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INVESTIGATION

Unconventional Recombination in the Mating Type Locus of Heterothallic Apple Canker Pathogen *Valsa mali*

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ABSTRACT Sexual reproduction in filamentous ascomycetes is controlled by the mating type (*MAT*) locus, including two idiomorphs *MAT1-1* and *MAT1-2*. Understanding the *MAT* locus can provide clues for unveiling the sexual development and virulence factors for fungal pathogens. The genus *Valsa* (Sordariomycetes, Diaporthales) contains many tree pathogens responsible for destructive canker diseases. The sexual stage of these ascomycetes is occasionally observed in nature, and no *MAT* locus has been reported to date. Here, we identified the *MAT* locus of the apple canker pathogen *Valsa mali*, which causes extensive damage, and even death, to trees. *V. mali* is heterothallic in that each isolate carries either the *MAT1-1* or *MAT1-2* idiomorph. However, the *MAT* structure is distinct from that of many other heterothallic fungi in the Sordariomycetes. Two flanking genes, *COX13* and *APN2*, were coopted into the *MAT* locus, possibly by intrachromosomal rearrangement. After the acquisition of foreign genes, unequal recombination occurred between *MAT1-1/2* idiomorphs, resulting in a reverse insertion in the *MAT1-2* idiomorph. Evolutionary analysis showed that the three complete *MAT1-1-2*, *COX13*, and *APN2* genes in this region diverged independently due to different selection pressure. Null hypothesis tests of a 1:1 *MAT* ratio of 86 *V. mali* isolates from four different provinces showed a relatively balanced distribution of the two idiomorphs in the fields. These results provide insights into the evolution of the mating systems in Sordariomycetes.

KEYWORDS
Cytospora sp.
sexual reproduction
MAT
unequal recombination

Sexual reproduction is important in pathogenic ascomycetes because new combinations of virulence alleles are created through outcrossing (McDonald and Linde 2002). Mating in filamentous ascomycetes is typically controlled by a single locus, termed the mating-type (*MAT*) locus, which includes two alleles called *MAT1-1* and *MAT1-2*. These alleles have been termed "idiomorphs" due to their high sequence divergence (Metzenberg and Glass 1990). Filamentous ascomycetes generally exhibit self-incompatible (heterothallic) or self-compatible (homothallic) lifestyles. In heterothallic ascomycete fungi, the *MAT* locus carries either the *MAT1-1* or the *MAT 1-2* idiomorph, which contains at least a *MAT1-1-1* α-domain gene or a *MAT1-2-1* high-mobility-group (HMG) domain gene, respectively. In addition, several additional genes are also found in various ascomycetes, although the functions of these genes remain obscure (Dyer *et al.* 2016). These mating-type genes function not only in controlling sexual development, but also in regulating fungal secondary metabolites and hyphal morphology (Kück and Böhm 2013). Thus, understanding the *MAT* locus can provide insights into the sexual development and virulence factors in filamentous ascomycetes.

The genus *Valsa* (Sordariomycetes, Diaporthales) contains >500 species, including many aggressive tree pathogens responsible for canker diseases. *Valsa* cankers affect >70 species of trees worldwide, and often cause extensive damage to trees (Agrios 2005). *V. mali* Miyabe et Yamada [anamorph *Cytospora sacculus* (Schwein.) Gvrit.], causing destructive canker on apple and resulting in severe yield losses in eastern Asia (Lee *et al.* 2006; Li *et al.* 2013), infects mainly apple by conidia,

