The Sclerotinia sclerotiorum Mating Type Locus (MAT) Contains a 3.6-kb Region That Is Inverted in Every Meiotic Generation

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Abstract

Sclerotinia sclerotiorum is a filamentous ascomycete in the Sclerotiniaceae (Pezizomycotina) and a necrotrophic pathogen of more than 400 hosts worldwide, including many important agricultural crops [1,2]. In California, the biggest lettuce producer in the United States, S. sclerotiorum is a causal agent of lettuce drop [3] that reduces overall annual lettuce yield by 15%, and losses in individual fields commonly amount to up to 60% [4].

S. sclerotiorum is very durable and survives in the soil in absence of a host for one or more years [5,6]. The survival structures are known as sclerotia that are clusters of cells surrounded by a melanized protective layer. New infections are initiated when sclerotia germinate to form hyphae, or when ascospores are released from fruiting bodies, known as apothecia that emerge from sclerotia [7]. Ascospores result from selfing [8] or outcrossing by heterokaryon formation and recombination [9]. Microconidia are formed and may play a role in fertilization [10].

In ascomycetes, mating system is determined by the mating type locus MAT that encodes transcription factors that regulate downstream gene expression [11]. There are two major mating systems which are heterothallic that is equivalent to obligate outcrossing, and homothallic that consists of selfing and outcrossing [12]. In heterothallic species, isolates generally carry one of two versions of MAT, and only isolates that differ at MAT are sexually compatible. In homothallic species, there is only one version of MAT that generally consists of all the MAT genes of heterothallic relatives, but assembled at a single locus [13]. The two versions of MAT in heterothallics are referred to as idiomorphs instead of alleles, because overall DNA sequence homology between idiomorphs is absent [14,15].

The nomenclature of MAT loci, idiomorphs and MAT genes is based on alphal and high mobility group (HMG) domain genes that encode transcription factors [16]. Most filamentous ascomycetes have a single MAT locus [12]. ‘MAT1-1’ refers to the idiomorph that contains an alphal gene, and ‘MAT1-2’ refers to...
the idiomorph that contains an HMG domain gene. All heterothallic ascomycete species examined in detail contain one MAT1-1 and one MAT1-2 idiomorph [12]. The alpha gene present at MAT1-1 is designated ‘MAT1-1-1’, and the HMG box gene at MAT1-2 is referred to as ‘MAT1-2-1’. Other genes are present at MAT depending on the species, and names for new genes are created taking idiomorphs and previously discovered genes into account. For instance, the second gene discovered at MAT1-1 was designated MAT1-1-2, the third gene MAT1-1-3, the fourth MAT1-1-4, and so on, and similarly at MAT1-2, with genes MAT1-2-2, MAT1-2-3, MAT1-2-4, and so on [16].

Sclerotinia sclerotiorum is a typical homothallic. Selfing [8] and outcrossing [9] have been demonstrated, and the MAT1-1 and MAT1-2 idiomorphs are fused end-to-end at MAT [17]. Sclerotinia sclerotiorum MAT thus consists of four genes, MAT1-1-1, MAT1-1-3, MAT1-2-1 and MAT1-2-4 and the same genes are present in the close relative and heterothallic Botrytis cinerea, but in a heterothallic MAT arrangement where MAT1-1 individuals contain MAT1-1-1 and MAT1-1-5, and MAT1-2 individuals MAT1-2-1 and MAT1-2-4 [17].

Whereas most filamentous ascomycete species are either heterothallic or homothallic, there are examples of mating type switching that occurs within a single generation, from heterothallic to homothallism [18,19,20], and vice versa [21,22,23]. Mating systems also change over longer evolutionary time scales, and in general homothallic species evolve from heterothallic ancestors [24,25,26,27].

The role of MAT as master regulator of downstream gene expression has been well studied in some ascomycetes including S. cerevisiae [28], but little is known about MAT regulated genes in filamentous ascomycetes. MAT target genes in filamentous ascomycetes include pheromone and pheromone receptors that play important roles in recognition of mating partners [29,30,31], and possibly in nuclear pairing following fertilization [32,33,34,35], and genes that are involved in heterokaryon incompatibility [36]. But the majority of genes under MAT regulation appear to have functions that are not directly related to mating [31,37,38,39]. These pleiotropic capabilities of MAT may explain the morphological changes that correlate with mating system in species where mating type switching has been documented [21,23,40].

In this study, we examined the mating type loci of 283 S. sclerotiorum isolates from lettuce in California and other states and hosts, and found that MAT contained a 3.6-kb region that is inverted between generations which correlates with changes in MAT gene expression. We show that the fused S. sclerotiorum MAT arrangement most likely evolved from heterothallic ancestors, and speculate whether a MAT inversion similar to the one in S. sclerotiorum may be responsible for mating type switching in several other filamentous ascomycetes.

Results

Sclerotinia sclerotiorum MAT locus

DNA sequencing coverage of three complete MAT regions was generated for S. sclerotiorum strains 44Ba1, 44Ba12 and 44Ba18 that are listed in Table S1. The DNA sequences measured 12,145 bp, 12,218 bp and 12,218 bp, respectively, and were submitted to GenBank (Accessions JQ815883, JQ815884, and JQ815885). The previously sequenced S. sclerotiorum strain 1980 MAT region from the recently completed genome sequencing project [17], was more than 98% similar overall across coding and non-coding regions to homologous regions of S. sclerotiorum strains 44Ba1, 44Ba12 and 44Ba18 (Table 1). The S. sclerotiorum strain 44Ba1 MAT region contained the same ORFs as S. sclerotiorum strain 1980 [17], notably MAT1-1-5, MAT1-1-1, MAT1-2-4 and MAT1-2-1, flanked by APN2 and SLA2, whereas S. sclerotiorum strains 44Ba12 and 44Ba18 differed by a 3.6-kb inversion that extended from MAT1-1-4 to MAT1-2-1, and resulted in the inversion of MAT1-1-4 and MAT1-2-4, and the truncation of MAT1-1-4 at the 3’-end (Figure 1; Alignment S1). Following the genetic nomenclature of plant pathogenic fungi on the designation of phenotypes [41], isolates with the MAT inversion were designated Inv+, and isolates without the inversion Inv-. The 3.6-kb MAT inversion in Inv+ isolates, and the homologous region in Inv- isolates, were referred to as the MAT inversion region.

There was little variation among the homologous MAT regions of the S. sclerotiorum isolates. Sclerotinia sclerotiorum strains 44Ba12 and 44Ba18 had identical sequences, and among the four MAT ORFs, MAT1-2-4 was most variable, and MAT1-2-1 was most conserved (Table S2). Phylogenetic analyses showed that irrespective of MAT gene, S. sclerotiorum strains 1980, 44Ba1, 44Ba12 and 44Ba18 were monophyletic (Figure 2). Due to the low number of parsimony informative characters (Table S3), branch supports were not evaluated. More details on the phylogenetic analyses are given in Table S3.

The non-coding regions of S. sclerotiorum MAT differed most notably by insertions and deletions. With respect to S. sclerotiorum strain 1980, two deletions of 152 and 26 bp, were present in S. sclerotiorum strains 44Ba1, 44Ba12 and 44Ba18 between APN2 and MAT1-1-5. Additionally, strain 44Ba1 included an 11 bp insertion between MAT1-2-4 and MAT1-2-1 (Table S4).

Comparisons between the mating type genes of S. sclerotiorum generated in this study to the closely related Botrytis cinerea [17] revealed 52 to 74% nucleotide identity depending on the MAT gene (Table 1). Among the four mating type genes, MAT1-1-5 was the most divergent with the lowest identity (52%), the remaining three MAT genes were more than 71% identical to B. cinerea homologs. Except for MAT1-2-4 where B. cinerea had an extra intron, intron numbers were conserved between S. sclerotiorum and B. cinerea (Table 1).

Comparison between the flanks of the MAT inversion region in the S. sclerotiorum Inv+ strains 44Ba12 and 44Ba18, to the homologous regions of the S. sclerotiorum Inv- strain 44Ba1, revealed the presence of a 250-bp inverted repeat, referred to as the 250-bp motif, on either side of the MAT inversion region. The MAT1-1-5 proximal 250-bp motif was part of MAT1-1-1, and the MAT1-1-5 distal 250-bp motif was inverted, and overlapped with the 3’-end of MAT1-2-1 (Figure 3A; Alignment S1). The DNA sequences of the two motifs were identical. Since the 250-bp motif was absent in B. cinerea MAT1-2-1, but present in MAT1-1-1 of both B. cinerea and S. homocarpa (Table S5; Alignment S2), this indicated that the 250-bp motif is a partial MAT1-1-1 that was integrated into MAT1-2-1 in an ancestor of S. sclerotiorum, possibly through a double crossing over (Figure 4A). The 250-bp motif consisted of 60% AT which is similar to the S. sclerotiorum strain 1980 genome overall AT content [17], lacked repeats, and did not match any transposon sequences in GenBank.

