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ORIGINAL ARTICLE

The cacao pathogen *Moniliophthora roreri* (Marasmiaceae) possesses biallelic *A* and *B* mating loci but reproduces clonally

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The cacao pathogen *Moniliophthora roreri* belongs to the mushroom-forming family Marasmiaceae, but it has never been observed to produce a fruiting body, which calls to question its capacity for sexual reproduction. In this study, we identified potential *A* (*HD1* and *HD2*) and *B* (pheromone precursors and pheromone receptors) mating genes in *M. roreri*. A PCR-based method was subsequently devised to determine the mating type for a set of 47 isolates from across the geographic range of the fungus. We developed and generated an 11-marker microsatellite set and conducted association and linkage disequilibrium (standardized index of association, I_A^S) analyses. We also performed an ancestral reconstruction analysis to show that the ancestor of *M. roreri* is predicted to be heterothallic and tetrapolar, which together with sliding window analyses support that the *A* and *B* mating loci are likely unlinked and follow a tetrapolar organization within the genome. The *A* locus is composed of a pair of *HD1* and *HD2* genes, whereas the *B* locus consists of a paired pheromone precursor, *Mr_Ph4*, and receptor, *STE3_Mr4*. Two *A* and *B* alleles but only two mating types were identified. Association analyses divided isolates into two well-defined genetically distinct groups that correlate with their mating type; I_A^S values show high linkage disequilibrium as is expected in clonal reproduction. Interestingly, both mating types were found in South American isolates but only one mating type was found in Central American isolates, supporting a prior hypothesis of clonal dissemination throughout Central America after a single or very few introductions of the fungus from South America.
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INTRODUCTION

Theobroma cacao L. is the source of chocolate and its production in Latin America is severely affected by two major diseases: frosty pod rot caused by *Moniliophthora roreri* and witches' broom disease, caused by the C-biotype of *M. perniciosa* (Phillips-Mora and Wilkinson, 2007), which together form a clade within the mushroom-forming family Marasmiaceae in the Marasmiineae, Agaricales (Aime and Phillips-Mora, 2005; Dentinger *et al.*, 2015). *Moniliophthora roreri* is believed to originate from Colombia and before the 1950s, it was confined to Colombia, Ecuador and western Venezuela (Phillips-Mora *et al.*, 2007). However, over the last 60 years, its geographical distribution has dramatically expanded throughout Central and South America (Phillips-Mora *et al.*, 2006a, b, 2007, 2015). Currently, *M. roreri* represents a threat to cacao production in Brazil, the only continental Latin American cacao-producing country yet to be invaded (Phillips-Mora and Wilkinson, 2007).

Sexual reproduction allows fungi to colonize new niches and to establish population structure through chromosomal recombination during meiosis (Milgroom, 1996). In basidiomycetes, meiosis occurs within specialized cells called basidia, which in Agaricales are typically produced within a mushroom-type fruiting body. While mushrooms typical of the Marasmiaceae are formed by *M. perniciosa* (Meinhardt *et al.*, 2008), no sexual fruiting body has ever been observed for *M. roreri* (Aime and Phillips-Mora, 2005). Multiple attempts to perform mating experiments with different isolates of *M. roreri* in laboratory conditions have never elicited a mating-compatible reaction (Phillips-Mora, 2003; Díaz-Valderrama, 2014), and in the field, the fungus has only been observed to produce billions of spores on the surface of infected pods (Campuzano, 1976), which are produced by a rhexolytic thallic mode of asexual sporogenesis (Díaz-Valderrama and Aime, 2016). However, the question of whether *M. roreri* undergoes a cryptic form of sexual reproduction necessitates further investigation.

In Agaricomycetes sexual reproduction may be homothallic—that is, capable of but not necessarily restricted to selfing—or heterothallic —that is, outcrossing (Billiard *et al.*, 2012); in addition, heterothallic strategies can be determined by either bipolar or tetrapolar mating systems (Heitman *et al.*, 2013). In bipolar mating systems, mating is governed by a single multiallelic locus, termed *A*; in tetrapolar species, mating is governed by the *A* and one additional unlinked multiallelic locus, termed *B* (Casselton and Olesnicky, 1998). A recently discovered intermediate mating system, referred to as pseudo-bipolar, occurs when there is partial linkage between the *A* and *B* mating loci causing some degree of multiallelism in both loci; however, this unusual mating system has only been seen in species outside the Agaricomycetes (Coelho *et al.*, 2010; Gioti *et al.*, 2013). Different combinations of alleles at each locus dictate the mating type of the individual, and to trigger the mating process, different mating types have to interact (Brown and Casselton, 2001). The *A* locus contains

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genes that code for HD1 and HD2 homeodomain transcription factors and the *B* locus, genes for pheromone precursors and STE3-like pheromone receptors (Riquelme *et al.*, 2005; James, 2007). Pheromone precursors are small lipopeptides containing a C terminal C*aa*X residue (where C is a cysteine, *a* is an aliphatic amino acid and X any amino acid) and a conserved charged doublet (usually ER, glutamic acid and arginine), which is the cleavage site of the mature pheromone (Casselton and Olesnicky, 1998). STE3-like pheromone receptors are G protein-coupled receptors with seven transmembrane (TM) domains that are homologous to the STE3 receptor for the pheromone 'a' factor required in mating of α cells of *Saccharomyces cerevisiae* (Hagen *et al.*, 1986). *Moniliophthora perniciosa* C-biotype is a primarily homothallic fungus and for which both *A* (partial *HD1* but not *HD2*) and *B* (five potential pheromone precursors and eight potential STE3-like receptors) mating-like genes have been annotated (Kües and Navarro-González, 2010). Whether *M. roreri* also possess a similar arrangement of *A* and *B* mating loci is yet to be determined.

Indirect evidence of sexual recombination in fungi can be assessed through population genetic approaches (Burt *et al.*, 1996; Halliday and Carter, 2003). In the case of *M. roreri*, these types of approaches have been attempted using random amplified polymorphic DNA (Grisales Ortega and Kafuri, 2007), amplified fragment length polymorphism and inter-simple sequence repeat (SSR) markers (Phillips-Mora *et al.*, 2007). However, both studies attributed the low levels of genetic diversity observed as consistent with a model of clonal propagation at least within populations in Central America and Antioquia, Colombia (Grisales Ortega and Kafuri, 2007; Phillips-Mora *et al.*, 2007). Evidence of sexual reproduction in fungi can be inferred using microsatellite or SSR markers (Razavi and Hughes, 2004), and in *M. perniciosa*, these have been developed for analyzing the genetic variability among populations (Silva *et al.*, 2008); however, such methods have not yet been applied in *M. roreri*.

The objective of this study was to analyze the reproductive biology of *M. roreri* by direct assessment of the mating genes, and by developing and using more powerful markers. Herein we: (i) utilize genomic data for *M. roreri* to locate and characterize potential *A* and *B* mating genes; (ii) assay and determine the mating type distributions for a set of *M. roreri* isolates from across its geographic range; and (iii) develop and analyze a set of 11 SSR markers in this set of isolates.

MATERIALS AND METHODS

Isolates and DNA extraction

We used 47 isolates of *M. roreri* from 10 Latin American countries across the geographic range of the disease, collected from four hosts and over a 13-year period (Supplementary Table S1). Many were available as previously extracted DNA from prior studies (Evans *et al.*, 2003; Aime and Phillips-Mora, 2005; Phillips-Mora *et al.*, 2006a, b, 2007) and others represented new field collections (Supplementary Table S1). Cultures were maintained on 3.9% w/v Potato Dextrose Agar and kept at room temperature with periodic subculturing. DNA samples were extracted as in Aime and Phillips-Mora (2005).

A and *B* mating loci identification

We used a draft genome of *M. roreri* CBS 138632 (Díaz-Valderrama and Aime, unpublished; GenBank accession number LATX00000000) to search for *A* and *B* mating type-like loci using homologous sequences from related species (Supplementary Table S2) as queries in blast searches against the CBS 138632 genome using Geneious 7.0.4 (Biomatters Ltd, Auckland, New Zealand). We compared our findings with a recently published *M. roreri* genome (Meinhardt *et al.*, 2014).

