Whole-Genome Analysis Illustrates Global Clonal Population Structure of the Ubiquitous Dermatophyte Pathogen *Trichophyton rubrum*

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**ABSTRACT** Dermatophytes include fungal species that infect humans, as well as those that also infect other animals or only grow in the environment. The dermatophyte species *Trichophyton rubrum* is a frequent cause of skin infection in immunocompetent individuals. While members of the *T. rubrum* species complex have been further categorized based on various morphologies, their population structure and ability to undergo sexual reproduction are not well understood. In this study, we analyze a large set of *T. rubrum* and *T. interdigitale* isolates to examine mating types, evidence of mating, and genetic variation. We find that nearly all isolates of *T. rubrum* are of a single mating type, and that incubation with *T. rubrum* “morphotype” *megninii* isolates of the other mating type failed to induce sexual development. While the region around the mating type locus is characterized by a higher frequency of SNPs compared to other genomic regions, we find that the population is remarkably clonal, with highly conserved gene content, low levels of variation, and little evidence of recombination. These results support a model of recent transition to asexual growth when this species specialized to growth on human hosts.

**KEYWORDS** *Trichophyton rubrum; Trichophyton interdigitale; dermatophyte; genome sequence; MLST; mating; recombination; LysM*

**DERMATOPHYTE** species are the most common fungal species causing skin infections. Of the > 40 different species infecting humans, *Trichophyton rubrum*, the major cause of athlete’s foot, is the most frequently observed (Achterman and White 2013; White et al. 2014). Other species are more often found on other skin sites, such as those found on the head, including *T. tonsurans* and *Microsporum canis*. Some dermatophyte species only cause human infections, including *T. rubrum*, *T. tonsurans*, and *T. interdigitale*. Other species, including *T. benhamiae*, *T. equinum*, *T. verrucosum*, and *M. canis*, infect mainly animals and occasionally humans, while others such as *M. gypseum* [Nannizzia gypseae (G. S. de Hoog et al. 2017)] are commonly found in soil and rarely infect animals. In addition to the genera *Trichophyton* and *Microsporum*, *Epidermophyton* and *Nannizzia* are other genera of dermatophytes that commonly cause infections in humans (S. de Hoog et al. 2017). The species within these genera are closely related phylogenetically and are within the Ascomycete order Onygenales, family Arthrodermataceae (White et al. 2008; G. S. de Hoog et al. 2017).

The *T. rubrum* species complex includes several morphotypes, many of which rarely cause disease, and *T. violaceum*, a
species that causes scalp infections (Gräser et al. 2000; G. S. de Hoog et al. 2017). Some morphotypes display phenotypic variation, though these differences can be modest. For example, the T. rubrum morphotype raubitscheckii differs from T. rubrum in production of urease, and in colony pigmentation and colony appearance under some conditions (Kane et al. 1981). T. rubrum morphotype megninii, which is commonly isolated in Mediterranean countries, requires L-histidine for growth unlike other T. rubrum isolates (Gräser et al. 2000). However, little variation has been observed between these and other morphotypes in the sequence of individual loci, such as the internal transcribed spacer (ITS) ribosomal DNA (rDNA) locus; additionally, some of the morphotypes do not appear to be monophyletic (Gräser et al. 2000, 2007; G. S. de Hoog et al. 2017), complicating any simple designation of all types as separate species. Combining morphological and multilocus sequence typing (MLST) data has helped to clarify relationships of the major genera of dermatophytes and resolved polyphyletic genera initially assigned by morphological or phenotypic data.

Mating has been observed in some dermatophyte species, although not to date in strict anthropophiles including T. rubrum (Metin and Heitman 2017). Mating type in dermatophytes, as in other Ascomycetes, is specified by the presence of one of two idiomorphs at a single mating type (MAT) locus; each idiomorph includes either an α-box domain or a high mobility group (HMG) domain transcription factor gene (Li et al. 2010). In the geophilic species M. gypseum, isolates of opposite mating type (MAT1-1 and MAT1-2) undergo mating and produce recombinant progeny (Li et al. 2010). In the zoophilic species T. benhamiae, both mating types are detected in the population and mating assays produced fertile cleistothecia (Symoens et al. 2013), structures that contain meiotic ascospores. In a study examining 600 isolates of T. rubrum, only five appeared to produce structures similar to cleistothecia (Young 1968), suggesting inefficient development of the spores required for mating. Sexual reproduction experiments of T. rubrum with tester strains of T. simii, a skin-infecting species that is closely related to T. mentagrophytes, have been reported and one recombinant isolate was characterized, consistent with a low frequency of mating of T. rubrum (Anzawa et al. 2010). Further, sexual reproduction of T. rubrum may be rare in natural populations, as a single mating type (MAT1-1) has been noted in Japanese isolates (Kano et al. 2013), matching that described in the T. rubrum reference genome of CBS 118892 (Li et al. 2010).

Here, we describe genome-wide patterns of variation in T. rubrum, revealing a largely clonal population. This builds on prior work to produce reference genomes for T. rubrum (Martinez et al. 2012) and other dermatophytes (Burmester et al. 2011; Martinez et al. 2012). Genomic analysis of two divergent morphotypes of T. rubrum, T. megninii, and T. souadanense reveal hotspots of variation linked to the mating type locus suggestive of recent recombination. While nearly all T. rubrum isolates are of a single mating type (MAT1-1), the sequenced megninii morphotype isolate contains a MAT1-2 locus, suggesting the capacity for infrequent mating in the population. Additionally, we examine variation in gene content across dermatophyte genomes including the first representatives of T. interdigitale.