In order to identify the S. sclerotiorum MAT1-1-1 – MAT1-2-1 fusion junction, we aligned S. sclerotiorum MAT with homologous B. cinerea MAT1-1 and MAT1-2 regions (Alignment S3), and found that the fusion of MAT1-1 and MAT1-2 most likely occurred in a region of 10 bp encompassing nucleotides 239 to 248 downstream of S. sclerotiorum MAT1-1-1. This is because DNA sequence homology between B. cinerea and S. sclerotiorum MAT1-1 idiomorphs extended 248 nucleotides downstream of S. sclerotiorum MAT1-1-1, and among the ten nucleotides in positions 239 to 248 downstream of S. sclerotiorum MAT1-1-1, five were identical between B. cinerea
# Table 1. Mating type gene statistics of *Sclerotinia sclerotiorum* strains 44Ba1, 44Ba12, and 44Ba18 in relation to *S. sclerotiorum* strain 1980 and *Botrytis cinerea* [17].

| Gene name | Species            | Strain identifier | GenBank / Broad Institute accession number | Gene length, bp | Similarity, % | Intron length, bp (position) | Coding sequence, bp | Protein length, aa |
|-----------|--------------------|-------------------|--------------------------------------------|----------------|---------------|-------------------------------|-------------------|-------------------|
| MAT1-1-1  | *S. sclerotiorum*   | 1980              | XM_001594147.1/ SS1G04004                  | 826            | NA            | Intron 1 50 (761–809)          | 777               | 259               |
|           |                     | 44Ba1             | JQ815883                                   | 1106           | 99.6          | 49 (224–272) 50 (1040–1089)   | 1005              | 335               |
|           |                     | 44Ba12            | JQ815884                                   | 354            | 98.6          | Absent                          | 354               | 117               |
|           |                     | 44Ba18            | JQ815885                                   | 354            | 98.6          | Absent                          | 354               | 117               |
|           | *B. cinerea*        | B05.10            | XM_001546388.1/ BC1G_15148                 | 1161           | 72            | Intron 1 50 (260–309) 49 (1096–1144) | 1002              | 354               |
| MAT1-1-5  | *S. sclerotiorum*   | 1980              | XM_001594146.1/ SS1G04003                  | 1303           | NA            | Intron 1 64 (878–941) 59 (969–1027) | 1131              | 377               |
|           |                     | 44Ba1             | JQ815883                                   | 1303           | 99.7          | 49 (281–329) 64 (878–941) 59 (969–1027) | 1131              | 377               |
|           |                     | 44Ba12            | JQ815884                                   | 1303           | 99.8          | 49 (281–329) 64 (878–941) 59 (969–1027) | 1131              | 377               |
|           |                     | 44Ba18            | JQ815885                                   | 1303           | 99.8          | 49 (281–329) 64 (878–941) 59 (969–1027) | 1131              | 377               |
|           | *B. cinerea*        | B05.10            | XM_001546387.1/BC1G_15147.1               | 1301           | 52.4          | Intron 1 62 (879–940) 59 (968–1026) | 1131              | 377               |
| MAT1-2-1  | *S. sclerotiorum*   | 1980              | XM_001594149.1                             | 1289           | NA            | Intron 1 50 (283–332) 54 (703–756) | 1185              | 395               |
|           |                     | 44Ba1             | JQ815883                                   | 318            | 99.7          | Absent                          | 318               | 105               |
|           |                     | 44Ba12            | JQ815884                                   | 318            | 99.7          | Absent                          | 318               | 105               |
|           |                     | 44Ba18            | JQ815885                                   | 318            | 99.7          | Absent                          | 318               | 105               |
|           | *B. cinerea*        | T4                | FQ790352.1                                 | 1248           | 74            | Intron 1 50 (283–332) 55 (706–760) | 1143              | 380               |
| MAT1-2-4  | *S. sclerotiorum*   | 1980              | XM_001594148.1/ SS1G04005                  | 944            | NA            | Intron 1 49 (171–219) 48 (459–506) | 846               | 282               |
|           |                     | 44Ba1             | JQ815883                                   | 944            | 99.5          | 49 (171–219) 48 (459–506)      | 846               | 282               |
|           |                     | 44Ba12            | JQ815884                                   | 944            | 99.6          | 49 (171–219) 48 (459–506)      | 846               | 282               |
|           |                     | 44Ba18            | JQ815885                                   | 944            | 99.6          | 49 (171–219) 48 (459–506)      | 846               | 282               |
|           | *B. cinerea*        | T4                | FQ790352.1                                 | 1517           | 72            | 48 (192–239) 48 (480–527) 190 (680–869) | 1230              | 409               |

*a* Gene lengths, intron lengths and positions, transcript lengths and amino acid translations for *S. sclerotiorum* strains 44Ba1, 44Ba12 and 44Ba18 were conceptually deduced by comparison to *S. sclerotiorum* strain 1980 [17] unless indicated otherwise.

*b* Intron 1, 2 and 3 refer to the relative positions of the introns in each gene from 5'- to 3'-end.

*c* Confirmed by RT-PCR and DNA sequencing.

*d* More than one transcript was obtained and all were listed by transcript size, see Figures 6, 7.

[17] doi:10.1371/journal.pone.0056895.t001
**MAT** gene expression analyses

RT-PCR was performed for all four **MAT** genes in the eight *S. sclerotiorum* strains 1B331-1 – 1B331-8 that represented an ordered tetrad. Our results agreed with previous studies with respect to gene boundaries and intron positions [17], with the following exceptions. **MAT1-1-1** was extended by 280 bp at the 5’-end and contained an additional, 49-bp intron. This conclusion was based on the fact that we obtained an RT-PCR product (Figure 6) with a forward primer situated approximately 200 bp upstream of the previously predicted start codon [17], and a **MAT1-1-1** internal reverse primer (Figure 6). Since an in-frame start codon was present 280 bp upstream of the earlier predicted start codon, conceptual translation indicated that **MAT1-1-1** measured at least 1106 bp and contained an additional, 49-bp intron, 223 bp from the 5’-end. The 49-bp intron was spliced in Inv- isolates, but not in Inv+ isolates, resulting in a frame shift, a premature stop codon and a truncated **MAT1-1-1** measuring 354 bp (Figure 7). This is opposed to the 1106 bp-**MAT1-1-1** in Inv- isolates (Table 1). The **MAT1-1-1** alpha1 domain in Inv+ isolates was thus shortened to 15 residues, from the 197 residues in Inv- isolates (Figure 7).

A further difference between **MAT** loci was the presence of three **MAT1-2-1** transcript variants in Inv- isolates, only one of the variants was detected in Inv+ isolates (Figure 6). The first variant was unspliced, the second variant was spliced as **MAT1-2-1** in *S. sclerotiorum* strain 1980 [17], and the third variant was spliced differently from the other two variants (Figure 7). Assuming identical start codons for all variants, conceptual translation indicated that variants 1 and 3 encoded identical proteins measuring 105 aa in length and lacking known functional domains, whereas variant 2, the only variant detected in both Inv- and Inv+ isolates, encoded a 395 aa protein containing an HMG domain.

The 3′-fragment of **MAT1-1-1** was also expressed despite the absence of an in-frame start codon (Figure 6) and did not contain any known functional domains.

**MAT** locus copy number

In order to assess whether the *S. sclerotiorum* **MAT** locus was single- or multi-copy, Southern hybridization was performed with a **MAT1-2-1** probe and all eight *S. sclerotiorum* strains 1B331-1 – 1B331-8 of the ordered tetrad. **MAT1-2-1** was targeted due to the detection of three transcript variants differing in length, and thus potentially derived from more than one **MAT** region. The results obtained were consistent with the presence of a single **MAT** locus, as all isolates had only one Southern band, diagnostic of the presence or absence of the **MAT** inversion (Figure 8).

**Segregation and DNA sequencing of the **MAT** inversion region in ordered tetrads**

There was 1:1 segregation of Inv- and Inv+ among the progeny of the two ordered tetrads examined. PCR screening for presence and absence of the inversion in the tetrads showed that among the eight *S. sclerotiorum* strains 1B331-1 – 1B331-8 of the complete tetrad, each sibling strain was PCR-positive for either the presence, or absence of the inversion (Figure 9). The *S. sclerotiorum* strains 1B331-1, 1B331-2, 1B331-5 and 1B331-6 originating from ascospores 1, 2, 5 and 6 numbered from top to bottom of the ascus, were Inv+, whereas *S. sclerotiorum* strains 1B331-3, 1B331-4, 1B331-7 and 1B331-8 corresponding to ascospores 3, 4, 7 and 8 were Inv- (Figure 9), indicative of crossing over and second division segregation [42,43]. Similarly, among the four *S. sclerotiorum* strains 1B321-2, 1B321-4, 1B321-6 and 1B321-8 of the incomplete tetrad, *S. sclerotiorum* strains 1B321-2 and 1B321-4 were Inv+, whereas *S. sclerotiorum* strains 1B321-6 and 1B321-8 were Inv- (Figure 10F), in agreement with first division segregation [42].

