incurvatum* separately from *M. fulvum*, *M. gypseum* / *A. gypseum*, *M. persicolor*, and also *E. floccosum; v)* finally, *T. ajelloi* as an independent clade.

Clinically important *Microsporum* species can be differentiated by a PCR-electrophoresis assay

Finally, as a practical application, taking advantage of the above-mentioned polymorphism in the HE region, two PCR-electrophoresis assays were designed for distinguishing species among the genus *Microsporum*. The tested strains belong to species with proven clinical importance, related mostly to the *Microsporum* genus.[1,4,10,20,58–60] Figure 4 shows an electrophoresis for identifying *M. canis* / *M. audouinii* from other clinically important dermatophytes by using the primers HE1F and HE2R. The primer HE1F anneals in a region next to Block C whereas the

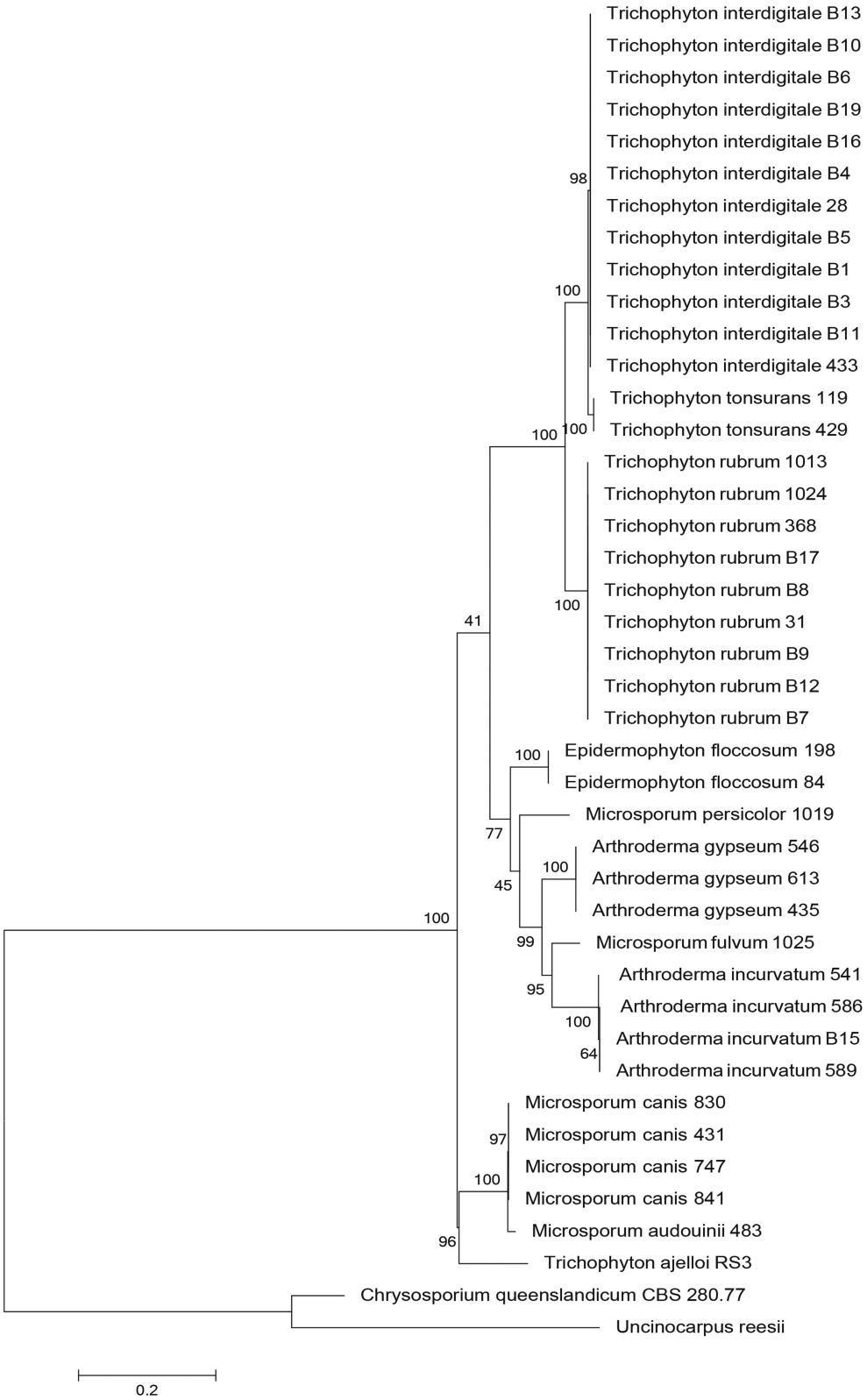

Figure 2. Phylogenetic construction using DNA sequences of the PRP8 intein in dermatophytes. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood is shown. The percentage of trees, in which the associated taxa are clustered together, is displayed next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis involved 42 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were 1310 positions in the final dataset.