Materials and Methods
Isolate selection, growth conditions, and DNA isolation
Isolates analyzed are listed in Supplemental Material, Table S1, including the geographic origin, site of origin, and mating type for each. Isolates selected for whole-genome sequencing were chosen to maximize diversity by covering the main known groups. For whole-genome sequencing, 10 T. rubrum isolates and 2 T. interdigitale isolates were selected, including representatives of the major morphotypes of T. rubrum (Table S2). Growth and DNA isolation for whole-genome sequencing were performed as previously described (Martinez et al. 2012).

For MLST analysis, a total of 80 T. rubrum isolates and 11 T. interdigitale isolates were selected for targeted sequencing. Isolates were first grown on potato dextrose agar (PDA) medium (Difco, Detroit, MI) for 10 days at 25°C. Genomic DNA was extracted using an Epicentre Masterpure Yeast DNA purification kit (catalog number MPY08200). Fungal isolates were harvested from solid medium using sterile cotton swabs, transferred to microcentrifuge tubes, and washed with sterile PBS. Glass beads (2 mm) and 300 μl yeast cell lysis solution (Epicentre) were added to the tube to break down fungal cells, and the protocol provided by Epicentre was then followed. The contents of the tube were mixed by vortexing and incubated at 65°C for 30 min, followed by addition of 150 μl Epicentre MPC Protein Precipitation Solution. After vortexing, the mixture was centrifuged for 10 min, followed by isopropanol precipitation and washing with 70% ethanol. The DNA pellet was dissolved in TE buffer.

For mating assays, we investigated 55 T. rubrum and 9 T. interdigitale isolates recovered from Adana and Izmir, Turkey. T. simii isolates CBS 417.65 MT +, CBS 448.65 MT +, and morphotype megninii isolates CBS 389.58, CBS 384.64, and CBS
of denaturation at 94°C. Amplifications were carried out using the following conditions for each isolate, three loci [the TruMDr1 ABC transporter (Cervelatti et al. 2006), an intergenic region (IR), and an α-1,3-mannosyltransferase (CAP59 protein domain)], with high sequence diversity between isolates (Table S5). Alternatively, sequences were obtained from genome assemblies (Table S4). Electropherograms of Sanger sequencing were examined and assembled using Sequencher 4.8 (Gene Codes). A total of 108 isolates were subjected to MLST analysis (Table S5). The petri dishes were examined under light microscopy for sexual structures. The petri dishes were examined under light microscopy for sexual structures.

**Mating assays**

Mating assays were performed using both Medium E [12 g/liter oatmeal agar (Difco), 1 g/liter MgSO4·7H2O, 1 g/liter NaOH3, 1 g/liter KH2PO4, and 16 g/liter agar (Weitzman and Silva-Hutner 1967)] and Takashio medium (1/10 Sabouraud containing 0.1% neopeptone, 0.2% dextrose, 0.1% MgSO4·7H2O, and 0.1%. KH2PO4). MAT1-1 and MAT1-2 isolates grown on Sabouraud Dextrose Agar for 1 week were used to inoculate both Medium E and Takashio medium plates pairwise 1 cm apart from each other. The plates were incubated at room temperature without parafilm in the dark for 4 weeks. The petri dishes were examined under light microscopy for sexual structures.

**Genome sequencing, assembly, and annotation**

For genome sequencing, we constructed a 180-base fragment library from each sample by shearing 100 ng of genomic DNA to a median size of ~250 bp using a Covaris LE instrument and preparing the resulting fragments for sequencing as previously described (Fisher et al. 2011). Each library was sequenced on the Illumina HiSeq 2000 platform. Roughly 100× of 101-bp Illumina reads were assembled using ALLPATHS-LG (Gnerre et al. 2011) run with an assisting mode utilizing T. rubrum CBS118892 as a reference. For most genomes, assisting mode 2 was used (ASSISTED_PATCHING = 2) with version R42874; for T. interdigitale H6 and T. rubrum MR1463, version R44224 was used. For T. rubrum morphotype megninii CBS 735.88 and T. rubrum morphotype rauubitschekii CBS 202.88, mode 2.1 was used (ASSISTED_PATCHING = 2.1) with version R47300. Assemblies were evaluated using GAEMR (http://software.broadinstitute.org/software/gaemr/); contigs corresponding to the mitochondrial genome or contaminating sequence from other species were removed from assemblies.

The Trichophyton assemblies were annotated using a combination of expression data, conservation information, and ab
"initio" gene-finding methods as previously described (Haas et al. 2011). Expression data included Illumina reads (SRX123796) from one RNA-sequencing (RNA-Seq) study (Ren et al. 2012) and all EST data available in GenBank as of 2012. RNA-Seq reads were assembled into transcripts using Trinity (Grabherr et al. 2011). PASA (Haas et al. 2003) was used to align the assembled transcripts and ESTs to the genome and identify open reading frames (ORFs); gene structures were also updated in the previously annotated T. rubrum CBS118892 assembly (Martinez et al. 2012). Conserved loci were identified by comparing the genome with the UniRef90 database (Wu et al. 2006) (updated in 2012) using BLAST (Altschul et al. 1997). The BLAST alignments were used to generate gene models using GeneWise (Birney et al. 2004). The T. rubrum CBS 118892 genome was aligned with the new genomes using NUCmer (Kurtz et al. 2004). These alignments were used to map gene models from T. rubrum to conserved loci in the new genomes.