Since the parent of the two tetrads was unknown, the two contending parental *S. sclerotiorum* strains were screened. *Sclerotinia sclerotiorum* strain BS001 was Inv-, and strain BS014 was Inv+ (Figure 10F).

The orientation of the **MAT** inversion regions and the flanking 250-bp motifs was confirmed by DNA sequencing in two isolates of the complete tetrad, *S. sclerotiorum* strain 1B331-1 that was Inv+, and strain 1B331-3 that was Inv-. The DNA sequences of the two **MAT** inversion regions and the flanking 250-bp motifs were...
identical, but the orientation of the MAT inversion regions differed between Inv- and Inv+ isolates as expected (Alignment S1).

Proportions of Inv- and Inv+ isolates among random ascospore progeny

Random ascospore progeny consisted of equal proportions of Inv- and Inv+ isolates (Table 2). Progeny of four different parents were screened, including S. sclerotiorum strains BS001, BS013, BS017 and BS028 that were either Inv- or Inv+, and PCR analysis of 18 to 20 progeny for each parent showed that the ratio of Inv- to Inv+ isolates was 1:1 for the progeny of individual parents, and for all progeny combined (Figure 10, Table S6).

Proportions of Inv- and Inv+ isolates among field populations

PCR screening for presence of the MAT inversion in S. sclerotiorum field populations in twelve different states showed that both types of MAT loci were present in California and Nebraska in equal proportions (Table 3). All isolates from Washington were Inv+, and for the remaining states only one or two isolates were screened (Table 3). Inv+ and Inv- isolates occurred on the same hosts that included lettuce, dry bean and canola, whereas in any given state, isolates from cauliflower, pepper, potato, soybean, sunflower and tobacco were either Inv- or Inv+. However, across states, soybean hosted both Inv+ and Inv- isolates (Table 3).

Comparison between inversion breakpoints of Inv- and Inv+ isolates

To confirm that the 250-bp motif was consistently present and intact on either side of the MAT inversion region, the inversion breakpoints were PCR amplified and sequenced in 32 different isolates of S. sclerotiorum, including 13 Inv- and 19 Inv+ isolates. We found that for each isolate, the inversion breakpoints contained the 250-bp motif as in the Inv- S. sclerotiorum strain 44Ba1 and the Inv+ strains 44Ba12 and 44Ba18 for which the entire MAT regions were sequenced (Alignment S4). All 250-bp motifs generated in this study and of S. sclerotiorum strain 1980 [17] were aligned after reverse complementing the MAT1-1-5 distal 250-bp motifs, and the DNA sequences of all 250-bp motifs were identical (Figure 11, Alignment S5).

Impact of the MAT inversion on self fertility

We investigated the relationship between MAT inversion and self fertility, and found that among the 36 S. sclerotiorum Inv+ isolates tested, 35 formed fully expanded apothecia which are the sexual fruiting bodies, and of the 21 Inv- isolates, 20 formed fully expanded apothecia (Table 4). Ascospore viability was not assessed in detail, but the ascospores of S. sclerotiorum strains BS001 and BS014 that are Inv- and Inv+, respectively, were viable [44], as were the ascospore progeny used for inversion screening (Table S6) and the 13 S. sclerotiorum strains used for assessment of mycelial compatibility groups (see below). In an apothecial stalk formation assay with another group of isolates, we found that of the 29 S. sclerotiorum Inv+ isolates, 18 produced apothecial stalks indicative of self-fertility (Table S7). All nine S. sclerotiorum Inv- isolates formed apothecial stalks (Table S7). However, the apothecial stalk assay may not necessarily reflect the ability to form mature apothecia [45].

Mycelial compatibility groups

All 13 S. sclerotiorum isolates investigated (BS001, BS002, BS003, BS011, BS013, BS014, BS017, BS028, BS047, BS058, BS071, BS095, BS096) (Table S1) were successfully paired with their progeny, and thus, mycelial compatibility groups were shared between parents and offspring in all instances. The S. sclerotiorum strains 1B331-1 – 1B331-8 representing the ordered tetrad were paired in all possible combinations among one another, and all pairings were compatible.

Discussion

We investigated the Sclerotinia sclerotiorum mating type locus (MAT) in a collection of 283 isolates from lettuce in California and from other states and hosts, and found that S. sclerotiorum has two MAT alleles that differ in the orientation of a 3.6-kb region that is undergoing an inversion in every meiotic generation. The MAT inversion changes the orientation of two genes, truncates another gene and correlates with altered MAT gene expression. MAT inversions have also been reported in other ascomycetes including Cochliobolus kusanoi [24], Peyronellaea spp. [27] and Stemphylium spp. [25], but it is unknown whether in these fungi the inverted MAT regions are stable, or are inverted in every meiotic generation as in S. sclerotiorum.

The MAT inversion region is present in half the progeny

The process associated with the change in orientation of the 3.6-kb MAT inversion region in S. sclerotiorum MAT may be highly regulated, because it results in a 1:1 ratio of ascospores that have the inversion and that are referred to as Inv+, to ascospores without the inversion designated Inv-. The 1:1 ratio was observed

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**Figure 2.** Most parsimonious trees obtained from Sclerotinia sclerotiorum MAT1-1, MAT1-1-5, MAT1-2-1 and MAT1-2-4 single locus datasets (Alignments S6, S7, S8, S9). The trees were rooted with B. cinerea which is not shown, numbers refer to alignment positions. Nucleotide substitutions along the branches are given for the strain identifiers, for S. sclerotiorum and B. cinerea with locus datasets (Alignments S6, S7, S8, S9). Positions. Nucleotide substitutions along the branches are given for the strain identifiers, for S. sclerotiorum and B. cinerea with locus datasets (Alignments S6, S7, S8, S9).
among random progeny and in ordered tetrads. Half of the 18 to 20 randomly selected ascospores of each of the four apothecia examined, were Inv+ and half were Inv-, regardless of whether the parental isolate was Inv+ or Inv- (Figure 12A). And the ratio of Inv+ to Inv- ascospores in a single ascus was 1:1. The two asci examined displayed inversion distribution patterns among the sibling ascospores indicative of segregation and recombination (Figure 12B).

The 1:1 ratio of Inv+ to Inv- isolates was also found at the population level in California and Nebraska suggesting that the transition between Inv- and Inv+ that we documented in the laboratory, may also commonly occur in nature (Table 3). Inv+ isolates were widespread, and in addition to California and Nebraska, were also found in Georgia, Missouri, North Dakota, Ohio, South Dakota and Washington State, but with the exception of Washington where all 15 isolates were Inv+ (Table 3).

MAT inversion does not occur during mycelial growth and sclerotia formation

We did not investigate in detail when during the S. sclerotiorum life cycle and under which conditions the MAT inversion occurs, but based on PCR results it appears that the rearrangement of the MAT inversion region does not occur during vegetative hyphal growth or during sclerotium formation. This is because the mycelia used for DNA extraction were grown from sclerotia that were generated in the laboratory, and all strains were either Inv- or Inv+ based on PCR assays, and none were both (Figure 10). Also, the inversion phenotype of S. sclerotiorum strain 1B331-8 did not change following subculturing (data not shown). This is dissimilar to human blood cells where DNA regions flanked by inverted repeats similarly to the MAT inversion region, undergo recurrent inversions that result in cell populations that are mixed.

Figure 3. Inversion of the 3.6-kb MAT inversion region in Sclerotinia sclerotiorum by means of crossing over between the two 250-bp motifs. Genes are colored boxes, white and dotted boxes within genes correspond to alpha1 and HMG domains, respectively, flanking genes are white boxes, directions of transcription are indicated by black arrows, gene names are inside or by the boxes, red arrows mark the orientations of the 250-bp motifs for which the 5’- and 3’-end sequence is given. Dashed box and arrow represent MAT1-1-1 3’-fragment lacking an in frame start codon. 3A) MAT region of S. sclerotiorum Inv- isolates. 3B) Crossing over between the two 250-bp motifs of a S. sclerotiorum Inv- isolate gives rise to 3C), the inversion of S. sclerotiorum Inv+ isolates. Figures are not to scale. The Inv+ alpha1 box is truncated after 45 bp and is not illustrated, for details see text.

doi:10.1371/journal.pone.0056895.g003
with regard to the orientations of inversion regions [46]. However, we expect a mixed population of nuclei within the same structure following rearrangement of the MAT inversion region, as *S. sclerotiorum* ascii harbor both Inv- and Inv+ ascospores (Figures 8, 10, 12).