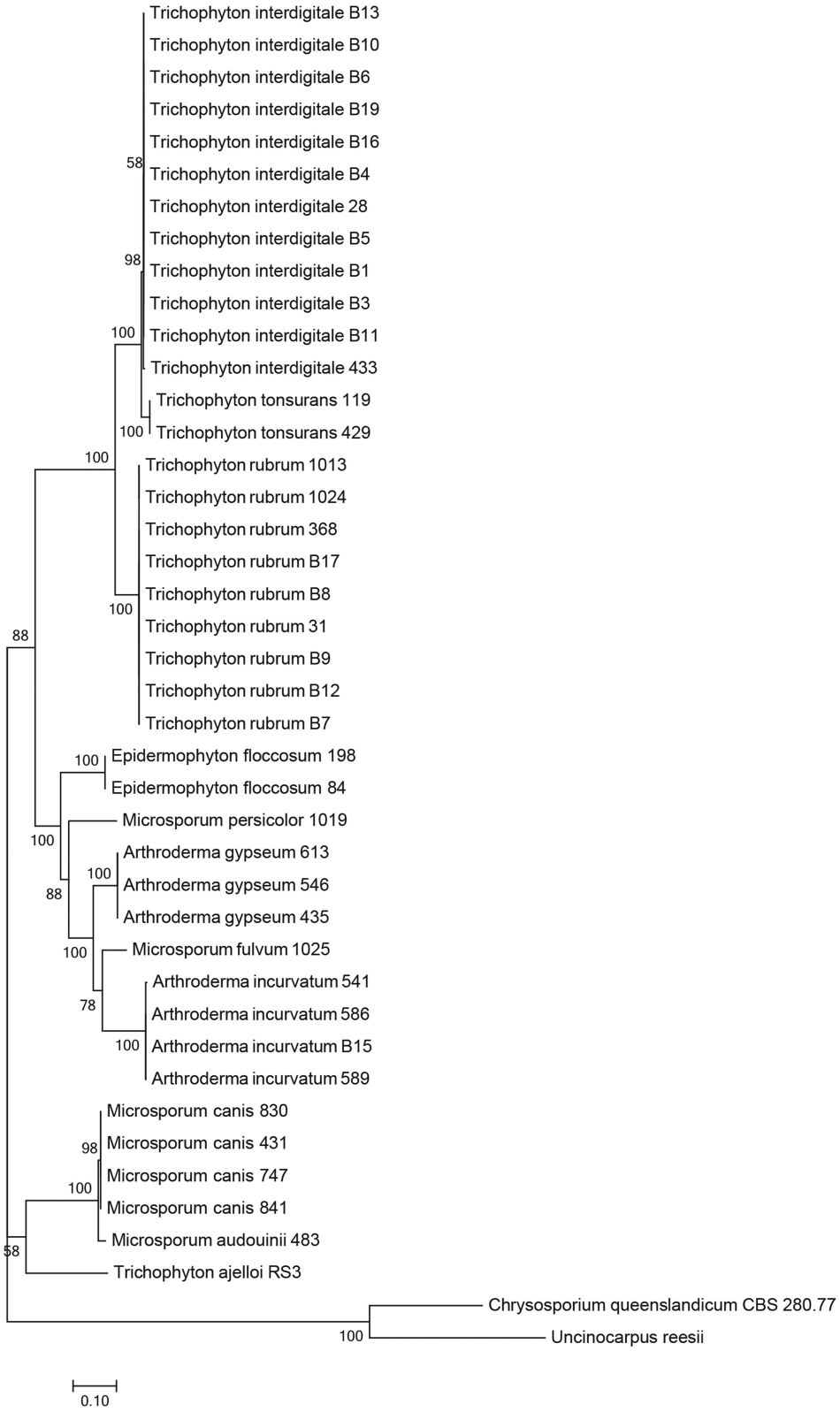

Figure 3. Phylogenetic construction using protein sequences of the PRP8 intein in dermatophytes. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood is shown. The percentage of trees, in which the associated taxa are clustered together, is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis involved 42 amino-acid sequences. There were 591 positions in the final dataset.