To predict gene structures, GeneMark, which is self-training, was applied first; GeneMark models matching GeneWise ORF predictions were used to train the other "ab initio" programs. Ab initio gene-finding methods included GeneMark (Borodovsky et al. 2003), Augustus (Stanke et al. 2004), SNAP (Korf 2004), and Glimmer (Majoros et al. 2004). Next, EvidenceModeler (EVM) (Haas et al. 2008) was used to select the optimal gene model at each locus. The input for EVM included aligned transcripts from Trinity and ESTs, gene models created by PASA and GeneWise, mapped gene models, and ab initio predictions. Rarely, EVM failed to produce a gene model at a locus likely to encode a gene. If alternative gene models existed at such loci, they were added to the gene set if they encoded proteins longer than 100 amino acids, or if the gene model was validated by the presence of a Pfam domain or expression evidence. Finally, PASA was run again to improve gene model structure, predict splice variants, and add UTRs.

Gene model predictions in repetitive elements were identified and removed from gene sets if they overlapped TransposonPSI predictions (http://transposonpsi.sourceforge.net), contained Pfam domains known to occur in repetitive elements, or had BLAST hits against the Repbase database (Jurka et al. 2005). Additional repeats were identified using a BLAT (Kent 2002) self-alignment of the gene set to the genomic sequence (requiring \( \geq 90\% \) nucleotide identity over 100 bases aligned); genes that hit the genome more than eight times using these criteria were removed. Genes with Pfam domains not found in repetitive elements were retained in the gene set, even if they met the above criteria for removing likely repetitive elements from the gene set.

Lastly, the gene set was inspected to address systematic errors. Gene models were corrected if they contained in-frame stop codons, had coding sequence overlaps with coding regions of other gene models, predicted transfer or ribosomal RNAs, contained exons spanning sequence gaps, had incomplete codons, or had UTRs overlapping the coding sequences of other genes. Transfer RNAs were predicted using tRNAscan (Lowe and Eddy 1997), and ribosomal RNAs were predicted with RNAmmer (Lagesen et al. 2007).

All annotated assemblies and raw sequence reads were available at the National Center for Biotechnology Information (NCBI) database (Table S5).

**SNP identification and classification**

To identify SNPs within the T. rubrum group, Illumina reads for each T. rubrum isolate were aligned to the T. rubrum CBS 118892 reference assembly using BWA-MEM (Li 2013); reads from \( H6 \) T. interdigitale were also aligned to the T. interdigitale MR 816 assembly. The Picard tools (http://picard.sourceforge.net) AddOrReplaceReadGroups, MarkDuplicates, CreateSequenceDictionary, and ReorderSam were used to preprocess read alignments. To minimize false-positive SNP calls near insertion/deletion (indel) events, poorly aligned regions were identified and realigned using GATK RealignmentTargetCreator and IndelRealigner (GATK version 2.7-4 [McKenna et al. (2010), page 201]. SNPs were identified using the GATK UnifiedGenotyper (with the haploid genotype likelihood model) run with the SNP genotype likelihood models (GLM). We also ran BaseRecalibrator and PrintReads for base quality score recalibration on sites called using GLM SNP and recalled variants with UnifiedGenotyper emitting all sites. VCFTools (Danecek et al. 2011) was used to count SNP frequency in windows across the genome (–SNPdensity 5000) and to measure nucleotide diversity (–site-pi), which was normalized for the assembly size. For comparison, the nucleotide diversity was calculated for the SNPs identified in a set of 159 isolates of Cryptococcus neoformans var. grubii, a fungal pathogen that undergoes frequent recombination (Rhodes et al. 2017).

SNPs were mapped to genes using VCFannotator (http://vcfannotator.sourceforge.net/), which annotates whether a SNP results in a synonymous or nonsynonymous change in the coding region. The total number of synonymous and nonsynonymous sites across the T. rubrum CBS 118892 and T. interdigitale MR 816 gene sets were calculated across all coding regions using codeml in PAML (version 4.8) (Yang 2007); these totals were used to normalize the ratios of nonsynonymous to synonymous SNPs.

**Copy number variation**

To identify regions of T. rubrum that exhibit copy number variation between the isolates, we identified windows showing significant variation in normalized read depth using CNVnator (Abyzov et al. 2011). The realigned read files used for SNP calling were input to CNVnator version 0.2.5, specifying a window size of 1 kb. Regions reported as deletions or duplications were filtered requiring \( P\text{-val1} < 0.01 \).

**Phylogenetic and comparative genomic analysis**

To infer the phylogenetic relationship of the sequenced isolates, we identified single-copy genes present in all genomes using OrthoMCL (Li et al. 2003). Individual orthologs were aligned with MUSCLE (Edgar 2004) and then the alignments...
were concatenated and input to RAxML (Stamatakis 2006), version 7.3.3 with 1000 bootstrap replicates and model GTRCAT. RAxML version 7.7.8 was used for phylogenetic analysis of SNP variants in seven T. rubrum isolates, with the same GTRCAT model.