Under appropriate conditions, sclerotia in *S. sclerotiorum* give rise to apothecia that are sexual fruiting bodies where meiosis occurs within the asci, and since we did not detect rearrangement of the MAT inversion region during vegetative growth, inversion appears to occur during the sexual cycle. The arrangement of Inv- and Inv+ ascospores inside the asci is consistent with first and second

**Figure 4. Evolutionary origin of the MAT locus in *Sclerotinia sclerotiorum* Inv- isolates from hypothetical ancestors by means of a double crossing over, an inversion and a single crossing over.** Genes are boxes, directions of transcription are indicated by arrows, gene names are inside the boxes, positions of the 250-bp motif are indicated by red horizontal lines, crossing overs are marked by 'X', the inversion by a curved arrow. 4A) A double crossing over between ancestral MAT1-1 (blue) and MAT1-2 (green) transferred a 250-bp fragment, the ‘250-bp motif’, from MAT1-1-1 to MAT1-2-1 and flanking region. 4B) A subsequent inversion (gray) in the ancestral MAT1-2 (green), followed by a crossing over, 4C) with an ancestral MAT1-1 (blue), results in the MAT arrangement in *S. sclerotiorum* Inv- isolates. The asterisk indicates the location of the DNA sequence alignment provided in Figure 5.

doi:10.1371/journal.pone.0056895.g004

**Figure 5. Proposed MAT1-1 – MAT1-2 border in *S. sclerotiorum* MAT inferred from an alignment of *S. sclerotiorum* MAT, *B. cinerea* MAT1-1 and MAT1-2.** The location of the alignment reproduced above is indicated in Figure 4 by an asterisk, for entire alignment see Alignment S3. *Sclerotinia sclerotiorum* strain 44Ba1 MAT (GenBank Accession JQ815883) and *B. cinerea* MAT1-1 (AAPD1003685) were alignable up to 248 bp downstream of *S. sclerotiorum* MAT1-1-1 as indicated by the top left arrow, homology ceases thereafter. The site where a crossover between ancestral MAT1-1 and MAT1-2 represented here by *B. cinerea* MAT1-1 and MAT1-2, might have occurred, is underlined. The border and crossover site positions are tentative, since *S. sclerotiorum* MAT and *B. cinerea* MAT1-2 (reverse complement of FQ790352) were too divergent to be aligned in the MAT1-2-4 downstream region (Figure 1), the top right arrow demarcates the MAT1-2 region in *S. sclerotiorum* based on lack of homology to *B. cinerea* MAT1-1, not based on homology to *B. cinerea* MAT1-2.

doi:10.1371/journal.pone.0056895.g005
division segregation during meiosis (Figure 12B), which suggests that Inv- and Inv+ nuclei were paired up prior to meiosis, and thus, the inversion precedes meiosis. Furthermore, pairing of Inv- and Inv+ nuclei may require nuclear recognition, a process that in heterothallic ascomycetes involves MAT and the production of nucleus-specific hormones and hormone receptors [32,33,34,35]. Sclerotinia sclerotiorum is homothallic, but Inv+ and Inv- MAT regions differ in gene expression (Figure 6) and may be functionally equivalent to MAT1-1 and MAT1-2 of heterothallics, also because Inv+ MAT appears to lack a functional alpha1 box (Figure 7). Thus, we hypothesize that rearrangement of the MAT inversion region occurs before meiosis but following sclerotium formation, and is involved in recognition between Inv- and Inv+ nuclei.

We do not know what triggers the MAT inversion, but in human blood cells, the recurrent inversion events are caused by non-allelic homologous recombination [46], a process that is initiated through DNA double strand breaks [47]. Non-allelic homologous recom-
Figure 7. Alignment of MAT1-1-1 (top) and MAT1-2-1 (bottom) transcript variants (left) and deduced protein variants (right). Transcript variants are colored boxes, black lines between the boxes represent alignment gaps with respect to other variants, the 5′- and 3′-ends are indicated, variant lengths are given underneath the boxes in base pairs, variant designation and presence in S. sclerotiorum Inv- and Inv+ isolates is indicated on the left. Protein variants are boxes, N and C terminals are indicated, white boxes mark alpha1 and dotted boxes mark HMG domains, black boxes represent unalignable regions, variant designation and presence in S. sclerotiorum Inv- and Inv+ isolates is indicated on the left. Lengths of protein variants are given underneath the boxes in residues. Protein variants were deduced conceptually taking transcript variants into account. Diagrams are not to scale. 7A) Schematic alignments of MAT1-1-1 transcript variants from Figure 6B and the corresponding inferred MAT1-1-1 variants. Only transcript variant 2 of S. sclerotiorum Inv- isolates implies the presence of a complete alpha1 box in MAT1-1-1. 7B) Schematic alignments of MAT1-2-1 transcript variants from Figure 6B and the corresponding inferred MAT1-2-1 variants. Only transcript variant 2 of S. sclerotiorum Inv- and Inv+ isolates contains an HMG box.

doi:10.1371/journal.pone.0056895.g007

Figure 8. Analysis of MAT1-2-1 copy number using Southern blotting. 8A) Gene diagrams of Sclerotinia sclerotiorum Inv- (left) and Inv+ (right) MAT loci illustrating the positions of the Southern probe with respect to the restriction sites used for Southern analyses. Boxes represent genes, white and dotted boxes correspond to alpha1 and HMG domains, respectively, dashed box represents MAT1-1-1 3′-fragment lacking an in frame start codon. Gene names are indicated above or within the boxes. The positions of the BsaHI restriction sites (black triangles) and the distances between the BsaHI sites are indicated above the boxes. The position of the Southern probe is marked by a horizontal black line beneath MAT1-2-1. The Inv+ alpha1 box is truncated after 45 bp and is not illustrated, for details see text. 8B) Southern blot of BsaHI-digested genomic DNA visualized with the digoxigenin-labeled MAT1-2-1 specific probe. Wells 1 - 8 correspond to S. sclerotiorum strains 1B331-1 – 1B331-8 that represent an ordered tetrad, band sizes are indicated on the right. Lanes 1, 2, 5 and 6 have the pattern reflective of an Inv+ MAT locus, lanes 3, 4, 7 and 8 have the pattern expected for an Inv- MAT locus.

doi:10.1371/journal.pone.0056895.g008
bination that results in genome rearrangements is also known from *Saccharomyces cerevisiae* [48].

**MAT** inversion is facilitated by two 250-bp motifs

The trigger for the **MAT** inversion is unknown. But the inversion event is most likely caused by non-allelic homologous recombination [46,47] between the 250-bp motifs that flank the 3.6-kb **MAT** inversion region. The **MAT1-1-1** 5′ distal 250-bp motif is inverted with respect to the **MAT1-1-1** 5′ proximal 250-bp motif, and juxtaposition of the two motifs and recombination results in a 3.6-kb inversion without altering the DNA sequence composition of the 250-bp motifs (Figure 3). Since one 250-bp motif is located near the center of **MAT1-1-1**, and the other 250-bp motif overlapped with the 3′-end of **MAT1-2-1**, only **MAT1-1-1** is truncated by the inversion (Figure 3; Alignment S1). In all 36 isolates examined, the two 250-bp motifs had identical DNA sequence composition (Figure 11). We sequenced across the two 250-bp motifs and the 3.6-kb **MAT** inversion region in *S. sclerotiorum* strains 1B331-1 (Inv+) and 1B331-3 (Inv−), two progeny of the ordered tetrad, and did not find any differences between the two isolates apart from the orientation of the **MAT** inversion region (Alignment S1).

**MAT** locus is single copy

There is just one **MAT** locus in *S. sclerotiorum*, based on the analysis of strain 1980 [17]. And the results from Southern blotting with a **MAT1-2-1** probe (Figure 8) were consistent with the presence of either Inv+ or Inv− **MAT** as documented by PCR screening (Figure 10). Thus, the transition between Inv+ and Inv− isolates is not due to the exchange of the **MAT** inversion region between two **MAT** loci, and thus differs from mating type switching in baker’s yeast, *Saccharomyces cerevisiae* [49].

**Evolutionary origin of the **MAT** inversion region**

*Sclerotinia sclerotiorum* is closely related to *B. cinerea* [50,51], but the two fungi differ in mating system and **MAT** gene arrangement. Whereas *B. cinerea* is heterothallic and each individual has a **MAT1-1** or a **MAT1-2** idiomorph at **MAT** [17], *S. sclerotiorum* is homothallic and the two **MAT** idiomorphs are fused, similarly to *Cochliobolus* [24], *Crivellia* [26], *Didymella* and *Peyronellaea* [27], *Gibberella* [52] and *Stemphylium* [25]. Homothallic species are generally thought to have evolved from heterothallic ancestors [24,25,26,27], and a similar scenario is possible for *S. sclerotiorum* (Figure 4C).