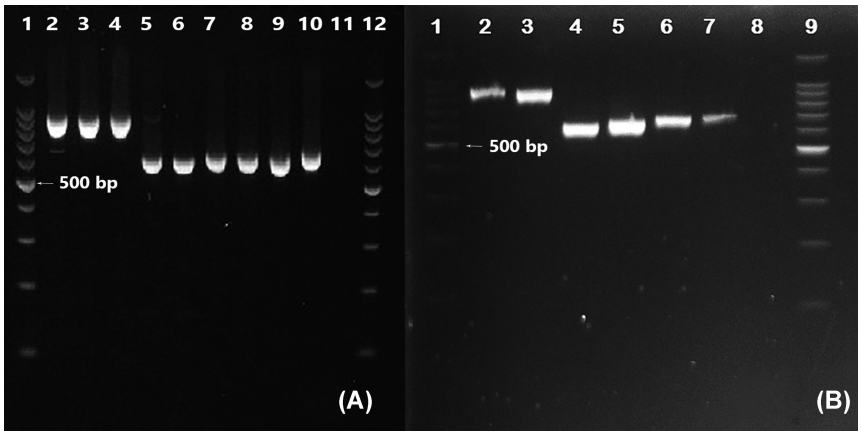

Figure 4. Electrophoresis of PCR products from PRP8 intein HE region for identifying *M. audouinii* and *M.canis*. A) HE1F/HE2R PCR products of *Microsporum* genus. Lanes 2 to 11 correspond to *M. canis* (strain 747), *M. canis* (strain 841), *M. audouinii* (strain 483), *A. incurvatum* (strain 586), *A. incurvatum* (strain 589), *A. gypseum* (strain 546), *A. gypseum* (strain 613), *M. persicolor* (strain 1019), *M. fulvum* (strain 1025) and negative control, respectively. B) Electrophoresis of clinically important species of *Microsporum* and *Trichophyton* genus. Lane 2 to 8: correspond with *M. canis* (strain 841), *M. audouinii* (strain 483), *A. incurvatum* (strain 589), *A. gypseum* (strain 546), *T. rubrum* (strain 31), *T. interdigitale* (strain 28) and negative control, respectively. Lanes 1 and 12 of Figure 4A and lanes 1 and 9 of Figure 4B correspond with the 100 bp Molecular Marker (Thermo Scientific, Carlsbad, USA).

HE2R primer anneals in a region between blocks D and E. Both were designed for amplifying all herein evaluated dermatophyte species. *M. canis* and *M. audouinii* were separated from other dermatophytes, with amplicons of about 930 bp, while *A. gypseum*, *A. incurvatum*, *M. persicolor*, and *M. fulvum* showed amplicons of about 600 to 700 bp (Fig. 4A). In addition, *T. rubrum* and *T. interdigitale* were slightly separated from *A. gypseum* and *A. incurvatum* with amplicons of about 680 bp (Fig. 4B).

Figure 5 shows a second electrophoresis for differentiating species from the *A. gypseum* complex. A third reverse primer HE3R was designed using the HE1F as the forward primer. The primer HE3R anneals in a region between blocks C and D and it is specific for species of the *M. gypseum* complex herein evaluated. Strains belonging to *A. incurvatum* with amplicons of 300 bp were separated from strains of *A. gypseum* with amplicons of 327 bp. The last two species, *M. persicolor* and *M. fulvum*, were also evaluated, being separated from *A. gypseum* and *A. incurvatum*, showing amplicons of about 350 bp.