For each gene set, HMMER3 (Eddy 2011) was used to identify Pfam domains using release 27 (Finn et al. 2014); significant differences in gene counts for each domain were identified using Fisher’s exact test, with P-values corrected for multiple testing (Storey and Tibshirani 2003). Proteins with LysM domains were identified using a revised hidden Markov model (HMM) as previously described (Martinez et al. 2012); this HMM includes conserved features of fungal LysM domains, including conserved cysteine residues not represented in the Pfam HMM model, and identified additional genes with this domain.

**Construction of paired allele compatibility matrix**

To construct SNP profiles, SNPs shared by at least two members of the T. rubrum data set were selected. Private SNPs are not informative for a paired allele compatibility test because they can never produce a positive result. These profiles were then counted across the genome to construct SNP profiles via a custom Perl script. We required profiles to be present at least twice, to minimize the signal from homoplastic mutations. Pairwise tests were then conducted between each of the profiles to look for all four possible allele combinations, which would only occur via either mating or homoplastic mutations.

**Linkage disequilibrium calculation**

Linkage disequilibrium was calculated for T. rubrum SNPs in 1-kb windows of all scaffolds with VCFtools version 1.14 (Danecek et al. 2011), using the–hap-r2 option with a minimum minor allele frequency of 0.2.

**Data availability**

All genomic data are available in NCBI under the Umbrella BioProject PRJNA186851 and can be accessed via the accession numbers in Table S2. The NCBI GenBank accession numbers of the three MLST loci are listed in Table S3.

**Results**

**Relationship of global Trichophyton isolates using MLST**

To examine the relationship of global isolates of T. rubrum, we sequenced three loci in each of 104 Trichophyton isolates and carried out phylogenetic analysis. The typed isolates included 91 T. rubrum isolates, 11 T. interdigitale isolates, and 2 T. benhamiae isolates (Table S1). In addition, data from the genome assemblies of additional dermatophyte species (T. verrucosum, T. tonsurans, T. equinum, and M. gypseum) were also included. Three loci—the TruMDR1 ABC transporter (Cervelatti et al. 2006), an IR, and an α-1,3-mannosyltransferase (CAP59 protein domain)—were sequenced in each isolate.

Phylogenetic analysis of the concatenated loci can resolve species boundaries between the seven species (Figure 1). A large branch separates a T. benhamiae isolate (MR857) from the previously described genome sequenced isolate (CBS 112371) (Figure 1), and the sequences of two loci of a second T. benhamiae isolate (MR827) were identical to those of MR857 (Table S3). Sequencing of the ITS region of the MR857 and MR827 isolates revealed high sequence similarity to isolates from the T. benhamiae African race (Materials and Methods), which is more closely related to T. bullosum than isolates of T. benhamiae Americano-European race including CBS112371 (Heidemann et al. 2010). Otherwise, the species relationships and groups are consistent between studies.

MLST analysis demonstrated that the T. rubrum isolates were nearly identical at the three sequenced loci. Remarkably, of the 84 T. rubrum isolates sequenced at all three loci, 83 were identical at all positions of the three loci sequenced (genotype 2, Table S3). Only one isolate, 1279, displayed a single difference at one site in the TruMDR1 gene (genotype 3, Table S3). For the remaining six isolates, sequences at a subset of the loci were generated and matched that of the predominant genotype. Thus, MLST was not sufficient to discern the phylogenetic substructure in the T. rubrum population that included six isolates representing different morphotypes (Table S3). Similarly, the 11 T. interdigitale isolates were highly identical at these three loci; two groups were separated by a single-nucleotide difference in the IR and the third group contained a six-base deletion overlapping the same base of the IR (genotypes 1, 5, and 6, Table S3). Although most species can be more easily discriminated based on the MLST sequence, T. equinum and T. tonsurans isolates differed only by a single transition mutation in the IR, which illustrates the remarkable clonality of these species.

**Genome sequencing and refinement of phylogenetic relationships**

As MLST analysis was insufficient to resolve the population substructure of the T. rubrum species complex, we sequenced the complete genomes of T. rubrum isolates representing worldwide geographical origins and five morphotypes: fischeri, kanei, megnini, raubitschekii, and soudanense. We generated whole-genome Illumina sequences for 10 T. rubrum and 2 T. interdigitale isolates (Table S2). The sequence of each isolate was assembled and utilized to predict gene sets. The T. rubrum assembly size was very similar across isolates, ranging from 22.5 to 23.2 Mb (Table S5). The total predicted gene numbers were also similar across the isolates, with between 8616 and 9064 predicted genes in the 10 T. rubrum isolates, and 7993 and 8116 predicted genes in the two T. interdigitale isolates (Table S5).

To infer the phylogenetic relationship of these isolates and other previously sequenced Trichophyton isolates, we identified 5236 single-copy orthologs present in all species and estimated a phylogeny with RAxML (Stamatakis 2006) (Figure 2A). This phylogeny more precisely delineates the species
groups than that derived from the MLST loci and also illustrates the relationship between the *T. rubrum* isolates (Figure 2B). The results of this analysis suggest that the *fischeri* morphotype is not monophyletic, as one *fischeri* isolate (CBS100081) is more closely related to the *raubitschekii* isolate than to the other *fischeri* isolate (CBS 288.86). While a subset of seven *T. rubrum* isolates appear closely related, others show much higher divergence, including the *soudanense* isolate, the *megninii* isolate, the MR1459 isolate, and the CBS 118892 isolate representing the reference genome. The *soudanense* isolate (CBS 452.61) was placed as an outgroup relative to the other *T. rubrum* isolates; this is consistent with this isolate being part of a clade more closely related to *T. violaceum* than to *T. rubrum* (Gräser et al. 2000) and with the reestablishment of *soudanense* isolates as a separate species (G. S. de Hoog et al. 2017).