The *S. sclerotiorum* **MAT** arrangement resembles most closely the **MAT** arrangement of homothallic *Stemphylium* species where the **MAT1-1** idiomorph is inverted with respect to heterothallic relatives, and is fused to **MAT1-2** [25]. The fused **MAT** arrangement in *Stemphylium* evolved following the inversion of **MAT1-1** that created a short stretch of DNA sequence identity between idiomorphs, facilitating a crossing over that resulted in the **MAT1-1** – **MAT1-2** fusion [25]. Similarly to *Stemphylium*, the orientation of **MAT1-2** differs between *S. sclerotiorum* and *B. cinerea* [17]. To investigate whether in an ancestor of *S. sclerotiorum* the **MAT1-2** inversion could have facilitated crossing over resulting in **MAT** fusion, we looked for a crossover site and aligned the **MAT1-1** – **MAT1-2** fusion junction in *S. sclerotiorum* Inv+ isolates with homologous regions of *B. cinerea*, a proxy for the unknown ancestor. We found that the fusion of **MAT1-1** and **MAT1-2** most likely occurred in a region of 10 bp encompassing nucleotides 239 to 248 downstream of *S. sclerotiorum* **MAT1-1-1**. This is because DNA sequence homology between *B. cinerea* and *S. sclerotiorum* **MAT1-1-1** idiomorphs extends 248 nucleotides downstream of *S. sclerotiorum* **MAT1-1-1**, and among the ten nucleotides in positions 239 to 248 downstream of *S. sclerotiorum* **MAT1-1-1**, five were identical between *B. cinerea* **MAT1-1-1**, *S. sclerotiorum* **MAT1-1-1** and *B. cinerea** **MAT1-2** (Figure 5; Alignment S3). The relatively high DNA sequence identity between *B. cinerea* **MAT1-1-1** and **MAT1-2** idiomorphs at the *S. sclerotiorum* **MAT1-1** – **MAT1-2** fusion junction is consistent with the presence of an ancient crossover site. Unlike **MAT1-1**, the *B. cinerea* and *S. sclerotiorum** **MAT1-2** regions were generally too divergent to be aligned with confidence outside of the presumptive crossover site (Alignment S3).

In other homothallic ascomycetes, **MAT1-1** – **MAT1-2** fusion junctions with similarities to homologous regions of related heterothallics are also known. Between the homothallic *Cochliobolus lutrellii* and its heterothallic relative, the fusion junction consisted of seven consecutive nucleotides shared between the two species [24]. Similarly, in *Crivellia* there were four shared consecutive nucleotides [26], in *Didymella* three [27] and in *Stemphylium* four [25]. An alternative explanation for the evolution of the *S. sclerotiorum* and *B. cinerea** **MAT** loci has been proposed. It was suggested that the fused idiomorphs of *S. sclerotiorum* were ancestral, and the separate idiomorphs of *B. cinerea* were derived [17]. This evolutionary scenario is less likely, since it requires at least three different steps to occur in an ancestor of *B. cinerea*, including a loss of **MAT1-1**, a loss of **MAT1-2** and an inversion of **MAT1-2**. Our scenario is more parsimonious since it requires only two steps which are inversion of **MAT1-2**, and fusion of **MAT1-2** to **MAT1-1**. The presence of partial **MAT1-1-1** and **MAT1-2-1** genes in *B. cinerea** **MAT**, proposed remnants of incompletely deleted **MAT1-1-1** and **MAT1-2-1** [17], likely resulted by crossing over between *B. cinerea** **MAT1-1** and **MAT1-2** after evolutionary separation from the *S. sclerotiorum** lineage.

A further difference between *B. cinerea* and *S. sclerotiorum** **MAT** is the presence of the 250-bp motifs delimiting the 3.6-kb **MAT** inversion region in *S. sclerotiorum*. Since a 250-bp motif is absent in *B. cinerea** **MAT1-2-1**, but present in **MAT1-1** of both *B. cinerea* and *S. homoeocarpa* (Table S5; Alignment S2), the 250-bp motif is most likely a partial **MAT1-1-1** that was integrated into **MAT1-2-1** in an ancestor of *S. sclerotiorum*, possibly through a double crossing...
Figure 10. Orientation of MAT inversion region in Sclerotinia sclerotiorum isolates as evaluated by Inv+ and Inv- specific PCR reactions. Each isolate has one PCR band and is thus either Inv- or Inv+, weak bands in isolates with strong bands are false positives due to cross contamination. Isolates used are parental strains BS001, BS011, BS013, BS014, BS017 and BS028 (Table S1), and their progeny (Table S6), including the tetrads in Figure 10F. The top gel in each part figure shows the PCR bands obtained with primer pair Type-II/F / Type-II/R specific to Inv+, and the
bottom gel the bands with primer pair MAT1-1/F - MAT1-1-R specific to Inv- (Figure 5). Lanes are numbered, numbers marked with asterisks contain negative controls, the first and last wells of each gel are DNA size standards, arrow heads in Figure 10A indicate positions of 1.2 kb and 0.6 kb bands, the remaining wells are as follows for S. sclerotiorum strains and negative controls in sequential order. 10A) BSO11, BSO11sa01, BSO11sa02, BSO11sa03, BSO11sa04, BSO11sa05, BSO11sa06, BSO11sa07, BSO11sa08, BSO11sa09, BSO11sa10, BSO11sa11, BSO11sa12, BSO11sa14, BSO11sa15, BSO11sa16, BSO11sa17, BSO11sa18, 10B) BSO11sa19, BSO11sa20, negative control, BSO13, BSO13sa01, BSO13sa02, BSO13sa04, BSO13sa05, BSO13sa06, BSO13sa07, BSO13sa08, BSO13sa09, BSO13sa10, BSO13sa11, BSO13sa12, BSO13sa13, BSO13sa14, BSO13sa15. 10C) BSO13sa16, BSO13sa17, BSO13sa18, BSO13sa19, BSO13sa20, negative control, BSO17, BSO17sa01, BSO17sa02, BSO17sa03, BSO17sa04, BSO17sa05, BSO17sa06, BSO17sa07, BSO17sa08, BSO17sa09, BSO17sa10, BSO17sa11. 10D) BSO17sa12, BSO17sa13, BSO17sa14, BSO17sa15, BSO17sa16, BSO17sa17, BSO17sa18, BSO17sa19, BSO17sa20, negative control, BSO28, BSO28sa03, BSO28sa04, BSO28sa05, BSO28sa06, BSO28sa07, BSO28sa08, BSO28sa09. 10E) BSO28sa10, BSO28sa11, BSO28sa12, BSO28sa13, BSO28sa14, BSO28sa15, BSO28sa16, BSO28sa17, BSO28sa18, BSO28sa19, BSO28sa20, negative control. 10F) BSO01, BSO11, BSO11sa01, BSO11sa02, BSO11sa03, BSO11sa04, BSO11sa05, BSO11sa06, BSO11sa07, BSO11sa08, BSO11sa09, BSO11sa10, BSO11sa11, BSO11sa12, BSO11sa14, BSO11sa15, BSO11sa16, BSO11sa17, BSO11sa18, 10. doi:10.1371/journal.pone.0056895.g010

over (Figure 4A). However, no potential crossover sites were found when comparing the flanks of the 250-bp motif in S. sclerotiorum to homologous regions of B. cinerea MAT-1 and MAT-2. Transposons are known to mediate inversions, for instance in bacteria [53] and in Drosophila [54,55], but the 250-bp motif did not contain any transposon sequences and lacked repeats.

**MAT inversion correlates with MAT gene expression**

Gene expression was examined in the eight S. sclerotiorum strains 1B331-1 – 1B331-8 of the complete tetrad, and differences in expression of MAT-1-1 and MAT-2-1 were found between Inv- and Inv+ isolates (Figures 6, 7). In each of the four Inv- isolates, there were three MAT-1-2-1 transcript variants, two of which did not encode any known proteins. The third variant encoded an HMG protein, and was the only MAT-1-2-1 gene expression variant. The Inv+ MAT-1-2-1 variant was found in the S. sclerotiorum strain 1980 transcriptome from the recent S. sclerotiorum genome sequencing project [17]. Inv- isolates contained a single MAT-1-1-1 transcript variant encoding an alpha1 protein (Figures 6, 7), but only the 3′-end of the transcript was found in the S. sclerotiorum strain 1980 transcriptome. The four Inv+ isolates had only one of the three MAT-1-2-1 transcript variants of Inv- isolates, and a different MAT-1-1-1 variant. The Inv+ MAT-1-2-1 variant encoded an HMG protein, and the Inv+ MAT-1-1-1 variant encoded a truncated alpha1 protein, lacking all but the first N-terminal 15 alpha1 box residues of the 197 residues that are present in Inv- isolates. As expected, no full-length match to the Inv+ MAT-1-1-1 variant was found in the S. sclerotiorum strain 1980 transcriptome that was Inv-. Reasons for the absence of MAT-1-1-1 expression of S. sclerotiorum were known to mediate inversions, for instance in bacteria [53] and in Drosophila [54,55], but the 250-bp motif did not contain any transposon sequences and lacked repeats.

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**Mating type switching in filamentous fungi**

In several species of filamentous ascomycetes, only half the progeny derived from a selfing parent is able to self, and the other half is self-sterile but is able to outcross to self-fertile strains. Thus, in these filamentous ascomycetes, there is a transition in life style from homothallism to heterothallism that occurs within a single generation, a process known as mating type switching [58] that differs from mating type switching in baker’s yeast, Saccharomyces cerevisiae [28]. Examples of filamentous ascomycetes where mating type switching has been demonstrated include Chromospora spinulosa [21], S. trifoliorum [22] and certain Ceratocystis species [23].