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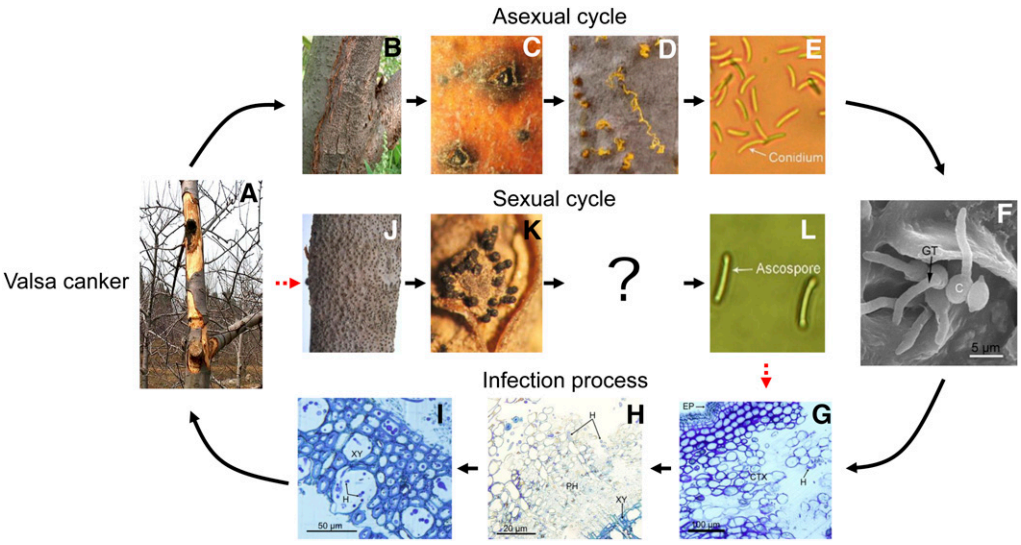

Figure 1 (A–L) Disease cycle of Valsa canker caused by *V. mali*. (A) Lesions on apple bark caused by *V. mali*. The infected bark is scraped. (B) Conidiomata formed in the canker. (C) Pycnidia. (D) Discharged conidia. (E) Conidium. (F) Conidial germination. (G–I) Light micrographs of infected bark. (J) Ascostromata formed in the canker. (K) Ascostromata. (I) Ascospore. The pathogen infects mainly apple by conidia, and sexual reproduction is occasionally observed in nature. Successful infection often occurs on wounded tissues, and infection hyphae develop mainly in the cortex and phloem. C, conidia; CTX, cortex; EP, epidermis; GT, germ tube; H, hyphae; PH, phloem; XY, xylem.

while ascospores are not common (Wang *et al.* 2014; Wang *et al.* 2016) (Figure 1). Of the 150 infected apple bark samples from different regions in China, only eight from dead trunks have ascostromata with ascospores (Wang 2007). In addition, the sexual reproduction of *Valsa* spp. cannot be induced in the laboratory to date, and no *MAT* locus in *Valsa* has been reported. Thus, in this study, we identified and delineated the structure of the *MAT* locus of *V. mali* using comparative genomic approaches, and investigated the evolution of *MAT* genes and loci in *V. mali* and closely related species.

MATERIALS AND METHODS

Strains and culture conditions

All strains used in this study were deposited at the Laboratory of Integrated Management of Plant Diseases in College of Plant Protection, Northwest A&F University, Yangling, PR China. Cultures were grown on potato dextrose agar (PDA) medium with a layer of cellophane at 25°.

Identification of the V. mali MAT locus

The mating type locus of *V. mali* strain 03-8 was identified by BLASTP searches against *V. mali* proteome (>1E−5) using protein sequences of *MAT1-1-1* and *MAT1-2-1* from the closely related species *Cryphonectria parasitica* as query sequences, which suggests that *V. mali* is heterothallic, and that strain 03-8 carries the *MAT1-1* idiomorph. *MAT1-2* candidate isolates were then identified by PCR using a *VmMAT1-1-1* specific primer pair. To identify the *MAT1-2* idiomorph, the genome of isolate SXLC146 was sequenced using Illumina HiSeq technology. Filtered paired-end reads were assembled by ABySS v1.9.0 (Simpson *et al.* 2009), and gene models were predicted using MAKER v2.31.8 (Holt and Yandell 2011). Primer pairs of *VmMAT1-1-1* (F: 5′-GAAAGGTCGGAAAGGCAAAG-3′ and R: 5′-AGAGTCGGGTCGGGCAAT-3′), and *VmMAT1-2-1* (F: 5′-CAACATTGGCATTCAACTCA-3′ and R: 5′-CTTGCTTC GTCGCTTCAC-3′), were used for PCR detection of isolates from different geographic regions.