To further classify the two *T. interdigitale* isolates, we assembled the ITS region of the ribosomal DNA locus and compared the sequences to previously classified ITS sequences, as *T. interdigitale* isolates differ from *T. mentagrophytes* at the ITS locus (Gräser et al. 2008; G. S. de Hoog et al. 2017). For the two genomes of these species that we sequenced, MR816 was identical to *T. interdigitale* at the ITS1 locus, whereas the H6 isolate appears intermediate between *T. interdigitale* and *T. mentagrophytes*, containing polymorphisms specific to each group (Figure S1). Genomic analysis of allele sharing across a wider set of *T. interdigitale* and *T. mentagrophytes* isolates could be used to evaluate the extent of hybrid genotypes and genetic exchange between these two species.

**MAT1-1 prevalence and clonality in *T. rubrum***

To address if the *T. rubrum* population is capable of sexual reproduction, we surveyed the MAT locus of all isolates. Using either gene content in assembled isolates or a PCR assay to assign mating type, we found that 79 of the 80 *T. rubrum* isolates contained the α-domain gene at the MAT locus (MAT1-1). In addition, a set of 55 isolates from Turkey were found to harbor the MAT1-1 allele based on a PCR assay (Figure S2). However, the *T. rubrum* morphotype *megninii* isolate contained an HMG gene at the MAT locus (MAT1-2).
The presence of both mating types (Figure 3 and Table S1). The presence of both mating types suggests that this species could be capable of mating under some conditions. However, the high frequency of a single mating type strongly suggests that *T. rubrum* largely undergoes clonal growth, although other interpretations are also possible (see Discussion). In further support of this, a study of 206 *T. rubrum* clinical isolates from Japan noted that all were of the MAT1-1 mating type (Kano et al. 2013).

A closer comparison of the genome sequences of *T. rubrum* isolates also supports a clonal relationship of this population. Phylogenetic analysis of the seven most closely related *T. rubrum* isolates using SNPs between these isolates (see below) suggests that the isolates have a similar level of divergence from each other (Figure S3). This supports the idea that these MAT1-1 *T. rubrum* isolates have likely undergone clonal expansion.

To test for recombination that could reflect sexual reproduction within the *T. rubrum* population sampled here, we conducted a genome-wide paired allele compatibility test to look for the presence of all four products of meiosis (Figure 4). This test is a comparison between two paired polymorphic sites in the population. While the presence of three of the four possible allele combinations at two sites in a population is possible through a single mutation and identity-by-descent, the presence of all four combinations requires either recombination, or less parsimoniously, a second homoplastic mutation. Four positive tests resulted from this analysis (out of 21 possible), including allele combinations that occurred a minimum of 13 times. This may suggest that recombination is a rare event arising through infrequent sexual recombination occurring in this population, although the same mutations and combinations arising via homoplasmy (or selection) are difficult to exclude. Based on the number of triallelic sites in the data set (19), we would predict 9.5 homoplastic sites to have occurred by random chance, which is similar to the number of sites responsible for the positive signals in the compatibility test. In addition, linkage disequilibrium does not decay over increasing distance between SNPs in *T. rubrum* (Figure S4), which further supports a low level of recombination in this species; sequencing additional diverse isolates would help to address whether some isolates or lineages were more prone to recombination.

We also characterized the MAT locus of the newly sequenced *T. interdigitale* isolates (H6 and MR816) and found that both contain an HMG-domain gene. These *T. interdigitale* isolates were more closely related to *T. equinum* (MAT1-2) and *T. tonsurans* (MAT1-1) than *T. rubrum* (Figure 2A). To survey the mating type across a larger set of *T. interdigitale* isolates, a set of 11 additional isolates from Turkey were typed. Based on PCR analysis, all *T. interdigitale* isolates harbor the MAT1-2 allele (Figure S2).
Figure 3 Alignment of the mating type locus of selected isolates. Mating type genes of *T. rubrum* morphotype *megninii* (CBS 735.88) and *T. rubrum* (CBS 188992) are shown along the x- and y-axes, respectively, with regions aligning by NUCmer show in the dot plot. The alignment extends into two hypothetical proteins (HP) immediately flanking the α- or high mobility group (HMG)-domain gene that specifies mating type. Most *T. rubrum* (MAT1-1) isolates contain an α-domain protein (blue) at the MAT locus. In contrast, the *T. rubrum* morphotype *megninii* isolate contains an HMG-domain protein (green) representing the opposite mating type (MAT1-2). All sequenced *T. interdigitale* isolates are also of MAT1-2 mating type including MR816. Gene locus identifiers are shown for the genes flanking each locus (prefix TERG, H106, and H109).