In progeny of C. fimbriata and S. trifoliorum, mating type correlates with ascospore size dimorphism. Heterothallic progeny show a decrease in ascospore size with respect to homothallic progeny [21,40]. Similarly, heterothallic strains of C. fimbriata grow more slowly than homothallic strains [23]. Thus, altered ascospore size or growth rate may represent pleiotropic effects of MAT, similarly to Coniochaeta tetraspora where half of the eight ascospores in each ascus are aborted [59].
Details on mating type switching in filamentous ascomycetes are unknown, but the involvement of a chromosomal inversion has previously been proposed [58]. A MAT inversion region similar to the one in S. sclerotiorum could account for mating type switching and the other phenotypic changes observed in half the progeny of Ch. spinulosa [21], S. trifoliorum [22,40] and in C. fimбриata [23]. Upon inversion, MAT genes that are situated on an inversion breakpoint are truncated, and MAT genes that are near an inversion breakpoint may undergo a change in gene expression, as observed in Drosophila [55]. Since MAT genes are pleiotropic and encode transcription factors that regulate more than 150 target genes, the majority of which have unknown functions [31], altered expression of just one MAT gene may affect multiple phenotypic traits, including mating system, ascospore size and viability, and growth rate. Thus, a MAT inversion region has the potential to act as a switch that regulates different sets of genes depending on its orientation. In the S. sclerotiorum MAT inversion region, the inversion is reversible, as the progeny of both Inv- and Inv+ parents is 50% Inv+. But in other ascomycetes, a one-time transition from Inv- to Inv+ in every meiotic generation would be sufficient to account for the mating type switching and the other phenotypic changes that have been documented. The inversion process may be mediated by inverted, non-repetitive DNA as is present at the inversion breakpoints of the S. sclerotiorum MAT inversion region. Alternatively, transposons are known to mediate inversions, including in bacteria [53] and in Drosophila [54,55].

Molecular data on mating type switching does not contradict involvement of an inversion. In Ceratocystis spp., Witthuhn et al. [60] used HMG box specific PCR primers and found that PCR amplification failed in heterothallic isolates. This would be expected if the HMG box comprised an inversion breakpoint. In Ch. spinulosa, homothallic strains have a MAT1-2-1 adjacent to a MAT1-1 idiomorph, the MAT1-2-1 is missing in that position in heterothallic isolates [61]. There is evidence for inversion breakpoints in homothallic Ch. spinulosa isolates in the form of repeats situated on either side of MAT1-2-1. One of the repeats is present upstream of MAT1-2-1, the other downstream of MAT1-2-1, inside MAT1-1. Since the distance between upstream repeat and MAT1-2-1 is greater than the distance between downstream repeat and MAT1-2-1, inversion of the region between the repeats would increase the distance between MAT1-2-1 and MAT1-1. This could explain why MAT1-2-1 was not detected near MAT1-1 in heterothallic isolates. However, more research on the nature of mating type switching in Ch. spinulosa and other species should include comparisons of genomes and gene transcription profiles between parents and offspring.

**Further research**

Given the importance to agriculture, it is surprising how little is known about sexual processes in S. sclerotiorum. Future research should focus on elucidating when and how the inversion occurs, whether there are differences in target gene expression between Inv- and Inv+ strains, and whether the inversion phenotype plays a role in fertilization and outcrossing.

**Materials and Methods**

**Fungal strains and culture conditions**

This study was based on 283 Sclerotinia sclerotiorum isolates of which 214 were from lettuce in California. The remaining 69 isolates were from nine other substrates (canola, cauliflower, dry bean, field soil, pepper, potato, soybean, sunflower, and tobacco) from twelve states (California, Georgia, Illinois, Kansas, Minnesota, Missouri, North Dakota, Nebraska, Ohio, South Dakota, Washington and Wisconsin), or were part of ordered tetrads obtained under laboratory conditions (Table S1). Fungi were grown on potato dextrose agar medium (PDA) (Sigma-Aldrich, St. Louis, MO) at room temperature. California isolates were derived from sclerotia collected from infected plants. One sclerotium per plant was surface sterilized in 10% bleach for 3 min, plated on PDA, and hyphal tips were transferred to fresh PDA plates to obtain pure cultures. Stocks were maintained as sclerotia at 4°C. Additional strains used in this study were progeny obtained as random ascospore isolates from strains listed in Table S1. Ascospore suspensions were prepared as described by Wu et al. [44], diluted, spread out on PDA, and single germinated ascospores were transferred to new PDA plates by excising the

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**Table 3. Occurrence of Sclerotinia sclerotiorum Inv+ isolates in twelve states and on different hosts in the United States based on PCR screening of all isolates in Table S1.**

| Origin      | Isolates screened | Inv+ isolates* | Hosts of Inv+ isolates | Hosts of Inv- isolates |
|-------------|-------------------|----------------|------------------------|------------------------|
| California  | 220               | 99 (45%)*      | Lettuce, dry bean, cauliflower | Lettuce, pepper |
| Georgia     | 2                 | 1 (50%)        | Canola                  | Canola                |
| Illinois    | 1                 | 0              | -                       | Soybean               |
| Kansas      | 1                 | 0              | -                       | Sunflower             |
| Minnesota   | 1                 | 0              | -                       | Sunflower             |
| Missouri    | 1                 | 1              | Soybean                 | -                     |
| Nebraska    | 26                | 16 (61%)*      | Dry bean                | Dry bean, soybean     |
| North Dakota| 1                 | 1              | Dry bean                | -                     |
| Ohio        | 1                 | 1              | Soybean                 | -                     |
| South Dakota| 1                 | 1              | Soybean                 | -                     |
| Wisconsin   | 1                 | 0              | -                       | Tobacco               |
| Washington  | 15                | 15 (100%)      | Potato                  | -                     |

*Percentage is given when more than one isolate was screened.

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doi:10.1371/journal.pone.0056895.t003
Strains (3) with sequenced MAT region

Strains (2) in contention as tetrad parents

Strains (4) of incomplete tetrad

Strains (8) of complete tetrad

Strain BS011 and progeny (3)

Strain BS013 and progeny (3)

Strain BS017 and progeny (3)

Strain BS028 and progeny (3)

Non-California isolates (7)
Figure 11. Phylogram inferred from 250-bp motifs of 36 *S. sclerotiorum* isolates. The two 250-bp motifs of each of the 36 isolates in Alignment S4 were aligned after the MAT1-1-5 distal motifs were reverse complemented (Figure 3), resulting in a 72 taxa, 250 bp dataset (Alignment S5). Taxon names consist of strain identifier, inversion phenotype, and location of 250-bp motif with ‘left’ referring to MAT1-1-5 proximal, ‘right’ to MAT1-1-5 distal motifs (Figure 3). For sequences deposited or obtained from other sources, database accession numbers are also included. All isolates are listed in Table S1, except the isolates representing the random progeny of *S. sclerotiorum* strains BS011, BS013, BS017 and BS028 which are in Table S6. Groups of isolates are delimited on the right by vertical lines, numbers of isolates in each group are in parentheses. Included isolates were *S. sclerotiorum* strains 44Ba1, 44Ba12, 44Ba18 for which the entire MAT region was sequenced, *S. sclerotiorum* strains BS001 and BS014 that are in contention for parent of the two tetrads, *S. sclerotiorum* strains 18321-2, 18321-4, 18321-6 and 18321-8 of the incomplete tetrad, *S. sclerotiorum* strains 18331-1 – 18331-6 of the complete tetrad, *S. sclerotiorum* strains BS011, BS013, BS017 and BS028 with one Inv- and one Inv+ random progeny each, and the non-California isolates. There are no substitutions in the tree, illustrating that all 250-bp motifs have identical DNA sequences.

Aliquots of PCR products (6μl) were separated on a 1% agarose gel by electrophoresis, gels were stained with ethidium bromide and visualized under a UV trans-illuminator (Ultra-Violet Products Ltd, Cambridge, UK). PCR products were sequenced with the PCR primers and additional internal primers were designed to generate complete sequencing coverage in both directions for all three isolates. Primer sequences are given in Tables S8, S9, and S10. DNA sequencing was performed at the UC Davis DNA Sequencing Facility, using ABI BigDye Terminator v3.1 Cycle Sequencing chemistry on an ABI 3730 Capillary Electrophoresis Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were assembled with Geneious v4.8.5 [64]. The *S. sclerotiorum* strains 44Ba1, 44Ba12 and 44Ba18 MAT locus sequences were deposited in GenBank [JQ815883, JQ815884, and JQ815885, respectively].

Phylogenetic analyses of the four MAT genes were inferred using PAUP v.4.0b 10 [65] using parsimony with default settings including treating gaps as missing data. Alignments were generated in Geneious v4.8.5 [64] invoking CLUSTAL X version 2.1 [66,67], and are available as Alignment S6, Alignment S7, Alignment S8 and Alignment S9. All four alignments contained five taxa, including *B. cinerea* as outgroup, as well as *S. sclerotiorum* strains 1980, 44Ba1, 44Ba12 and 44Ba18.