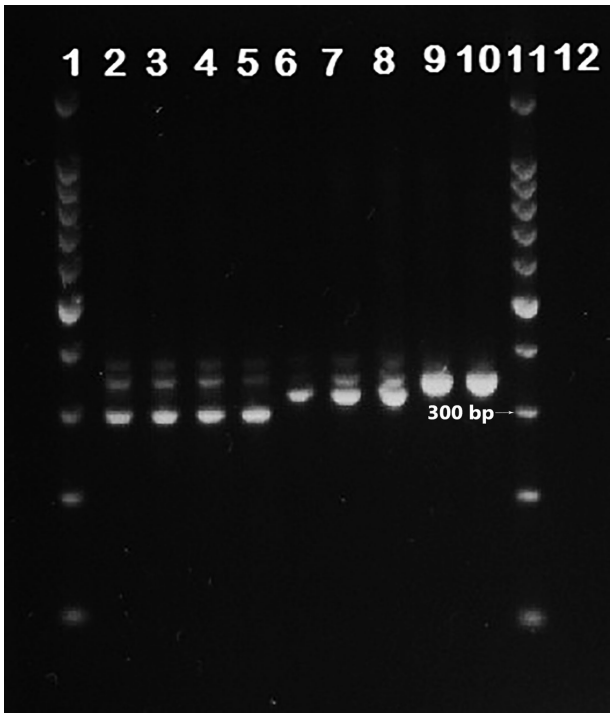

Figure 5. Electrophoresis of HE1F/HE3R PCR products from PRP8 intein HE for identifying *A.gypseum* complex species. Lanes 2 to 5 correspond to *A. incurvatum* (strains 589, 586, 541, B15); lanes 6 to 8 correspond to *A. gypseum* (strains 613, 435, 546); and lanes 9 and 10 correspond to *M. persicolor* (strain 1019) and *M. fulvum* (strain 1025), respectively. Lanes 1 and 11 contain the 100 bp Molecular Marker (Thermo Scientific, Carlsbad, USA) and lane 12 the negative control.

Discussion

Our results indicate that PRP8 intein is suitable for species identification among dermatophytes. Intein sequences correctly identified, with accuracies equivalent to rDNA sequences, the species *M. canis*, *A. gypseum*, *T. interdigitale*, *T. tonsurans* and *T. rubrum* that present genome data already deposited at Genbank. Some sequences showed low similarity values (<99%) in relation to other species (different from their identification by rRNA regions) because their sequences for *PRP8* gene or PRP8 intein were not deposited in the GenBank database. Once additional PRP8 intein dermatophytes sequences are deposited in databases, further research studies might employ this genomic region

for identifying dermatophyte species or to confirm previous identifications by ribosomal DNA regions.

The studied dermatophytes showed a full-length PRP8 intein with a LAGLIDADG HE. As previously mentioned, a significant polymorphism was found related to the HE domain. Butler et al. (2006), by comparing PRP8 inteins from different fungi, observed that domains between the conserved blocks C and D might range from 212 to 300 residues.[22] This polymorphism-length pattern was observed repeatedly in more than one isolate of the species *M. canis* (four isolates), *T. tonsurans* (two isolates), *A. incurvatum* (four isolates), *A. gypseum* (three isolates), *E. floccosum* (two isolates), *T. rubrum* (nine isolates), *T. interdigitale* (12 isolates), indicating that this region reflects the species phylogeny and might be used as a molecular marker to differentiate them.

The HE region tends to be more polymorphic because it is actually not necessary for intein splicing, having no role in Prp8 protein function and therefore in cell survival. Since this polymorphism is reflected in a phylogeny that corroborates the topology previously obtained for nuclear ribosomal regions,[3,19] we can infer that the PRP8 intein has evolved vertically among the dermatophytes, which means no lateral transfer was detected. Even for very close species, such as those within the *A. gypseum* complex, the PRP8 intein, due to HE polymorphisms, was sufficiently informative for species distinction. In this way, *A. gypseum*, *A. incurvatum*, *M. persicolor*, and *M. fulvum* were completely distinguished in the PRP8 phylogeny.

During evolution, dermatophytes underwent adaptation to different hosts, passing from soil to humans and other animals.[10,12,15] This adaptation to new hosts led to changes in reproduction, with the geophilic dermatophytes being the species retaining sexual reproduction.[16] This flux of genes caused by sexual reproduction during the speciation process in geophilic dermatophytes could lead to a higher variability and polymorphism even within closer species (species of the *A. gypseum* complex). This is reflected by the accuracy of both markers, the rRNA region (sequence polymorphism) as well as PRP8 intein (sequence and size polymorphisms) (Supplementary Material 1) in distinguishing these species.