The number of TM domains and the orientation of the N terminus with respect to the cell membrane of the identified *B*-like receptors were predicted with HMMTOP v2.0 (http://www.enzim.hu/hmmtop/). In addition, the genomic surrounding areas of receptors were explored to find linked potential *B* pheromone precursor sequences by manually identifying the conserved motifs (Riquelme *et al.*, 2005) in small polypeptides detected by FGENESH (http://www.softberry.com/). FGENESH was also used to explore the area surrounding the *A* loci to locate conserved mating type-linked genes (James, 2007).

A and *B* mating loci screening

Primers were designed to amplify the identified *A* and *B* mating genes using the NCBI Primer-BLAST tool (Supplementary Tables S3 and S4). In addition, a PCR-based restriction enzyme assay was designed to rapidly determine the *A* alleles for some isolates (Supplementary Table S4 and Supplementary Figure S1). Enzymes *Stu*I and *Bsi*HKAI (New England Biolabs, Ipswich, MA, USA) were used for digestion of the PCR products (Supplementary Figure S1). Dot plots of alignments between *A* and *B* alleles found were built with PLALIGN (http://fasta.bioch.virginia.edu/) with BLOSUM50 as the scoring matrix. A chi-square test was conducted to test the null hypothesis of random mating based on the identified mating types.

TAIL-PCR, assembling contiguous scaffolds and sliding window analysis

To identify scaffolds from both available genomes contiguous to the ones where *A* and *B* mating loci are located, we used thermal asymmetric interlaced (TAIL)-PCR as specified in Singer and Burke (2003) with some modifications (Supplementary Tables S2–S4). Blast searches between both genomes was performed to detect overlapping scaffolds. Sequencher 5.2.3 (Gene Codes Co., Ann Arbor, MI, USA) was used to assemble sequences from TAIL-PCR and scaffolds using 60% of minimum match percentage and 20% of minimum overlap as parameters. Overlapping scaffolds were compared with a sliding window analysis using DnaSP 5.10.01 (http://www.ub.edu/dnasp/) with a window length and step size of 200 and 25 nucleotides, respectively.

Inference of ancestral mating system

To infer the mating system of the ancestor of *M. roreri*, we constructed a phylogenetic tree using internal transcribed spacer sequences (Supplementary Table 2) from species in the most representative families within the Marasmiineae for which the mating system has been determined (Guillaumin, 1973; Ullrich and Anderson, 1978; Mallett and Harrison, 1988; Petersen and Gordon, 1994; Griffith and Hedger, 1994; Petersen, 1995; Abomo-Ndongo *et al.*, 1997; Johnson and Petersen, 1997; Murphy and Miller, 1997; Chillali *et al.*, 1998; Mata *et al.*, 2004a, b; Tan *et al.*, 2007, 2009; Chew *et al.*, 2013). We noticed that the results from these studies reveal that most of the species within the suborder have a heterothallic tetrapolar mating system, but owing to unavailability of sequences, many of them were not included in the analysis. However, it is estimated that in the Agaricomycetes, 10% of species are homothallic, 25–35% are bipolar and 55–65% tetrapolar (Raper, 1966), and so we tried to have a final taxon sample similar to this distribution. Sequences were aligned with MEGA 5.2.1 (http://www.megasoftware.net/) using the muscle algorithm followed by a selection of conserved blocks within the alignment using GBlocks Server (http://molevol.cmima.csic.es/castresana/Gblocks.html) with the less stringent conditions; maximum likelihood/rapid bootstrapping was carried out with the RAxML Black Box tool via the Cipres Science Gateway portal (https://www.phylo.org/). Inference of ancestral stage was performed using the likelihood reconstruction method in Mesquite 3.04 (http://mesquiteproject.wikispaces.com/).

Phylogenetic analysis of the pheromone receptors

The putative *M. roreri STE3*-like receptor sequences were translated into amino acids, removing introns at splicing sites, to perform a phylogenetic analysis with *STE3*-like receptors from other Basidiomycota species (Supplementary Table S2). Alignment and phylogenetic tree construction was carried out as for the Marasmiineae phylogeny but with default parameters for protein sequences.

RT-PCR and transcriptome

Transcription of the *A* and *B* mating genes was corroborated by RT-PCR (Supplementary Table S4). RNA was extracted with the E.Z.N.A. Fungal RNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's guidelines. DNase treatment was carried out after RNA extraction with the RQ1 RNase-Free DNase (Promega Corp., Madison, WI, USA) protocol followed by the construction of cDNA using the Maxima Universal First-Strand Synthesis Kit (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA). In addition, the published transcriptome of *M. roreri* (Meinhardt *et al.*, 2014) was screened to confirm the transcription of putative mating genes.

Microsatellite marker detection and analysis

A set of Perl scripts that integrates Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) was used to design 30 pairs of primers (Supplementary Table S3) able to amplify microsatellite loci on different scaffolds in the CBS 138632 genome. An initial screening of the 30 microsatellite loci was performed (Supplementary Table S4). From this, 11 microsatellite markers showing polymorphic bands were selected for genotyping and full screening; because of limited material, SSR analyses could only be conducted on 40 of the isolates (Supplementary Tables S1 and S3) using a modified M13 method (Schuelke, 2000; Supplementary Table S4).

For association analyses, a binary set of data was produced from the SSR allele scoring (1 and 0 standing for presence and absence of the allele, respectively). An Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis using a distance matrix (Jaccard coefficient) was generated with XLSTAT 2013.5.05. A 2000-iteration approximately unbiased *P* value (Shimodaira, 2002) was used as a cluster support. The principal component analysis (PCA) was performed with XLSTAT 2013.5.05 and plotted in RStudio 0.97.551. As indicators of random mating and/or clonality, the standardized Index of Association, I_A^s, was calculated with LIAN 3.6 (http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl/query); 100 000 random Monte Carlo iterations were employed.

RESULTS

A mating locus identification

Potential *HD1* and *HD2* genes were found on the ~376 Kb-long scaffold kmer90.96619 next to each other in the CBS 138632 genome; open reading frames read in opposite directions (Figure 1a). After removing likely introns, translating the nucleotide sequence into amino acids and blastp searches, the putative HD1 and HD2 mating proteins are 79.1% and 81.6% identical to their homologs (ESK81811 and ESK81812) in the Meinhardt *et al.* (2014) genome (Figure 1a).

The mitochondrial intermediate peptidase (*MIP*) and β-flanking (*βFG*) genes were located on the same scaffold, kmer90.96619, in the area surrounding the putative *A* mating locus. Other syntenic non-mating type genes, such as the para-amino benzoic acid synthetase (*PAB1*) and the glycine dehydrogenase (*GLYDH*) genes, among others, were also located in its vicinity (Figure 1a; Supplementary Table S5).

B mating locus identification

Ten potential *STE3*-like pheromone receptor genes were identified on four scaffolds in the CBS 138632 draft genome (Figures 1b–f).