The 250-bp motif sequence was analyzed for the presence of repeats using REPFIND [68], available at http://zlab.bu.edu/repfind/index.shtml.

**PCR screening for MAT inversion and sequencing of inversion breakpoints**

All *S. sclerotiorum* isolates in Table S1 and the progeny of *S. sclerotiorum* strains BS011, BS013, BS017 and BS028 listed in Table S6 were screened for the presence and absence of the MAT inversion using two sets of PCR primers. The Inv+ specific primer pair Type-IIF / Type-IIR (Table S11) targeted a 1306 bp fragment spanning the MAT1-1-5 proximal inversion breakpoint (Figure S1), and the primer pair MAT1-1-F / MAT1-1-R targeted a 671 bp fragment of the intact MAT1-1-1 of Inv- isolates [69]. Primer binding sites are illustrated in Figure S1. PCR conditions were as described for MAT locus cloning, except that the extension temperature and time were 72°C and 60 sec, respectively. PCR reactions were performed on a Bio-Rad DNA Engine thermocycler (Bio-Rad Laboratories, Hercules, CA). Aliquots of PCR products (6 μl) were separated on a 1% agarose gel by electrophoresis, stained with ethidium bromide and visualized under UV trans-illuminator (Ultra-Violet Products Ltd, Cambridge, UK). Selected PCR products were sequenced to confirm target-specific amplification.

Both inversion breakpoints were PCR amplified and sequenced for the 18 *S. sclerotiorum* strains BS001, BS011, BS013, BS014, BS017, BS028, 1B321-2, 1B321-4, 1B321-6, 1B321-8, and 1B331-1 – 1B331-8 from California (Table S1), and from one Inv- and one Inv+ randomly selected progeny of strains BS011, BS013, BS017 and BS028, respectively (Table S6). Also sequenced were...
Table 4. Apothecia production in 57 *Sclerotinia sclerotiorum* isolates from California.

| Strain identifier | MAT inversion | Apothecium production, % A |
|-------------------|---------------|----------------------------|
| BS001             | Inv−          | 100                        |
| BS002             | Inv−          | 95                         |
| BS003             | Inv−          | 95                         |
| BS009             | Inv+          | 100                        |
| BS010             | Inv−          | 100                        |
| BS011             | Inv−          | 100                        |
| BS012             | Inv−          | 100                        |
| BS013             | Inv+          | 95                         |
| BS014             | Inv+          | 100                        |
| BS015             | Inv+          | 100                        |
| BS016             | Inv+          | 100                        |
| BS017             | Inv−          | 75                         |
| BS018             | Inv+          | 100                        |
| BS019             | Inv+          | 100                        |
| BS020             | Inv+          | 100                        |
| BS021             | Inv+          | 100                        |
| BS022             | Inv+          | 100                        |
| BS023             | Inv+          | 95                         |
| BS024             | Inv+          | 100                        |
| BS025             | Inv+          | 100                        |
| BS026             | Inv+          | 100                        |
| BS027             | Inv+          | 100                        |
| BS028             | Inv+          | 95                         |
| BS030             | Inv+          | 100                        |
| BS031             | Inv+          | 100                        |
| BS032             | Inv−          | 100                        |
| BS033             | Inv−          | 100                        |
| BS034             | Inv−          | 100                        |
| BS035             | Inv−          | 100                        |
| BS040             | Inv+          | 100                        |
| BS041             | Inv+          | 100                        |
| BS042             | Inv+          | 0                          |
| BS047             | Inv+          | 10                         |
| BS050             | Inv+          | 100                        |
| BS051             | Inv+          | 95                         |
| BS055             | Inv+          | 100                        |
| BS057             | Inv+          | 100                        |
| BS058             | Inv+          | 30                         |
| BS061             | Inv−          | 100                        |
| BS062             | Inv+          | 95                         |
| BS063             | Inv+          | 100                        |
| BS064             | Inv+          | 100                        |
| BS065             | Inv−          | 100                        |
| BS066             | Inv−          | 100                        |
| BS067             | Inv+          | 100                        |
| BS068             | Inv+          | 100                        |
| BS070             | Inv+          | 100                        |
| BS076             | Inv−          | 100                        |

*Referring to the proportions of 20 sclerotia that gave rise to fully expanded apothecia over a four week period.

doi:10.1371/journal.pone.0056895.t004

Figure 12. The *S. sclerotiorum* MAT inversion region changes orientation in every meiotic generation and segregates at the first or second division of meiosis. Small ovals are ascospores, large ovals are asci, yellow ascospores are Inv+, red ascospores are Inv-. Single ascospores represent parental isolates, asci containing eight ascospores represent progeny. 12A) Regardless of whether the parent is Inv− or Inv+, 50% of the progeny is Inv+, suggesting a highly regulated process during the sexual cycle ensures the 1:1 ratio. The transition of Inv− to Inv+ involves a change in orientation of the MAT inversion region. 12B) Meiotic segregation pattern of Inv− and Inv+ among ascospores in *S. sclerotiorum*. The pattern that is characteristic for first division segregation in *Neurospora crassa* is shown on the left, the pattern for second division segregation following recombination is shown on the right (42). Figure 12 is based on PCR screens for absence and presence of the MAT inversion region in tetrad and random progeny and their MAT inversion region changes.
1B331-1 (Inv+) and 1B331-3 (Inv-). PCR primers used were SS1f / SS2r, targeting 4.4 kb including the MAT inversion region and the 250-bp motifs, using GoTaq Colorless Master Mix (Promega Corp., Madison, WI, USA) and the following PCR conditions based on Garza et al. [63]: Initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 10 sec, 60°C for 20 sec, and 72°C for 6 min, with a 7 min final extension at 60°C. The resulting PCR bands were cut out from gel, melted in 50 μl PCR quality water, diluted 10 and 100 times, and used as templates in PCR reactions with three overlapping primer pairs, SS1f / SS5r, SS2r / SS2f, SS1 / SS1f, SS3 / SS2r, for S. sclerotiorum strain 1B331-1, and primer pairs SS1f / SS1, SS2r / SS2s, SS3 / SS2r, for S. sclerotiorum strain 1B331-3. The PCR primers, as well as primer SS7 for S. sclerotiorum strain 1B331-1, and primers SS5 and SS6 for S. sclerotiorum strain 1B331-3 were used for DNA sequencing as described above (Table S11).

**MAT gene expression analysis**

The expression of all MAT genes was assessed using primer pairs specific to each gene (Figure 6), in S. sclerotiorum strains 1B331-1, 1B331-2, 1B331-5 and 1B331-6 that are Inv+, and strains 1B331-3, 1B331-4, 1B331-7 and 1B331-8 that are Inv-. The primer pairs for each gene and approximate expected amplicon sizes were as follows. MAT1-1-5: MAT3F / MAT5R (350 bp); MAT1-1-1: MAT1F / MAT1R (250 bp); MAT1-2-4: MAT4F / MAT4R (310 bp); MAT1-2-1: MAT2F / MAT2R (1180 bp); MAT1-1-1 3’-end fragment: FusionF / FusionR (640 bp), and for the actin control (250 bp): F / R (Table S12). First-strand cDNA synthesis was done with DNase treated total RNA using Clontech SMARTer cDNA synthesis kit following the manufacturer’s instructions (Clontech Laboratories, Inc., CA). Double-stranded cDNA for all MAT genes was synthesized using the gene-specific primer pairs listed in Table S12.

When more than one transcript was obtained, PCR products were cloned into pCR2.1-TOPO following the manufacturer’s protocol (Invitrogen Life Technologies, Carlsbad, CA) and sequenced using both vector (M13F / M13R) and gene specific primers (Table S5). Exon-intron boundaries were determined by comparison of transcript and gene sequences using CLUSTALW V2.1 [66].

**Southern blotting of MAT region**

To assess the numbers of MAT loci in S. sclerotiorum genomes, Southern blotting was performed for S. sclerotiorum isolates derived from the ordered tetrad. These were the S. sclerotiorum Inv+ strains 1B331-1, 1B331-2, 1B331-5 and 1B331-6, and the S. sclerotiorum Inv- strains 1B331-3, 1B331-4, 1B331-7 and 1B331-8. Genomic DNA (5 to 8 μg) was digested with BamH1 that had restriction sites upstream, downstream and on the MAT inversion (Figure 8). Digests were run on 0.8% agarose gel and transferred to nylon membrane (Roche, Basel, Switzerland) following Selden [70]. A Southern probe specific to MAT1-2-1 (Figure 8) was generated by PCR using primers MAT1-2-2F / MAT1-2-R [69]. The probe was labeled with digoxigenin (Roche, Basel, Switzerland) according to the manufacturer’s instructions, and hybridization and detection was performed according to the manufacturer instructions (Roche, Basel, Switzerland). X-ray film (Kodak, Rochester, NY) was used and developed following a 30 min exposure. The probe was expected to hybridize to 1.16 and 1.5 kb fragments in Inv- and Inv+ isolates, respectively.