Evolutionary analyses of the MAT locus

Synteny of the *MAT* locus between *MAT1-1* and *MAT1-2* idiomorphs was analyzed using GATA (Nix and Eisen 2005). Protein sequences of mating type genes were aligned using MAFFT v7.245 (Katoh and Standley 2013), and poorly aligned regions were removed by trimAl v1.4 (Capella-Gutiérrez *et al.* 2009). Maximum likelihood trees were constructed by IQtree v1.3.11 (Nguyen *et al.* 2015), using the build-in best evolutionary model selection function. Branch supports were assessed with ultrafast bootstrap method (Minh *et al.* 2013) and SH-aLRT test (1000 replicates). Selection pressure on mating type genes were tested at the codon level using the ete evol tool in ETE package v3.0 (Huerta-Cepas *et al.* 2016). The coding sequence alignments of these genes were constructed by the ETE package using several build-in alignment tools, and CodeML and Slr analyses were then performed by the ete-evol program. Sites under selection were identified using the M2 and SLR models. The null hypothesis of a 1:1 *MAT* ratio of *V. mali* was tested using chi-square goodness-of-fit test using the online tool VassarStats (http://vassarstats.net/).

Transmission electron microscopy (TEM)

The perithecium, ascus, and ascospore of *V. mali* in the field were investigated by TEM. Ascostromata samples from the canker were processed for TEM as described by Ke *et al.* (2013). For TEM, ultrathin sections of specimens cut with a diamond knife were collected on copper grids. After contrasting with uranyl acetate and lead citrate, the grids were examined with a TEM 1230 (JEOL) at 80 kV.

Data availability

All strains used in this study are available upon request. The raw Illumina reads of isolate SXLC146 have been deposited at the Sequence Read Archive (SRA) database of NCBI (SRP075864). The nucleotide sequence of *MAT1-2* idiomorph has been deposited at the GenBank database (KX349090).

RESULTS AND DISCUSSION

Identification of MAT1-1 idiomorph

To identify the mating type locus in *V. mali*, protein sequences of core mating type genes *MAT1-1-1* (GenBank: AAK83346) and *MAT1-2-1* (AAK83343) of the closely related species *C. parasitica* were used to search against the genome of *V. mali* strain 03-8 (GCA_000818155.1) (Yin *et al.* 2015). By performing BLASTP searches, only *MAT1-1-1* was