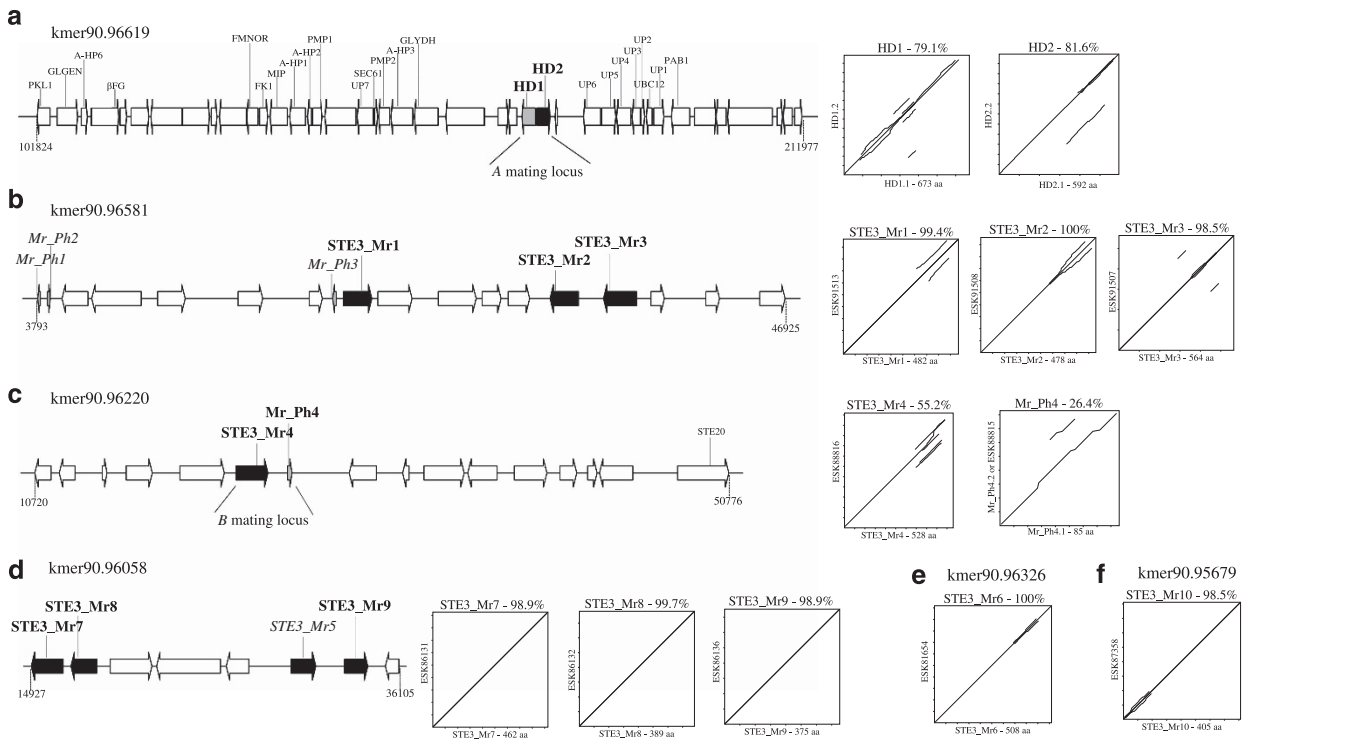

Figure 1 Genetic map of potential *A* and *B* mating genes in the draft genome sequence of *M. roreri* (LATX00000000) and dot plots of the alignments with their homologs from the Meinhardt *et al.* (2014) genome. (**a**) Potential homeodomain transcription factors (*HD1* = gray, *HD2* = black) and *A* mating locus-linked genes found on scaffold kmer90.96619. (**b**) Potential pheromone precursors (gray) and pheromone receptors (black) found on scaffolds kmer90.96581, (**c**) kmer90.96220, and (**d**) kmer90.96058, which are ~52, 103 and 37 Kb-long, respectively. Pheromone precursors and receptor not transcribed are in italics. Arrows indicate the direction of gene transcription and numbers indicate the position ranges of scaffolds. Only dot plots for receptors *STE3_Mr6* and *STE3_Mr10* found on scaffolds kmer90.96326 (58 Kb-long) and kmer90.95679 (49 Kb-long) are shown because no other *B* mating type-linked genes were found on these scaffolds (**e** and **f**). White arrows = putative non-mating type genes linked to homeodomain transcription factors (**a**) and pheromone precursors and receptors (**b**). Labeled non-mating type genes linked to *A* or *B* mating loci are conserved in Agaricomycetes. Notice that conserved genes *MIP* and *βFG* do not flank directly *HD1* and *HD2* (**a**) and conserved gene *STE20* is linked to *STE3_Mr4* and *Mr_Ph4* (**c**). See Supplementary Table S5 for information about non-mating type conserved genes. Percentage on dot plots indicate the identity at the amino acid level between the *A* and *B* genes with their homologs.

When the receptor genes are translated into amino acids and compared with annotated proteins from Meinhardt *et al.* (2014), eight of them share complete or almost complete identity with their homologous receptor (Figures 1b–f). However, the predicted STE3_Mr4 shares only 55.2% identity with another STE3-like receptor (ESK88816) (Figure 1c). Predicted receptor STE3_Mr5 did not have a protein match in the Meinhardt *et al.* (2014) genome, although an identical nucleotide sequence was found on contig AWSO01000972.

Four predicted pheromone precursor genes were identified in the CBS 138632 genome (Figures 1b and c). Two of these, *Mr_Ph1* and *Mr_Ph2*, do not appear to be tightly linked to pheromone receptor genes and are located ~17 Kb upstream from receptor gene *STE3_Mr1* on scaffold kmer90.96581. However, *Mr_Ph3* and *Mr_Ph4* are located 420 bp upstream from *STE_Mr1* and 1134 bp downstream from *STE3_Mr4* on scaffolds kmer90.96581 and kmer90.96220, respectively (Figures 1b and c). No pheromone precursor-like sequence or other conserved linked genes were located in the surrounding areas of any other predicted receptors.

A and *B* mating locus screening

After screening, all 47 isolates were found to carry one of only two versions of the *A* locus, herein termed *A1*, consisting of *HD1.1* and *HD2.1*, and *A2*, consisting of *HD1.2* and *HD2.2* (Supplementary Figure S3). Thirty-one isolates contained *A1* and 16 carried the *A2* locus (Supplementary Table S1). HD1.2 and HD2.2 are completely identical with proteins ESK81811 and ESK81812, respectively, from the Meinhardt *et al.* (2014) genome of *M. roreri*; that is, this genome has allele *A2*.

Owing to limited DNA material for some isolates, only 36 were available for *B* locus screening. Screening for potential alternate alleles of *STE3_Mr1* and *STE3_Mr4* was performed because only these two receptor genes were located next to a pheromone precursor gene (Figures 1b and c), making them the most likely candidates for the *B* locus. Two variants of *STE3_Mr1* were found; their predicted products share 99.4% identity and differing by only three amino acid substitutions. One of these variants shares 100% identity with a pheromone receptor ESK91513 from Meinhardt *et al.* (2014) (Figure 1b; Supplementary Figure S4a). However, two allelic versions of the *STE3_Mr4* receptor gene were found; their products STE3_Mr4.1 and STE3_Mr4.2 share 55.2% identity. The STE3_Mr4.2 receptor shares 100% identity with a STE3-like receptor ESK88816 in the Meinhardt *et al.* (2014) genome (Figure 1c; Supplementary Figure S4b).

As was true for *STE3_Mr4*, screening of *Mr_Ph4* revealed two alleles for this pheromone precursor gene; their products Mr_Ph4.1 and Mr_Ph4.2, share 26.4% amino acid identity (Figure 1c; Supplementary Figure S4c). Both contain typical conserved motifs of pheromone precursors from other Agaricales (Supplementary Figure S4c; Riquelme *et al.* 2005). Mr_Ph4.2 shares 100% identity with a pheromone precursor-like polypeptide (ESK88815) located next to a STE3-like receptor (ESK88816) in the Meinhardt *et al.* (2014) genome, which as mentioned previously also shares 100% identity to receptor allele STE3_Mr4.2. DNA sequences of *Mr_Ph1*, *Mr_Ph2* and *Mr_Ph3* from the CBS 138632 genome share 100%, 100% and 97% identity, respectively, with nucleotide sequences found on contig AWSO01000343 of the Meinhardt *et al.* (2014) genome, but these did not appear to be transcribed (Meinhardt *et al.*, 2014). In addition, the conserved *STE20* gene was found ~22 Kb downstream of this putative *B* locus (Figure 1c). Finally, as was also true for the *A* locus, two *B* alleles were found within our isolates, which harbor either one of the two alternatives: allele *B1* constituted by *STE3_Mr4.1* and *Mr_Ph4.1* (24/36 isolates) and allele *B2* constituted by *STE3_Mr4.2* and *Mr_Ph4.2* (12/36 isolates) (Supplementary Table S1). The Meinhardt *et al.* (2014) genome has allele *B2* and the CBS 138632 genome, allele *B1*.