In relation to HE activity, the dS/dN and phylogenetic analyses suggest that the majority of the HE of the PRP8 inteins in ascomycetes are active. This suggestion contrasts with the activity of the HE of the VMA intein, which has been proven to be inactive, in most of the species.[22,61] The dS/dN rate is a measure of the selective pressures acting on two compared gene sequences. Higher dS/dN rates imply an abundance of synonymous substitutions, which means that the encode protein is going through a structural and functional conservation. When dS/dN rates present very low or negative values it signifies an abundance of nonsynonymous substitutions implying that the referred protein is degenerating. The ratio of nonsynonymous to synonymous substitutions in a protein-coding gene reflects the relative influence of purifying, positive, or neutral selection.[49]

As the PRP8 gene is essential for mRNA splicing, the intein splicing domain must operate perfectly, to maintain a functional Prp8 protein.[62] So it is expected that dS/dN values for this domain are higher than those for the HE domain, even when it is active, because, as mentioned before, the HE is not crucial for intein splicing, and therefore extein functionality.[34,57,63] It seems that the HE of dermatophytes remains active since their dS/dN rates are high and nearly identical to the domains of splicing conserved blocks (Supplementary Material 2 and 3). *E. floccosum*, despite being an anthropophilic dermatophyte, shows high values because it is a species related to *A. gypseum* complex species proved by phylogenetic constructions employing ITS and D1/D2 regions[19,64] and confirmed by phylogenetic constructions using the PRP8 intein in the present work (Figs. 2 and 3). The HE region for this dermatophyte may have been conserved during evolution from an ancestral species originating the *A. gypseum* species complex and *E. floccosum*. Butler et al. (2006) by employing the dS/dN rates determined that HEs of PRP8 inteins are mainly active.[22] However, Theodoro et al. (2011) found by applying the same methodology that the HE might not be active for *P. brasilensis*.[24]

For the VMA intein, the importance of two aspartic acid residues (Asp) was demonstrated at the positions 218 and 326 for HE activity.[57] By aligning PRP8 intein sequences with the HE VMA intein, Butler et al. (2006) determined that two Asp residues located in the blocks C and E of the PRP8 intein HE are fundamental for its activity on account of finding that almost all PRP8-intein-containing species show these two aminoacids residues. Theodoro et al. (2011), analyzing the HE function of the PPR8 intein from *B. dermatitidis*, *E. parva,* and four cryptic species of *P. brasiliensis*, found a substitution of the aspartic acid in place of a glycine in block E in *P. brasiliensis* and *E. parva,* and a serine in *P. lutzii,* confirming previous results by the dS/dN method.[24] For species belonging to the *A. gypseum* complex and *E. floccosum*, a substitution of Asp for Glu was found in block E. However, as both Asp and Glu are very similar amino acids (acids and polar), this substitution may not disrupt the HE function. We can conclude that the HE of the PRP8 intein in dermatophytes may be active given the high dS/dN rates within conserved blocks of the HE domains and the presence of two Asp or Glu residues within blocks C and E. However, this *in silico* analysis cannot replace an experimental assay for HE activity.

Both phylogenetic constructions shown in Figures 2 and 3 were consistent with other previous phylogenies by using

other genomic regions. Cafarchia et al. (2012) and Gräser et al. (2008), by analyzing the phylogenetic relationships among dermatophytes using nuclear ribosomal regions (ITS region), found similar phylogenies but inconsistencies in relation to *Epidermophyton* genus clustering.[3,64] Our previous phylogenetic constructions were also inconclusive as to *E. floccosum* clustering, depending on the nuclear ribosomal region used.[19] For ITS-5.8S-ITS2, E. *floccosum* clustered with *A. gypseum* complex, specifically with *M. persicolor*, but when using the D1/D2 region it clustered with the *T. rubrum* / *T. violaceum* complex. However, by joining both regions ITS1-5,8S-ITS2 and D1/D2, *E. floccosum* maintained the same clustering observed by employing the PRP8 intein (DNA and protein sequences), validating the PRP8 intein phylogenetic constructions (Figs. 2 and 3). It seems that *E. floccosum* becomes anthropophilic starting from a species related to the *M. gypseum* complex or they share a common ancestor. An additional fact supporting this idea is the substitution pattern of the essential Asp for Glu within block E of the HE that occurs only in species of *M. gypseum* complex and *E. floccosum*.