Figure 4 Paired allele compatibility test suggests limited evidence for sexual reproduction. (A) A single example of a positive paired allele compatibility test from the *T. rubrum* population. In this test, two loci are examined and typed across the population. To perform a meaningful test, at least two individuals in the population must share a variant allele at each site. Here, alternative SNPs are depicted in red and the reference in white. Evidence for recombination is provided by any pairwise comparison of two loci in which isolates are present where red–red, white–white, red–white, and white–red combinations are all found (AB, Ab, aB, and ab), satisfying the allele compatibility test and providing evidence for recombination. (B) Paired allele compatibility tests were performed for all isolates in the *T. rubrum* population across the entire genome. SNP profiles were grouped into unique and informative allele patterns and collapsed, with the number of occurrences of each profile across the genome listed. Thus, the larger the number, the more common that SNP distribution is in the population. Pairwise tests were then conducted for each combination of SNP profiles. Reference nucleotides are indicated by white and variants by red. The pairwise matrix displays the results of all of these tests; a green square in the pairwise matrix is indicative of a positive test for the pairwise comparison and thus provides potential evidence of recombination.

were not observed (Figure S5). While it is possible that mating may occur under cryptic conditions (Heitman 2010), this data suggests that the conditions tested are not sufficient for the initiation of mating structures in *T. rubrum*.

**Genome-wide variation patterns in *T. rubrum***

SNP variants were identified between *T. rubrum* isolates to examine the level of divergence within this species complex (Table S8). On average, *T. rubrum* isolates contain 8092 SNPs compared to the reference genome of the CBS118892 isolate; this reflects a bimodal divergence pattern where most isolates, including three morphotypes (*fischeri, kanei,* and *raubitschekii*), have an average of 3930 SNPs and two more divergent isolates (morphotypes *megninii* and *soudanense*) have an average of 24,740 SNPs. The average nucleotide diversity (π) for all 10 *T. rubrum* isolates is 0.00054; excluding the two divergent morphotypes, the average nucleotide diversity is 0.00031. By comparison, the average nucleotide diversity of the fungal pathogen *C. neoformans* var. *grubii*, which is actively recombining as evidenced by low linkage disequilibrium (Desjardins et al. 2017; Rhodes et al. 2017), is 0.0074, a level ~24-fold higher than that in *T. rubrum* [Materials and Methods (Rhodes et al. 2017)]. Even higher levels of nucleotide diversity have been reported in global populations of other fungi (see Discussion). A similar magnitude of SNPs separate the two *T. interdigitale* isolates; 22,568 SNPs were identified based on the alignment of H6 reads to the MR 816 assembly. Across all isolates, SNPs were predominantly found in IRs for both species, representing 76 and 81% of total variants, respectively (Table 1 and Table S8). Within genes, the higher ratio of nonsynonymous relative to synonymous changes among the closely related *T. rubrum* isolates (Table 1) is consistent with lower purifying selection over recent evolutionary time (Rocha et al. 2006).

Examining the frequency of SNPs across the *T. rubrum* genome revealed high-diversity regions that flank the mating type locus in the two divergent isolates. Across all isolates, some regions of the genome are overrepresented for SNPs, including the smallest scaffolds of the reference genome (Figure 5); these regions contain a high fraction of repetitive elements (Martinez et al. 2012). The largest high-diversity window unique to the *T. rubrum* morphotype *megninii* was found in an ~810-kb region encompassing the mating type locus on scaffold 2; a smaller high-diversity region spanning the
regions include two genes classiﬁcation factors, NRPS, and LysM-domain proteins. in dermatophytes for a small set of genes including transcrip-

ferences between the morphotypes; no stop codons were result in new stop codons. These predicted loss-of-function isolates, an average of 8.1 SNPs are predicted to re-

Table 1 Variation in T. rubrum SNP rate and class

| Isolate              | Total number of SNPs | SNPs in CDS | SYN | NSY | pN/pS |
|----------------------|----------------------|-------------|-----|-----|-------|
| T. rubrum MR1448     | 4,283                | 374         | 83  | 287 | 1.15  |
| T. rubrum MR1459     | 2,188                | 436         | 103 | 317 | 1.02  |
| T. rubrum MR850      | 4,203                | 387         | 88  | 289 | 1.09  |
| T. rubrum D6         | 4,121                | 484         | 112 | 363 | 1.08  |
| T. rubrum (morphotype ﬁscheri) CBS 100081 | 4,199 | 409 | 94  | 307 | 1.09  |
| T. rubrum (morphotype ﬁscheri) CBS 288.86 | 4,147 | 375 | 84  | 283 | 1.12  |
| T. rubrum (morphotype kanel) CBS 289.86 | 4,491 | 474 | 116 | 350 | 1.00  |
| T. rubrum (morphotype raubitschekii) CBS 202.88 | 3,808 | 375 | 83  | 285 | 1.14  |
| T. rubrum (morphotype megnii) CBS 735.88 | 26,406 | 7,328 | 3,069 | 4,185 | 0.45  |
| T. rubrum (morphotype soudanense) CBS 452.61 | 23,073 | 6,253 | 2,377 | 3,808 | 0.53  |
| T. interdigitale MR816 | 1,223,298          | 591,173     | 395,250 | 194,498 | 0.16  |
| T. interdigitale H6  | 1,183,411           | 585,288     | 393,079 | 190,826 | 0.16  |

CDS, coding sequence; SYN, synonymous SNP sites; NSY, nonsynonymous SNP sites; pN/pS, (NSY/total NSY sites)/(SYN/total SYN sites).