TAIL-PCR, assembling contiguous scaffolds and sliding window analysis

Assembling of sequences from TAIL-PCR and overlapping scaffolds from both *M. roreri* genomes revealed other scaffolds contiguous to kmer90.96619 and kmer90.96220. This expands up to ~392.3 Kb and ~294.4 Kb the size of the region in which the *A* and *B* mating loci fall, respectively. From this, the minimum possible distance between the *A* and *B* mating loci is ~250.2 Kb, if they were in the same chromosome (Supplementary Figure S2). The sliding window analysis reveals that genomic sequences are very dissimilar at the mating loci but very similar in the flanking regions (Supplementary Figure S2).

Inference of ancestral mating system

We constructed a Marasmiineae phylogeny using taxa from four families within the suborder (Figure 2). Our topology is consistent with that of Dentinger *et al.* (2015). The ancestral reconstruction analysis shows that the ancestor of *M. roreri* is more likely to be heterothallic and tetrapolar, as are the ancestors of the families represented in the tree (Figure 2).

Geographical distribution of mating types

Even though each mating locus has two alleles, the screening of isolates found only two mating types, *A1_B1* and *A2_B2*, out of four possible combinations (Table 1). We also noticed that Central American isolates only harbor mating type *A1_B1* while South American ones harbored both mating types (Supplementary Table S1). The chi-square test did not support outcrossing and random mating in *M. roreri* ($P<0.0001$; Table 1). No additional alleles for either locus were found nor were any isolates found harboring both alleles, that is, none of the isolates appeared to be dikaryotic or diploid (Supplementary Table S1).

Phylogenetic analysis of the pheromone receptors

Phylogenetic analysis of the identified potential pheromone receptors was conducted as an additional means to infer which might be homologs of identified *B* locus receptors. Analysis was conducted with representatives of STE3-like pheromone receptors from other Agaricales and placed those from *M. roreri* within four groups, I–IV (Figure 3). Receptors STE3_Mr2, STE3_Mr7, STE3_Mr8, STE3_Mr9 and their homologs from the Meinhardt *et al.* (2014) genome belong to group I along with a receptor from *M. perniciosa* (Contig15640), which does not belong to any of the *Coprinopsis cinerea* subfamilies of functional *B* receptors (Riquelme *et al.*, 2005; Kües and Navarro-González, 2010). In addition, in this group, receptor gene *Slrcb5* from *Serpula lacrymans*, is known to not be part of the *B* locus (Skrede *et al.*, 2013), and receptor gene *Brl3* from *Schizophyllum commune*, is not linked to a pheromone and probably is not involved in *B* mating functions (Ohm *et al.*, 2010). Group II contains both alleles of receptor STE3_Mr4 and receptors STE3_Mr6 and STE3_Mr10; a 3' truncated *B* receptor from *M. perniciosa* (Contig 14531), which belongs to sub-family 1 of *B* receptors of *C. cinerea* (Kües and Navarro-González, 2010); receptor CDSTE3.1 from *Coprinellus disseminatus* that has a clear origin from mating type-specific Agaricomycetes receptors (James *et al.*, 2006); and functional *B* receptors Bbr2, Rcb1.3 and Slrcb1 from *S. commune*, *C. cinerea* and *S. lacrymans*, respectively (Fowler *et al.*, 1999; Riquelme *et al.*, 2005;

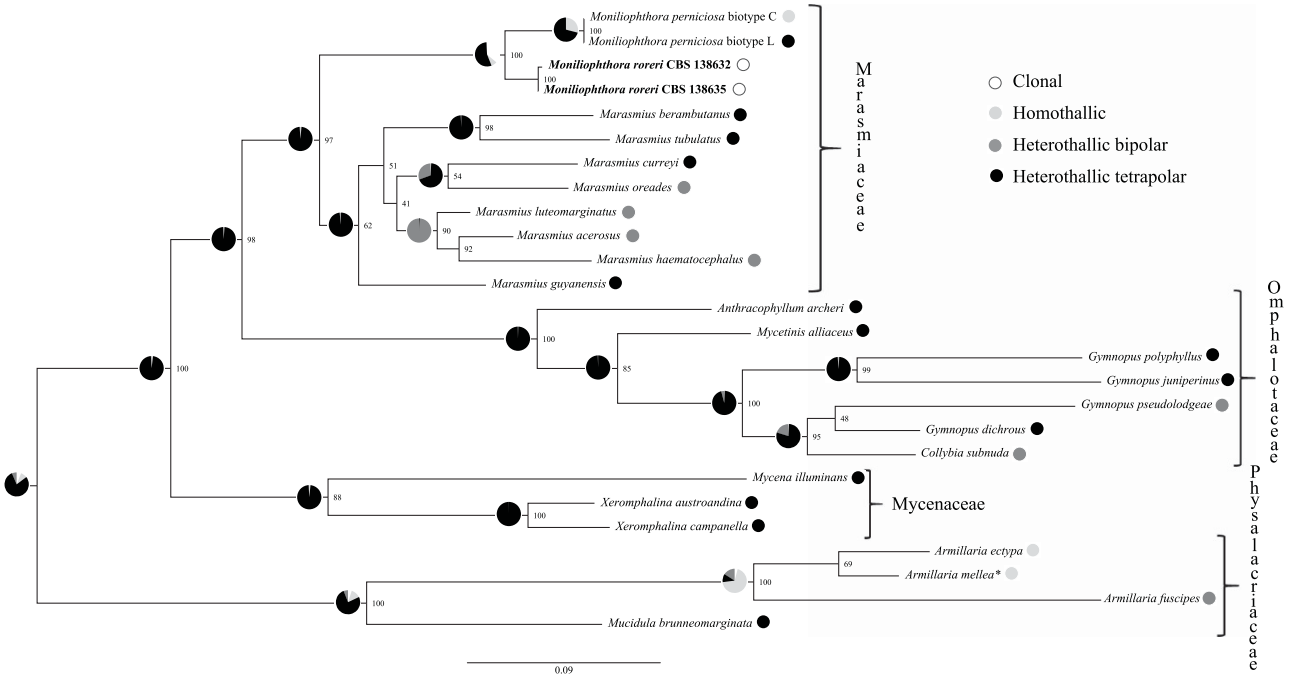

Figure 2 Ancestral reconstruction analysis of the mating system of *M. roreri* and other species within representative families of Marasmiineae. Pie charts contain the proportional likelihoods for a specific mating system in the ancestors across the Marasmiineae internal transcribed spacer phylogeny. The *Armillaria mellea* complex have both homothallic and heterothallic tetrapolar members, but for this phylogeny, we used a sequence from a homothallic specimen. Numbers on nodes indicate bootstrap values. For accession numbers, see Supplementary Table S2.

Table 1 Chi-square test (χ^2) and standardized index of association (I_A^s) analysis to evaluate the null hypothesis of random mating in *M. roreri* based on the comparison of expected and observed mating types and multilocus SSR data, respectively

Chi-square test (χ^2)	*Mating types*	*Observed (O)*	*Expected (E)* [a]	*O—E*	*$(O—E)^2$*	*$\frac{(O-E)^2}{E}$*
	A1_B1	24	9	15	225	25
	A1_B2	0	9	−9	81	9
	A2_B1	0	9	−9	81	9
	A2_B2	12	9	3	9	1
	Total of isolates	36[b]	36		χ^2 =	44
					P value <	0.0001

Standardized index of association (I_A^s) analysis	*Geographic origin*[c]	*Mating type*	*I_A^s*	P *value*[d]
	South America	*A1_B1* and *A2_B2*	0.467	$<1.00\times10^{-5}$
	South America	*A1_B1*	0.100	$<1.10\times10^{-4}$
	South America	*A2_B2*	0.076	1.40×10^{-4}
	Central America	*A1_B1*	0.084	1.37×10^{-2}
	South and Central America	*A1_B1*	0.097	1.00×10^{-5}
	South and Central America	*A1_B1* and *A2_B2*	0.468	$<1.00\times10^{-5}$

Abbreviation: SSR, simple sequence repeat.
[a]If random mating was true, expected values for the four mating types should be equally proportioned, assuming linkage equilibrium or *A* and *B* mating loci are located in different chromosomes.
[b]Analysis of only isolates for which full *A* and *B* mating type screening have been completed (see Supplementary Table S1 for details).
[c]Isolates are grouped by geographic origin and mating type for I_A^s analysis.
[d]In all cases, I_A^s are significantly greater than zero ($P<0.05$), rejecting a hypothesis of random mating and mating between isolates of the same mating type.