**Generation of the sexual state in culture**

Apothecium formation was evaluated following the method of Wu et al. [44]. Two different groups of isolates were used. The production of fully expanded apothecia was assessed for a group of 57 isolates based on 20 sclerotia per strain (Table 4). The formation of apothecial stalks, an approximate measure of apothecia formation, was assessed for another group of 38 isolates (Table S7), and repeated once for isolates that failed to produce apothecial stalks in the first repetition.

**Isolation of ordered tetrads**

Ordered tetrads were obtained by culturing all ascospores of a single ascus. Ascii were derived from a fully expanded apothecium grown in culture [44] and were released by placing apothecia in 1.5 ml centrifuge tubes, adding sterile water and gentle macerating by forceps. Random ascii containing eight mature ascospores were squeezed from the distal end to release the ascospores using a scalpel, and ascospores were transferred in sequence to individual PDA plates and stored at 4°C. Two tetrads were obtained, a complete tetrad was represented by the eight S. sclerotiorum strains 1B331 – 1B331-8, another tetrad was incomplete and consisted of the four S. sclerotiorum strains 1B321-2, 1B321-4, 1B321-6 and 1B321-8. The numbers following the dashes reflect the ascospore positions inside the ascus. For instance, S. sclerotiorum strain 1B331-1 was derived from the ascospore closest to the tip of the ascus. Tetrads were isolated from two different parental isolates, S. sclerotiorum strains BS001 and BS014. Our lab notes were inconclusive as to which of the two strains was the parent of the tetrads used in this study. Strains BS001 and BS014 were identical at the variable MAT regions that were sequenced and compared (data not shown).

**Mycelial compatibility group assessment**

Mycelial compatibility groups were assessed for 13 S. sclerotiorum isolates (BS001, BS002, BS003, BS011, BS013, BS014, BS017, BS028, BS047, BS058, BS071, BS095, BS096) (Table S1) and their progeny, at least 20 progeny were tested for each parent. Progeny were paired with their parent as described by Wu and Subbarao [3] following the methodology of Schafer and Kohn [71]. Sclerotinia sclerotiorum strains 1B331-1 – 1B331-8 representing the complete ordered tetrad were paired in all possible combinations among one another.

**Supporting Information**

Figure S1  Positions of primers used for MAT inversion screening (MAT1-1F / MAT1-1R, Type-IIF / Type-IIR), and PCR amplification and sequencing of the inversion breakpoints in S. sclerotiorum isolates S1A) without (Inv+) and, S1B) with inversion (Inv+). Genes are boxes, white and dotted boxes correspond to alpha1 and HMG domains, respectively, directions of transcription are indicated by arrows, gene names are inside or by the boxes. Dashed box and arrow represent MAT1-1-1 3’-end fragment lacking an in frame start codon. Primer sites are indicated by half arrows. Diagrams are to scale. The Inv+ alpha1 box is truncated after 45 bp and is not illustrated, for details see text. (TIF)

Table S1  Sclerotinia sclerotiorum isolates used in this study. Given are the isolate identifiers, the host’s scientific and common names, the location and year of collection, the source, as well as the orientation of the MAT inversion. (DOC)

Table S2 MAT gene nucleotide polymorphisms in Sclerotinia sclerotiorum strains 44Ba1, 44Ba12 and...
44Ba18 in comparison to S. sclerotiorum strain 1980 (Amselem et al. 2011).

Table S3 Phylogenetic analyses of Sclerotinia sclerotiorum and Botrytis cinerea MAT genes using maximum parsimony. For each analysis, the number of taxa, alignment length, the number of parsimony informative characters, the number and length of most parsimonious trees (MPT) and the consistency (CI) and retention indices (RI), are given. Respective alignments are available as Alignments S6, S7, S8 and S9.

Table S4 Length comparisons of homologous MAT intergenic spacer regions in Sclerotinia sclerotiorum strains 44Ba1, 44Ba12, 44Ba18 and S. sclerotiorum strain 1980 [17].

Table S5 Top blast matches at GenBank against the 250-bp Sclerotinia sclerotiorum motif query using blastn with e-values equal or smaller than 0.33, only a single representative match for each gene in each species is listed.

Table S6 Orientation of MAT inversion region among progeny of four different Sclerotinia sclerotiorum parental strains, 18 to 20 progeny derived from a single apothecium were screened for each parent. For details on parental isolates, see Table S1.

Table S7 Formation of apothelial stalks in 38 Sclerotinia sclerotiorum isolates, boldface highlights absence of apothecial stalk formation.

Table S8 Primers used for PCR and sequencing of the Sclerotinia sclerotiorum mating type locus in isolates 44Ba1, 44Ba12 and 44Ba18. The last letter in a primer name indicates the primer direction, forward and reverse, respectively.

Table S9 Sequencing primers targeting the MAT inversion in Sclerotinia sclerotiorum strains 44Ba12 and 44Ba18. The last letter in a primer name indicates the primer direction, forward and reverse, respectively.

Table S10 Sequencing primers used in Sclerotinia sclerotiorum strain 44Ba1 targeting the MAT inversion region in S. sclerotiorum strains 44Ba12 and 44Ba18. The last letter in a primer name indicates the primer direction, forward and reverse, respectively.

Table S11 Primer pairs used for Sclerotinia sclerotiorum Inv+ PCR screening, and PCR amplification and DNA sequencing of inversion breakpoints and MAT inversion region. The ‘f’ or ‘r’ in a primer name indicates the primer direction, forward and reverse, respectively.

Table S12 Primers used for Sclerotinia sclerotiorum MAT gene expression analysis.

Alignment S1 FASTA text file with alignment of Sclerotinia sclerotiorum strains 1980, 44Ba1, 44Ba12 and 44Ba18 MAT regions, the MAT1-1-1 and MAT1-2-1 transcript variants, and the MAT inversion region sequences of the tetrad progeny S. sclerotiorum strains 1B331-1 (Inv+) and 1B331-3 (Inv-) obtained with primers SS1f / SS2r. Sequence accession numbers are given as part of sequence names. Indicated are genes, the MAT inversion region and the 250-bp motifs. Sequence accession numbers are given as part of sequence names for sequences in public databases.

Alignment S2 FASTA text file with alignment of Sclerotinia sclerotiorum and Botrytis cinerea MAT1-1 and MAT1-2-1 demonstrating the absence of the 250-bp motifs in B. cinerea MAT1-2-1. Sequence accession numbers are given as part of sequence names.

Alignment S3 FASTA text file with alignment of Sclerotinia sclerotiorum MAT1-1 with Botrytis cinerea MAT1-1 and B. cinerea MAT1-2. The potential crossover site between ancestral MAT1-1 and MAT1-2 is marked and includes the positions where alignment between S. sclerotiorum and B. cinerea MAT1-1 is no longer possible, and a short stretch of B. cinerea MAT1-2 region aligns. Sequence accession numbers are given as part of sequence names.

Alignment S4 FASTA text file with alignment of Sclerotinia sclerotiorum strains 44Ba1 (Inv-), 44Ba12 (Inv+), 44Ba18 (Inv+) and 1980 (Inv-) and inversion breakpoint sequences obtained with primers SS1f, SS1r2, SS2f and SS2r (Table S11, Figure S1) for 32 S. sclerotiorum isolates demonstrating that the 250-bp motifs spanning the inversion breakpoints were identical in all isolates. Positions of strain 44Ba1 MAT1-1-1 and MAT1-2-1 and the 250-bp motifs are indicated. Sequence accession numbers, strain identifiers used in this study (Table S1, Table S6), and primers used to generate each sequence are part of the sequence names. None of the SS1f, SS1r2, SS2f or SS2r sequences was submitted to GenBank.

Alignment S5 FASTA text file with alignment of all 250-bp motifs from Alignment S4, used to generate Figure 11. The MAT1-1-5 distal 250-bp motifs were reverse complemented before alignment. Taxon labels are as in Figure 11.

Alignment S6 FASTA text file with alignment of Botrytis cinerea and Sclerotinia sclerotiorum strain MAT1-1-1 sequences used in phylogenetic analyses for Figure 2. Accession numbers are part of taxon names.

Alignment S7 FASTA text file with alignment of Botrytis cinerea and Sclerotinia sclerotiorum strain MAT1-1-5 sequences used in phylogenetic analyses for Figure 2. Accession numbers are part of taxon names.

Alignment S8 FASTA text file with alignment of Botrytis cinerea and Sclerotinia sclerotiorum strain MAT1-2-1 sequences used in phylogenetic analyses for Figure 2. Accession numbers are part of taxon names.

Alignment S9 FASTA text file with alignment of Botrytis cinerea and Sclerotinia sclerotiorum strain MAT1-2-4 sequences used in phylogenetic analyses for Figure 2. Accession numbers are part of taxon names.
sequences used in phylogenetic analyses for Figure 2. Accession numbers are part of taxon names.

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