Gene content variation in T. rubrum and T. interdigitale

To examine variation in gene content in the T. rubrum species complex, we ﬁrst measured copy number variation across the genome. Duplicated and deleted regions of the genome were identiﬁed based on signiﬁcant variation in normalized read depth (Materials and Methods). We observed increased copy number only for two adjacent 26-kb regions of scaffold 4 in two isolates (MR850 and MR1448) (Figure S6). Both of these regions had nearly triploid levels of coverage (Table S9). While ploidy variation is a mechanism of drug resistance in fungal pathogens, none of the 25 total genes in these regions (Table S10) are known drug targets or eﬄux pumps. These regions include two genes classiﬁed as fungal zinc cluster transcription factors; this family of transcription factors was previously noted to vary in number between dermatophyte species (Martinez et al. 2012). A total of 12 deleted regions (CNVnator P-val < 0.01) ranging in size from 4 to 37 kb were also identiﬁed in a subset of genomes (Table S11). Two of these regions include genes previously noted to have higher copy number in dermatophyte genomes, a nonribosomal peptide synthase (NRPS) gene (TERG_02711) and a LysM gene (TERG_02813) (Table S12). Overall, this analysis suggests recent gain or loss in dermatophytes for a small set of genes including transcription factors, NRPS, and LysM-domain proteins.

We next examined candidate loss-of-function mutations in the T. rubrum species complex. For the eight closely related T. rubrum isolates, an average of 8.1 SNPs are predicted to result in new stop codons, disrupting protein-coding regions; in the soudanense and megnii isolates, an average of 58.5 SNPs result in new stop codons. These predicted loss-of-function mutations do not account for previously noted phenotypic diﬀerences between the morphotypes; no stop codons were found in the seven genes involved in histidine biosynthesis (HIS1-HIS7) in the histidine auxotroph T. rubrum morphotype megnii or in urease genes in T. rubrum morphotype raubitschekii.

Comparison of the ﬁrst representative genomes for T. interdigitale (isolates MR816 and H6) to those of dermatophyte species highlighted the close relationship of T. interdigitale to T. tonsurans and T. equinum. These three species are closely related (Figure 2), sharing 7618 ortholog groups, yet there are also substantial differences in gene content. A total of 1253 ortholog groups were present only in T. equinum and T. tonsurans, and 512 ortholog groups were present only in both T. interdigitale isolates. However, there were no signiﬁcant diﬀerences in functional groups between these species based on Pfam domain analysis, suggesting no substantial gain or loss of speciﬁc protein families. Two Pfam domains were unique to the T. interdigitale isolates and present in more than one copy: PF00208, found in ELFV dehydrogenase family members and PF00187, a chitin-recognition protein domain. This chitin-binding domain is completely absent from the T. equinum and T. tonsurans genomes, while in T. interdigitale this domain is associated with the glycosyl hydrolase family 18 (GH18) domain (Davies and Henrissat 1995). GH18 proteins are chitinases and some other members of this family also contain LysM domains. We also examined genes in the ergosterol pathway for variation, as this could relate to drug resistance; while this pathway is highly conserved in dermatophytes (Martinez et al. 2012), T. interdigitale isolates had an extra copy of a gene containing the ERG4/ERG24 domain found in sterol reductase enzymes in the ergosterol biosynthesis pathway. The ERG4 gene encodes an enzyme that catalyzes the ﬁnal step in ergosterol biosynthesis, and it is possible that an additional copy of this gene results in higher protein levels to help ensure that this step is not rate-limiting.

These comparisons also highlighted the recently discovered dynamics of the LysM family members, which bind bacterial peptidoglycan and fungal chitin (Buist et al. 2008). Dermatophytes contain high numbers of LysM-domain proteins ranging from the 10 genes found in T. verrucosum to 31 copies found in M. canis (Table S13 [Martinez et al. 2012]). Both the class of LysM proteins with additional catalytic domains and the larger class consists of proteins with only LysM domains, many of which contain secretion signals and may represent candidate eﬀectors (Martinez et al. 2012), vary in number across the dermatophytes. Isolates from the T. rubrum species complex have 16–18 copies of
LysM proteins compared to the 15 found in the previously reported genome of the CBS 118892 isolate (Table S13). One of the additional LysM genes present in all of the newly sequenced isolates encodes a polysaccharide deacetylase domain involved in chitin catabolism. There is also an additional copy of a gene with only a LysM domain in 9 of the 10 new T. rubrum isolates (Table S13). The genomes of the T. interdigitale isolates have only 14 genes containing a LysM-binding domain and are missing a LysM gene encoding GH18 and Hce2 domains (Figure S7). Notably, this locus is closely linked to genes encoding additional LysM-domain proteins in some species (Figure S7). The variation observed in the LysM gene family suggests that recognition of chitin appears to be highly dynamic based on these differences in gene content and domain composition.

Discussion

In this study, we selected diverse T. rubrum isolates for genome sequencing, assembly, and analysis and surveyed a wider population sample using MLST analysis. These isolates include multiple morphotypes, which show noted phenotypic variation yet are assigned to the same species based on phylogenetic analyses (Gräser et al. 2008; G. S. de Hoog et al. 2017). The T. rubrum morphotype soudanense and T. rubrum morphotype megninii show higher divergence from a closely related subgroup that includes the kanei, raubitschekii, and fischerii morphotypes, as well as most other T. rubrum isolates.