Skrede *et al.*, 2013). Group III includes the two variants from receptor STE3_Mr1 and other *B* receptors from *S. commune* (Bar8 and Bbr1), *C. cinerea* (Rcb2.43), *Pleurotus djamor* (PDSTE3.3) and *S. lacrymans* (Slrcb4); it also includes the non-multiallelic *C. disseminatus* CDSTE3.4 and *S. lacrymans* Slrcb4 receptors (James *et al.*, 2006; Skrede *et al.*, 2013). Group IV consists of receptor STE3_Mr3 and its homolog (ESK91507) together with receptor Brl2 from *S. commune*, which, like the *STE3_Mr3* gene, is not linked to a pheromone precursor sequence (Ohm *et al.*, 2010). HMMTOP analysis predicts that two of the 10 potential receptors, STE3_Mr1 and STE3_Mr9, have six TM regions (Figure 3) and their N and C termini are both inside-oriented; the groups from the phylogenetic analysis in which these receptors are located also contain receptors that have six TM regions. The other seven predicted *M. roreri* receptors have seven TM regions each and their N and C termini are outside- and inside-oriented, respectively (Supplementary Table S6).

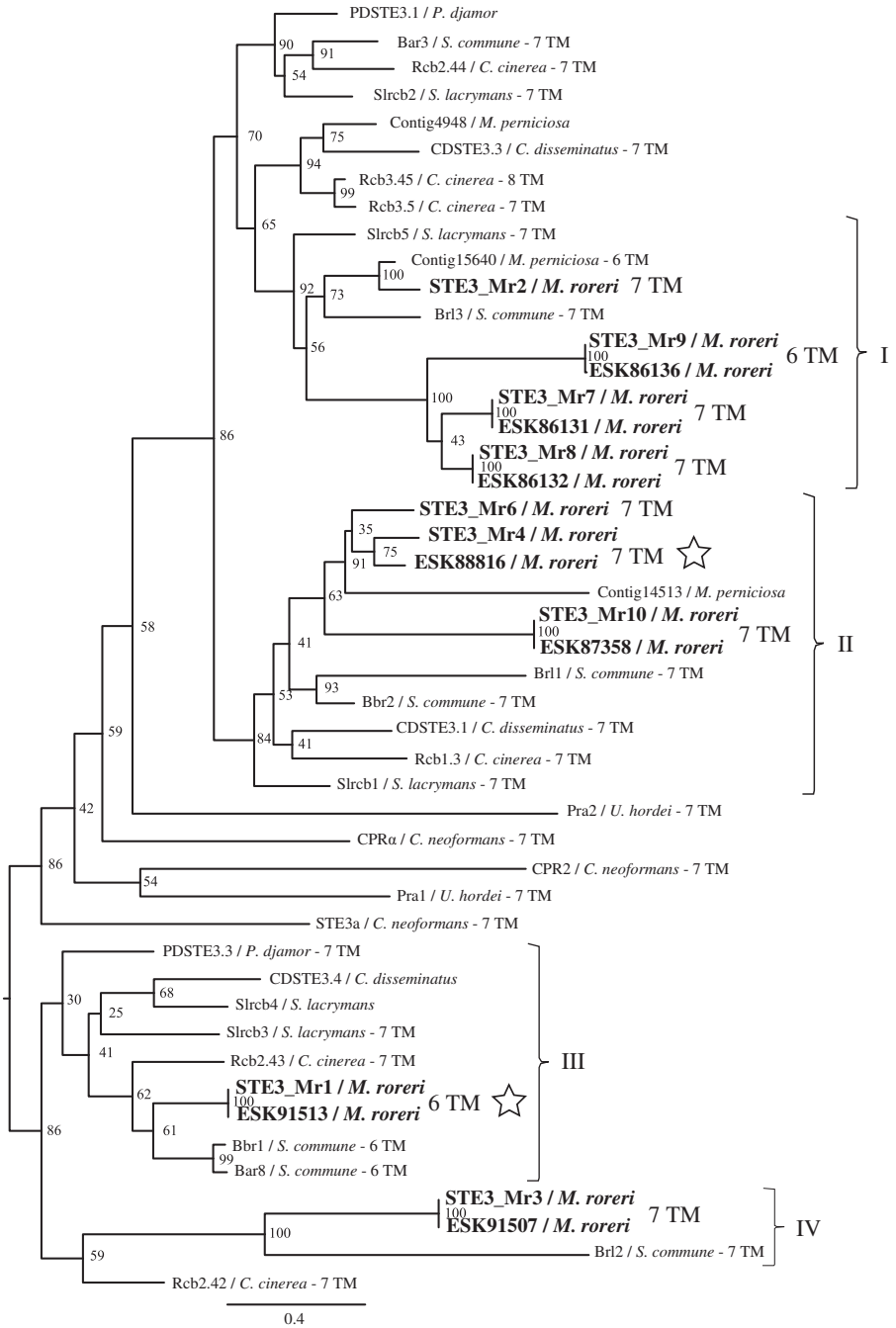

Figure 3 Phylogenetic tree of the STE3-like pheromone receptors from *M. roreri* and other Basidiomycota species. STE3-like receptors from *M. roreri* (in bold) are clustered into four groups (I, II, III and IV). Stars indicate *M. roreri* receptors that have a closely linked pheromone precursor. The number of TM domains the receptors have is also indicated; in some cases, TM number is not shown because of incompleteness of receptor sequence (for more details on TM analysis, see Supplementary Table S6). Numbers on nodes indicate bootstrap values. For accession numbers, see Supplementary Table S2.

RT-PCR and transcriptome

Both the *A* and *B* mating genes are transcribed (Supplementary Figure S1). Primer combination Mr_HD1_Int_B_F/ Mr_HD1_Int_B_R amplifies a 467 bp section from both *HD1* alleles from cDNA (Supplementary Figure S1c), and primer combination Mr_HD2_Int_B_F/ Mr_HD2_Int_R amplifies a 152 bp portion from both *HD2* alleles from cDNA (Supplementary Figure S1d). Likewise, primer combination Mr_Rec4_F2/ Mr_Rec4_R2 amplifies from cDNA a 572 bp portion of *STE3_Mr4.1* (Supplementary Figure S1e) and Mr_R4_A2_F/ Mr_R4_A2_R, amplifies an 879 bp fragment of *STE3_Mr4.2* (Supplementary Figure S1f).

The transcription of the other predicted receptor genes was corroborated on the basis of comparisons with the published transcriptome of *M. roreri* (Meinhardt *et al.*, 2014). However, there is no mRNA sequence for *STE3_Mr5* despite the presence of a corresponding nucleotide sequence on contig AWSO01000972, suggesting this might not be transcribed (Figure 1). Similarly, there were no homologous mRNA sequences for the predicted pheromone

precursor genes *Mr_Ph1*, *Mr_Ph2* and *Mr_Ph3* even though, similarly, corresponding DNA sequences were present on contig AWSO01000343 (Figure 1). However, allele *Mr_Ph4.2* is present in the Meinhardt *et al.* (2014) transcriptome (ESK88815), suggesting that, as with the *B* receptors *STE3_Mr4*, both *Mr_Ph4* alleles are transcribed.