Another remarkable difference can be observed in relation to the *A. vanbreuseghemii* complex that encompass several species belonging to the genus *Trichophyton* such as *T. mentagrophytes*, *T. interdigitale*, *T benhamiae,* and *T. tonsurans* among others. Gräser et al. (2008) based on a polyphasic study have proposed to group several former *T. mentagrophytes* complex species into the single species *T. interdigitale,* maintaining as *T. mentagrophytes* only the species *T. mentagrophytes* var *quinckeanum*.[3] However, a recent study conducted by De Hoog et al. (2017) proposed a new taxonomic classification by using a phylogenetic multilocus study and other characteristics by which they separated and recognized several *T. interdigitale* as *T. mentagrophytes*.[12] Both authors considered *A. vanbreugseghemii* to be a separated species. Previous nuclear ribosomal phylogenies using the same strains of this work separated strain B19 (*T. interdigitale*, zoophilic) and strain 433 (*A. vanbreuseghemii*),[19] which according to recent nomenclature are classified as different species of *T. interdigitale*.[12] Phylogenetic analysis of the PRP8 intein does not support this species separation since strains B19 and 433 clustered together with remaining *T. interdigitale* strains. In fact, there is no nucleotide polymorphism among these strains in the PRP8 intein. However, the related species *T. tonsurans* were efficiently clustered as a separated species. Although, a speciation process could be occurring in *T. interdigitale* species, the PRP8 intein phylogeny supports the idea of grouping all *T. mentagrophytes* species into the single species *T. interdigitale* proposed by Gräser et al. (2008).[3] Even the species *A. vanbreuseghemii* could be grouped into *T. interdigitale,* according to this particular genomic region.

T. rubrum from an African population (strain 1024), recognized nowadays as *T. soudanense,*[12] was separated from other *T. rubrum* strains by D1/D2 rDNA,[19] but using the PRP8 intein it was clustered among *T. rubrum* strains. The proposal made for Gräser et al. (2008) to consider *T. soudanense* as a single species within *T. rubrum*[3] is supported herein, since no differences were found when DNA of PRP8 intein sequences was analyzed for nucleotide changes.

M. canis and *M. audouinii* were efficiently separated showing results similar to those from ITS-5.8S-ITS2 and D1/D2 phylogenetic constructions.[19] Nucleotide changes were detected in *M. audouinii* PRP8 intein DNA sequences compared with *M. canis* (98% identity, Table 1). This finding suggests that *M. audoinii* and *M. canis* are supported as separate species. This fact validates PRP8 intein as a suitable additional marker for phylogenetic species recognition, in a multilocus approach. All remaining species were grouped according to ITS and D1/D2 rDNA genomic regions.[19]

As a practical application, we attempted to design two assays for distinguishing medically important *Microsporum* species (Fig. 1). The first PCR-electrophoresis assay can be used to identify *M. audouinii* and *M. canis* in a simple and accurate way with no need of sequencing. Although other dermatophytes herein sequenced and studied were not tested for amplification (ex: *E. floccosum*, *T. ajelloi,* and *T. tonsurans*), it was proved by bioinformatics that amplicons must range around 650 bp for all three species, so that *M. audouinii* and *M. canis* can still be distinguished when these species are involved in the diagnosis.

As to the second procedure involving the *A. gypseum* species complex, we should remark that *A. gypseum* and *A. incurvatum* were considered distinct teleomorph states for the single anamorph specie *M. gypseum*.[3] Nowadays, *A. incurvatum* is considered a new species with its own teleomorph *Nannizia incurvata,* while *A. gypseum* was renamed as *N. gypsea*.[12] Despite being different species, they are not well differentiated morphologically, demanding molecular methods, such as sequencing of nuclear ribosomal regions, for their distinction.[3] This assay might represent an alternative to differentiate *A. gypseum* (*N. gypsea*) species from *A. incurvatum* (*N. incurvata*) when morphological traits are not sufficient.

This is the first study to our knowledge on the PRP8 intein in dermatophytes. All studied species present a full-length PRP8 intein, with a HE belonging to the family LAGLIDADG. PRP8 inteins are hereby proven to constitute an efficient molecular marker in order to determine the phylogenetic relations among dermatophytes. Further studies can be performed by using other species such as some members from the *A. vanbreuseghemii* complex and *T. violaceum* for elucidating species borderlines and taxonomy. Because of the polymorphism difference in the HE

region, some *Microsporum* species can be easily identified by a PCR-electrophoresis assay, as herein proposed.

Supplementary material

Supplementary data are available at *MMYCOL* online.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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