Our MLST and whole-genome analyses provide strong support for the idea that T. rubrum is highly clonal and may be primarily asexual, or at least infrequently sexually reproducing. Across 135 isolates examined, 134 were from a single mating type (MAT1-1). Consistent with prior reports (Gräser et al. 2008; G. S. de Hoog et al. 2017), only the T. rubrum morphotype megninii isolates contain the opposite mating type (MAT1-2), while all other T. rubrum isolates are of MAT1-1 type. Direct tests of mating between these and other species did not find evidence for mating and sexual development. While mating was not detected, studies in other fungi have required specialized conditions and long periods of time to detect sexual reproduction (O’Gorman et al. 2009). As genes involved in mating and meiosis are conserved in T. rubrum (Martinez et al. 2012), gene loss does not provide a simple explanation for the inability to mate. Sexual reproduction might occur rarely under specific conditions such as specific temperatures as found for T. onychocola (Hubka et al. 2015), may be geographically restricted, as the opposite mating type megninii morphotype is generally found in the Mediterranean (Sequeira et al. 1991), or could be unisexual as in some other fungi such as C. neoformans (Lin et al. 2005).

As MLST data provided no resolution of the substructure of the T. rubrum population, we examined whole-genome sequences for eight diverse isolates. Analysis of the sequence read depth revealed that while some small regions of the genome show amplification or loss, there is no evidence for aneuploidy of entire chromosomes. Most of these T. rubrum isolates contain an average of only 3930 SNPs (0.01% of the genome) and phylogenetic
analysis revealed little genetic substructure. Two isolates were more divergent with an average of 24,740 SNPs (0.06% of the genome); one of these was of the recently proposed separated species *T. soudanense* (G. S. de Hoog et al. 2017) and the other was the *T. rubrum* morphotype *megninii* isolate. While the similar level of divergence raises the question of whether morphotype *megninii* isolates could also be a separate species, this has not yet been proposed when considering additional phenotypic data in addition to molecular data; however, further study would help clarify species assignments. The low level of variation is remarkable in comparison to other fungal pathogens; for example, while *T. rubrum* isolates are identical at 99.97% of positions on average, isolates of *C. neoformans* var. *grubii* isolates are 99.36% identical on average (Desjardins et al. 2017; Rhodes et al. 2017). Global populations of *Saccharomyces* have even higher reported diversity (Liti et al. 2009). The low diversity and the dependency on the human host for growth suggests that *T. rubrum* may have a low effective population size impacted by the reduction of intraspecies variation by genetic drift. In addition, direct tests for recombination found a low level of candidate reassortment that was not in excess of the estimated number of homoplasmic mutations; further, as there was no apparent decay of linkage disequilibrium over genetic distance, our analyses support the overall clonal nature of this species. The high clonality observed in *T. rubrum* is also supported by MLST analysis of eight microsatellite markers in ~230 *T. rubrum* isolates, including morphotypes from diverse geographic origins (Gräser et al. 2007). With additional genome sequencing, geographic substructure may become more apparent; the fungal pathogen *Talaromyces marneffei* also displays high clonality yet isolates from the same country or region are more closely related (Henk et al. 2012). While low levels of diversity seem surprising in a common pathogen, this is similar to findings in some bacterial pathogens including *Mycobacterium tuberculosis* and *My. leprae* (Monot et al. 2005; Comas et al. 2013), which also display high clonality despite phenotypic variation.

LysM-domain proteins are involved in dampening host recognition of fungal chitin (de Jonge et al. 2010) and can also regulate fungal growth and development (Seidl-Seiboth et al. 2013), yet their specific function in dermatophytes and closely related fungi is not well understood. We also observed variation in genes containing the LysM domain across the sequenced isolates, both in the gene number and domain organization. LysM genes have higher copy numbers in dermatophytes than related fungi in the Ascomycete order Onygenales (Martinez et al. 2012). Recent sequencing of additional nonpathogenic species in this order related to *Coccidioides* revealed that most LysM copies found in dermatophytes have a homolog (Whiston and Taylor 2015). Although this analysis excluded *M. canis*—the dermatophyte species with the highest LysM count—this suggests that dermatophytes have retained rather than recently duplicated many of their LysM genes. However, changes in the domain composition of both genes with catalytic domains and those with only LysM domains, many of which represent candidate effectors, highlight the dynamic evolution of the LysM family in the dermatophytes. Studies of LysM genes in dermatophytes are needed to determine whether these genes serve similar or different roles in these species.

*T. rubrum* is only found as a pathogen of humans, though this adaptation is more recent relative to the related species that infect other animals or grow in the environment. Unlike the obligate human fungal pathogen *Pneumocystis jirovecii* (Cissé et al. 2012; Ma et al. 2016), *T. rubrum* does not display widespread gene loss (Martinez et al. 2012) indicative of host dependency for growth; further, its genome size is also comparable to related dermatophyte species, supporting no overall reduction (Martinez et al. 2012). The presence of a single mating type in the vast majority of isolates and the limited evidence of recombination suggests that sexual reproduction of *T. rubrum* may have been recently lost or may be rarely occurring in specific conditions or geographic regions. This may be linked to the specialization as a human pathogen, as mating may be optimized during environmental growth in the soil (Gräser et al. 2008).

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