Microsatellite analysis

Only one allele per isolate per locus was found as expected for monokaryotic isolates (Supplementary Table S1). Forty alleles were found in total with locus *Mr_SSR1* being the most polymorphic (seven alleles) and loci *Mr_SSR5*, *Mr_SSR6*, *Mr_SSR10*, and *Mr_SSR23* the least polymorphic (two alleles each). There were three alleles found for locus *Mr_SSR27*; four for *Mr_SSR4*, *Mr_SSR22* and *Mr_SSR28*; and five for *Mr_SSR17* and *Mr_SSR30*. Loci *Mr_SSR1* and *Mr_SSR22* present rare alleles which are only harbored in one isolate ($f=0.025$) (Supplementary Table S7). Overall, same mating type isolates have similar multilocus SSR haplotypes which differ from those of opposite mating type isolates (Supplementary Table S1).

Although some individuals from different geographic locales contained the same genotype at every SSR locus (that is, clones such as CBS 138629 and DIS 371 from Panama and Ecuador, respectively; Supplementary Table S1), some genetic variability exists between isolates. The UPGMA cluster analysis revealed two main groups, which may primarily be distinguished based on the mating type of the isolate (Figure 4). The PCA shows the same results, wherein PC1, which explains 39% of the variability between isolates, correlates precisely with mating type (Figure 5). However, the source of the variation explained by PC2 and PC3 (which explain 11.3% and 10.2 % of the variation between isolates, respectively) is unclear as these do not appear to correlate with any variables relating to country of origin, host or year of collection (Figure 5; Supplementary Table S1). When we test the null hypothesis of random mating in *M. roreri* using the multilocus SSR data, we find that I_A^S values differ significantly from zero ($P<0.05$) in every tested geographic origin-mating type combination suggesting clonal reproduction (Table 1).

DISCUSSION

M. roreri possesses a full set of mating genes distributed in two loci, *A* and *B*, on different scaffolds (Figure 1). Both loci are biallelic (Supplementary Figures S3 and S4) and transcribed (Supplementary Figure S1). Characterization of the *A* mating locus in *M. roreri* shows that both *A* mating genes (*HD1* and *HD2*) are tightly linked and oppositely transcribed (Figure 1a), an orientation typical in other Agaricales (James, 2007). The annotation of the surrounding areas of the *A* locus revealed the syntenic genes *MIP* and *βFG*, which are largely known to directly flank the *A* mating locus in most Agaricales (James *et al.*, 2004a; James, 2007; Niculita-Hirzel *et al.*, 2008).

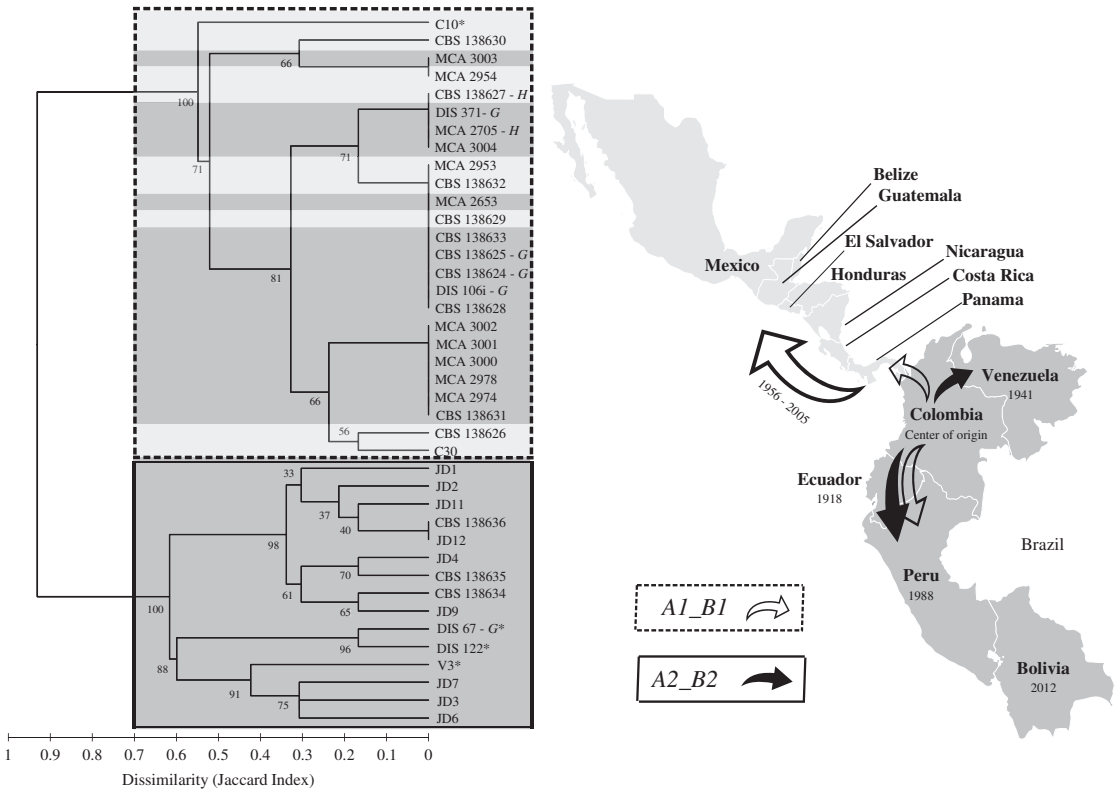

Figure 4 UPGMA cluster analysis based on the multilocus SSR haplotypes of 40 *M. roreri* isolates and map of the geographic distribution and expansion of mating types. Mating type *A1_B1* isolates are clustered in dashed box and *A2_B2* isolates in solid-line box. South and Central American isolates are represented by dark and light gray, respectively. Hosts other than *Theobroma cacao* are indicated with *H* for *Herrania* spp. or *G* for *T. gileri*. The mating types of isolates indicated by an asterisk were not fully determined by mating type screening (see Supplementary Table S1 for details) but were inferred from multilocus SSR data. Numbers on nodes indicate the bootstrap support value approximately unbiased (AU) at 2000 iterations. Note that in Central America, only mating type *A1_B1* isolates were found, while in South America, both mating types were found. Arrows indicate likely routes for geographic expansion of mating types throughout Central America and South America from the hypothetical center of origin in Colombia (Phillips-Mora *et al.*, 2007). Years constitute the times when *M. roreri* was first confirmed in each country (Phillips-Mora and Wilkinson, 2007). Isolates from the recently invaded Bolivia were not available for testing. *M. roreri* is not yet present in Brazil (Phillips-Mora and Wilkinson, 2007).

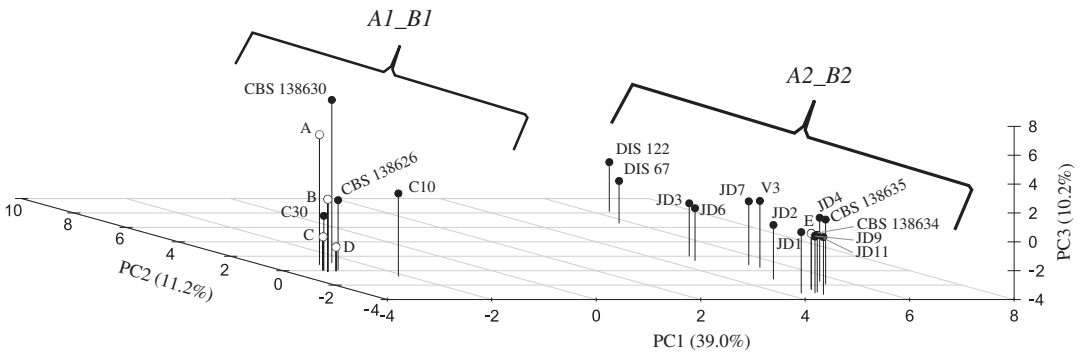

Figure 5 Plot of the first three components from the PCA from the microsatellite genotyping of *M. roreri* field isolates. *A1_B1* isolates are grouped to the left, whereas *A2_B2* isolates are to the right, along the component 1 axis (PC1) explaining 39.0% of the variability. Isolates are labeled with black dots; clones with white dots and a capital letter: (**A**) MCA 3003 and MCA 2954; (**B**) MCA 2974, CBS 138631, MCA 2978, MCA 3000, MCA 3001 and MCA 3002; (**C**) CBS 138628, CBS 138631, CBS 138625, CBS 138633, CBS 138629, MCA 2653, CBS 138632, MCA 2953 and DIS 106i; (**D**) MCA 2705, MCA 3004, DIS 371 and CBS 138627; (**E**) CBS 138636 and JD12.

However, in *M. roreri, MIP* and *βFG* are much more distant from the *A* locus, being ~40 and 60 Kb upstream of *HD1* and *HD2*, respectively (Figure 1a). Similarly, in the mushrooms *Flammulina velutipes* (Physalacriaceae) and *Lentinula edodes* (Omphalotaceae), *MIP* and *βFG* are distant to and do not directly flank the *A* locus (van Peer *et al.*, 2011; Au *et al.*, 2014). These two species also belong to the Marasmiineae suggesting that synteny disruption in the *A* mating region within the suborder might have occurred. Exploring this genomic region in other Marasmiineae species may eventually test this hypothesis.

Ten potential pheromone-like receptors and four pheromone-like precursors were located within the CBS 138632 *M. roreri* genome (Figures 1b–f). Of these, accumulated evidence from locus configuration (Figure 1), sequence divergence between the two alleles (Supplementary Figure S4), phylogenetic analysis (Figure 3) and HMMTOP modeling (Supplementary Table S6) indicate that the *B* locus in *M. roreri* is composed of the two tightly linked genes, *STE3_Mr4*, a *STE3*-like pheromone receptor gene, and *MrPh4*, a pheromone precursor gene. In the receptor phylogeny, STE3_Mr4 is located together with other mating type-specific *B* receptors in group II; all members of this group have seven TM domains and their N and C termini are outside- and inside-oriented (Figure 3), features of functional *B* mating receptors (Wu *et al.*, 2013). Additional evidence is provided by the presence of *STE20*, a conserved gene that is known to be linked to the *B* mating locus in other fungi, including Ascomycota, and to participate in the mating process as a p21-activated PAK kinase (James, 2007; James *et al.*, 2013), on the same scaffold as *STE3_Mr4* and *Mr_Ph4* (Figure 1c). Nevertheless, on scaffold kmer90.96581, the non-allelic predicted receptor gene, *STE3_Mr1*, is linked to the non-transcribed *Mr_Ph3* pheromone gene, which together also resemble a typical *B* mating locus of Agaricomycetes (Figure 1b). However, based on its predicted topology, STE3_Mr1 possesses six TM domains and its N terminus is inside-oriented (Supplementary Table S6). This suggests that STE3_Mr1 might have lost functionality as a fungal mating receptor, especially because the phylogenetic analysis groups it together with functional *B* receptors Rcb2.43 from *C. cinerea* and Bar8 and Bbr1 from *S. commune* in group III (Figure 3). This raises the possibility that the ancestor of *M. roreri* might have had a *B* locus composed of two sub-loci of functional pheromones and receptors but became separated during a relocation event. Other Marasmiineae members harbor two *B* sub-loci, *Bα* and *Bβ*, such as *S. commune* and *L. edodes* (Fowler *et al.*, 2004; Ohm *et al.*, 2010; Wu *et al.*, 2013). Its sister species, *M. perniciosa,* has at least seven putative pheromone receptors and five putative pheromone precursor genes (Kües and Navarro-González, 2010) which might be distributed in two *B* sub-loci, but more studies on *M. perniciosa* are needed to confirm this.

The annotation revealed that, excepting *STE3_Mr1* and *STE3_Mr4*, the other predicted *STE3*-like receptor genes do not have a pheromone precursor-like sequence or any other conserved linked genes in their surrounding areas nor do they appear to be multiallelic (Figures 1b–d). Phylogenetic analysis places receptors STE3_Mr2, STE3_Mr7, STE3_Mr8 and STE3_Mr9 together with non-*B* mating type receptors from *M. perniciosa*, *S. lacrymans* and *S. commune* in group I (Figure 3); these receptors are clustered in a lineage that might represent the diverging point of non-mating type STE3-like receptors from Agaricomycetes. In addition, group I contains receptors that have six TM regions, in support of their non-mating-type specificity (Figure 3). Similarly, STE3_Mr3 is clustered in group IV in a highly diverged branch with receptor Brl2 from *S. commune* that is also not linked to a pheromone precursor sequence (Ohm *et al.*, 2010), suggesting that STE3_Mr3 might also be a non-*B* mating type receptor (Figure 3). Interestingly, receptor STE3_Mr6 and STE3_Mr10 are clustered in the *B* functional receptor group II (Figure 3) and, although they are not multiallelic and do not have an associated pheromone precursor, they appear to possess seven TM regions with the N terminus outside-oriented (Supplementary Table S6), and thus to be functional. The presence of multiple copies of pheromone-free and non-allelic STE3-like receptors distributed in the genomes of Agaricomycetes is common, for example, in *S. lacrymans* (Skrede *et al.*, 2013), *C. disseminatus* (James *et al.*, 2006) and *F. velutipes* (van Peer *et al.*, 2011). Unfortunately, the function of these elements in the mating process remains poorly understood (James *et al.*, 2013). One of the few instances where the function of a non-mating type-specific receptor has been unveiled is the case of CPR2 from *Cryptococcus neoformans.* Owing to an amino acid substitution in its sixth TM domain, this receptor is constitutively activated and when expressed in *S. cerevisiae*, it can trigger the *B* mating pathway in the absence of a pheromone (Hsueh *et al.*, 2009). However, no *M. roreri* receptor is ortholog to CPR2, that is, they were not clustered together in the phylogeny (Figure 3). Therefore, this might not be the functional activity of non-mating type-specific receptors in *M. roreri* although we cannot discard it.

Screening of all 47 *M. roreri* isolates revealed only two alleles of the *A* mating locus, *A1* (*HD1.1* and *HD2.1*) and *A2* (*HD1.2* and *HD2.2*). Alleles HD1.1 and HD1.2 share 79.1% identity, whereas HD2.1 and HD2.2 alleles share 81.6% identity at the amino acid level (Figure 1). These values are higher than identities from other closed related species. For example, less than 70% amino acid identity between HD1 alleles and 60% between HD2 alleles in *C. cinerea* (Badrane and May, 1999) have been reported. In addition, in *S. commune*, Aα- HD1 alleles exhibit 42% identity and Aα- HD2, between 49 and 52% (Stankis *et al.*, 1992). Clearly, identities in *M. roreri A* mating alleles are higher but, importantly, the majority of amino acid difference between both *A* genes occurs in the N terminus of the protein (Supplementary Figure S3), which has been shown to be the primary determinant of mating specificity in HD proteins from *S. commune* and *C. cinerea* (Kronstad and Staben, 1997). This suggests that the *HD1.1* and *HD2.2* as well as *HD1.2* and *HD2.1* should be able to interact and form heterodimer complexes, which are considered the master regulators in mushroom sexual development (Brown and Casselton, 2001).

Screening of the *B* mating locus was completed in 36 isolates, revealing only two alleles, *B1* (*STE3_Mr4.1* and *Mr_Ph4.1*) and *B2* (*STE3_Mr4.2* and *Mr_Ph4.2*). STE3_Mr4.1 and STE3_Mr4.2 share 55.2% amino acid identity, whereas Mr_Ph4.1 and Mr_Ph4.1 share 26.4% identity. Similar values are found in *B* mating genes from other Agaricales. For example, in *C. cinerea, STE3*-like receptor allele identity values may vary from 18 to 81% (Riquelme *et al.*, 2005), and pheromone precursor allele identities from 22 to 50% (Kües, 2000). This suggests also that the *B* alleles found in *M. roreri* should function normally for the *B* mating pathway to initiate.

We show that the ancestor of *M. roreri* was highly likely to possess a heterothallic tetrapolar rather than bipolar or homothallic mating system (Figure 2). This is consistent with the hypothesis that the ancestral stage in the Basidiomycota was heterothallic tetrapolar and that transitions from tetrapolar to bipolar mating systems have occurred independently and multiple times during evolution of species within the phylum (Hsueh and Heitman, 2008; Maia *et al.*, 2015). This strongly supports a tetrapolar arrangement of *A* and *B* mating genes in *M. roreri*, supported by the fact that its sister species, *M. perniciosa* biotype L has a tetrapolar mating system (Figure 2; Griffith and Hedger, 1994).

Even though the genomic distance between the *A* and *B* mating loci in *M. roreri* was not determined because of incompleteness of available genomes, it is in any case greater than 250.2 Kb if they were on the same chromosome (Supplementary Figure S2). However, we did not find any evidence that scaffolds contiguous to one mating locus would match or be also contiguous to any of the scaffolds around the other mating locus, suggesting that the minimum possible distance can be much higher (Supplementary Figure S2). The presence of both mating loci on the same chromosome would imply two possible types of organization, depending on the actual separation distance. If both mating loci were linked as in *Ustilago hordei*, *Malassezia globosa*, *Microbotryum lychnidis-dioicae* and *C. neoformans* var. *neoformans* (Lee *et al.*, 1999; Xu *et al.*, 2007; Hsueh and Heitman, 2008; Badouin *et al.*, 2015), we would be talking about a bipolar organization of mating loci. On the other hand, if both mating loci were partially linked, as in *Sporidiobolus salmonicolor* and *Malassezia sympodialis* (Coelho *et al.*, 2010; Gioti *et al.*, 2013), they would follow a pseudo-bipolar arrangement within the chromosome. However, these two possibilities would imply a chromosomal translocation for the mating loci to become linked or partially linked, which has never been shown to occur in the Agaricomycetes. In fact, in all known instances, bipolarity in this class has always emerged not because of chromosomal translocation and physical linkage between the *A* and *B* mating loci but because of loss of mating type specificity and polymorphism in the *B* mating locus, remaining both loci unlinked or in different chromosomes, as has happened, for example, in the bipolar mushrooms *C. disseminatus*, *P. nameko* and the bipolar white rotter *Phanerochaete chrysosporium* (Aimi *et al.*, 2005; James *et al.*, 2006, 2011). Chromosomal translocation and linkage would also imply rearrangements in the mating loci flanking regions as seen in the bipolar *M. lychnidis-dioicae* (Badouin *et al.*, 2015); however, the sliding window analysis demonstrates the opposite, high levels of similarity and synteny among these regions (Supplementary Figure S2), as observed in the tetrapolar mushroom *P. djamor* (James *et al.*, 2004b). Moreover, pseudo-bipolarity has only been observed in species outside the Agaricomycetes (Coelho *et al.*, 2010; Gioti *et al.*, 2013). Therefore, it is less likely that *M. roreri* mating genes follow a bipolar or pseudo-bipolar organization.

Considering two unlinked and biallelic loci, four different mating types should be expected in a sexually reproducing species. However, only two mating types, *A1_B1* or *A2_B2* and no recombinant mating types (*A1_B2* or *A2_B1*) were identified (Table 1), suggesting that *M. roreri*, despite directly evolving from a heterothallic tetrapolar ancestor (Figure 2), appears to reproduce clonally ($P<0.0001$; Table 1). This explains why evidence of sexual reproduction and meiosis has not been found in previous studies (Grisales Ortega and Kafuri, 2007; Phillips-Mora *et al.*, 2007; Díaz-Valderrama and Aime, 2016) and why mating experiments have never been successful (Phillips-Mora, 2003; Díaz-Valderrama, 2014).

All analyses from SSR data—multilocus SSR haplotype, UPGMA cluster, PCA and I_A^s—are congruent with mating type screening. Isolates that harbor different mating types have a different multilocus SSR haplotype profile, whereas profiles are very similar among same mating type isolates (Supplementary Table S1). UPGMA cluster and PCA analysis strongly separate isolates into two well-defined groups on the basis of mating type (Figures 4 and 5), similar to what was observed in an Australian population of *C. neoformans* var. *gattii* (Halliday and Carter, 2003). Interestingly, the general distribution of isolates in our PCA plot (Figure 5) is very similar to the one found in the PCA from a amplified fragment length polymorphism/inter simple-sequence repeat analysis of *M. roreri* (Phillips-Mora *et al.*, 2007), suggesting it might be likely that the main differences found in that study are dictated by the mating type of isolates.

There is some variability within *A1_B1* and *A2_B2* isolates that can be explained by PC2 and PC3, which contain 11.3% and 10.2% of the overall diversity, respectively (Figure 5). However, this variability does not appear to correlate with geography, collection date or host. For instance, some isolates collected in different countries and years display identical genotypes (for example, CBS 138629 and DIS 371 from Panama and Ecuador, respectively), indicative of clonal reproduction. Likewise, host associations do not explain the variation observed in our dataset, wherein, for example, isolates from *T. gileri* are found in both main genetic groups (Figures 4 and 5). This variation may be explained most likely by the random mutations that probably occur during the massive production of spores that can reach up to seven billion per mature infected pod (Campuzano, 1976). Furthermore, I_A^s values were greater than zero rejecting a hypothesis of random mating of *M. roreri* mating types ($P<0.05$; Table 1).

Mating type *A1_B1* was only found in Central American isolates but both mating types were found in South American isolates (Supplementary Table S1; Figure 4). It has been proposed that the introduction of *M. roreri* to Central America took place first in Panama in 1956 when infected cacao pods were brought from the region of Antioquia, Colombia (Phillips-Mora and Wilkinson, 2007),

which was corroborated with highly similar amplified fragment length polymorphism/inter simple-sequence repeat fingerprints between isolates from Central America and Antioquia (Phillips-Mora *et al.*, 2007) and supported by our finding of the presence of only one mating type in Central American isolates (Figure 4). We found no evidence of mating between these Central American isolates (mating type *A1_B1*) ($I_A^s = 0.084$; $P = 1.37 \times 10^{-2}$; Table 1), discarding a possibility of mating between same mating type isolates—that is, homothallic same-sex mating system (Heitman *et al.*, 2013). These results are consistent to a prior hypothesis of clonal propagation of the fungus throughout Central America after one or very few introductions from South America (Phillips-Mora *et al.*, 2007). Therefore, our data support a model of introduction into Central America of a single mating type from a parental South American population that continues to harbor two mating types (Figure 4).

There are several possible explanations for the clonal behavior of *M. roreri*. First, even though both mating type loci contain the necessary conserved motifs to be functional (Supplementary Figures S1), the *A* and/or *B* mating alleles may have somehow lost their specificity and are plausibly no longer compatible enough to trigger the mating process pathway. This is consistent with the morphology and biology of *M. roreri* that seems to produce exclusively asexual spores (Díaz-Valderrama and Aime, 2016) and forms dolipore septa but does not produce clamp connections (Evans *et al.*, 1978), structures believed to maintain the dikaryotic condition during the mating process (Brown and Casselton, 2001). Another alternative is that receptors STE3_Mr6 and/or STE3_Mr10, which belong to the group II of known *B* mating receptors (Figure 3) and are present in both mating types, might be constantly activated by a pheromone, like Mr_Ph4.1 or Mr_Ph4.2, simulating a *B*-on situation. This has been demonstrated in *C. cinerea* transformants where the monokaryotic hyphae do not accept nuclei from different mating type hyphae upsetting the interaction so that clamp connections and fruiting body formation do not occur (Kües *et al.*, 2002).

This study fills several knowledge gaps regarding the reproductive biology of this cacao pathogen. *M. roreri* possesses a tetrapolar organization of mating genes but does not appear to outcross but rather persists and reproduces as a clonal homokaryon. These results might have direct implications on the strategies that cacao breeders may adopt in their attempts to look for resistance against the fungus. *M. roreri* represents an unusual case of an asexual species within the largely sexual Agaricomycetes.

DATA ARCHIVING

For GenBank accession numbers, see Supplementary Table S2.

Microsatellite data and sequence alignment of pheromone receptors and internal transcribed spacer sequences used in the ancestral reconstruction analysis: DOI:10.5061/dryad.ns657.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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