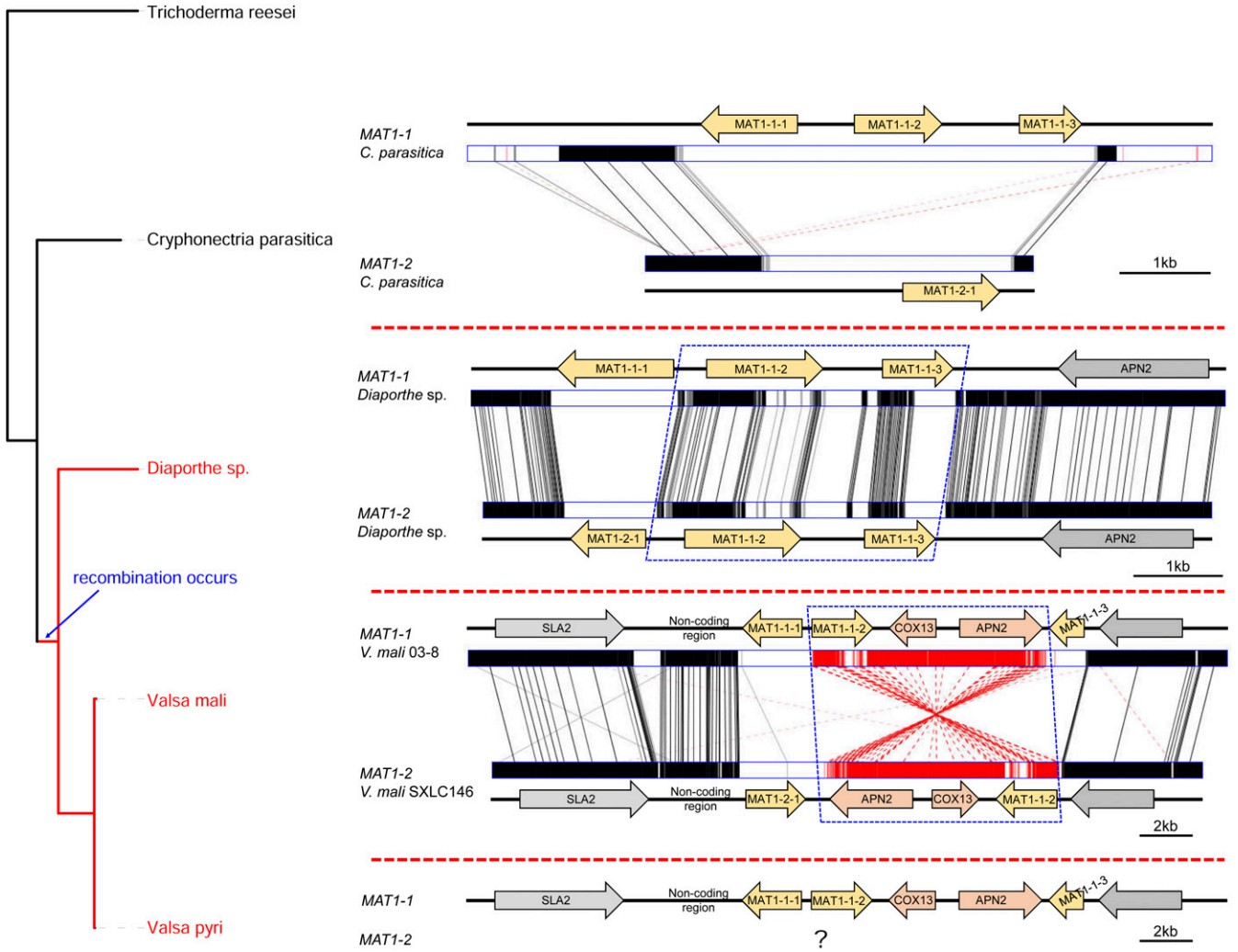

Figure 2 Mating type idiomorphs of *Valsa* spp. and closely related species. The phylogenetic tree on the left side was constructed on the basis of *MAT1-1-1*. Sequences of *C. parasitica* and *Diaporthe* spp. were retrieved from GenBank according to McGuire *et al.* (2001) and Kanematsu *et al.* (2007), respectively. The blue dashed box indicates recombination region.

found in strain 03-8. The absence of the *MAT1-2-1* HMG-box gene in the *MAT* locus of strain 03-8 suggests that *V. mali* is likely heterothallic. *VmMAT1-1-1* (VM1G_08160) contains the typical α-domains, and adjacent genes include *SLA2* (VM1G_08159), *MAT1-1-2* (VM1G_08161), *COX13* (VM1G_08162), *APN2* (VM1G_08163), and *MAT1-1-3* (VM1G_08164) (Figure 2). Intriguingly, *COX13* and *APN2* locate in the *MAT* locus, while the location of *SLA2* and *APN2* is fairly conserved, and often flanks the idiomorph among other mating type genes in many other filamentous ascomycetes (Dyer *et al.* 2016), such as *C. parasitica* (McGuire *et al.* 2001). Likewise, idiomorphs of two heterothallic species *Coccidioides immitis* (Fraser *et al.* 2007) and *Uncinocarpus reesii* (Mandel *et al.* 2007) also captured *COX13* and *APN2* into the *MAT* locus, while both these genes are adjacent to the *MAT* locus in closely related species. However, the role or influence of this kind of remodeling remains unknown.

As a heterothallic species, there must be *MAT1-2* isolates of *V. mali*. However, *MAT1-1* and *MAT1-2* isolates of filamentous ascomycetes are morphologically indistinguishable for most of their life cycles (Debuchy *et al.* 2010). To identify *MAT1-2* isolates, a specific primer pair for *VmMAT1-1-1* (F: 5′-GAAAGGTCGGAAAGGCAAAG-3′ and R: 5′-AGAGTCGGGTCGGGCAAT-3′) was used to detect *V. mali* isolates. Isolate SXLC146, without a PCR product, was then used to identify the *MAT1-2* idiomorph.

Identification of the MAT1-2 idiomorph

To determine the structure of the *MAT1-2* idiomorph, the genome of isolate SXLC146 was sequenced *de novo* by Illumina HiSeq-PE150 platform. A total of 20,860,413 clean reads (5.2G, effective rate 96.46%) were subjected to assembly. Nucleotide sequences of genes

Table 1 ***MAT1-1/MAT1-2*** **ratio tests on populations of** ***V. mali***

Population	Total Number	*MAT1-1*	*MAT1-2*	χ^2, *P* Value
Baoji, Shaanxi	10	4	6	0.4, *P* = 0.7518
Yuncheng, Shanxi	24	9	15	1.5, *P* = 0.3078
Tianshui, Gansu	13	6	7	0.08, *P* = 1
Sanmenxia, Henan	30	12	18	1.2, *P* = 0.3594
All isolates	86	35	51	2.98, *P* = 0.1055

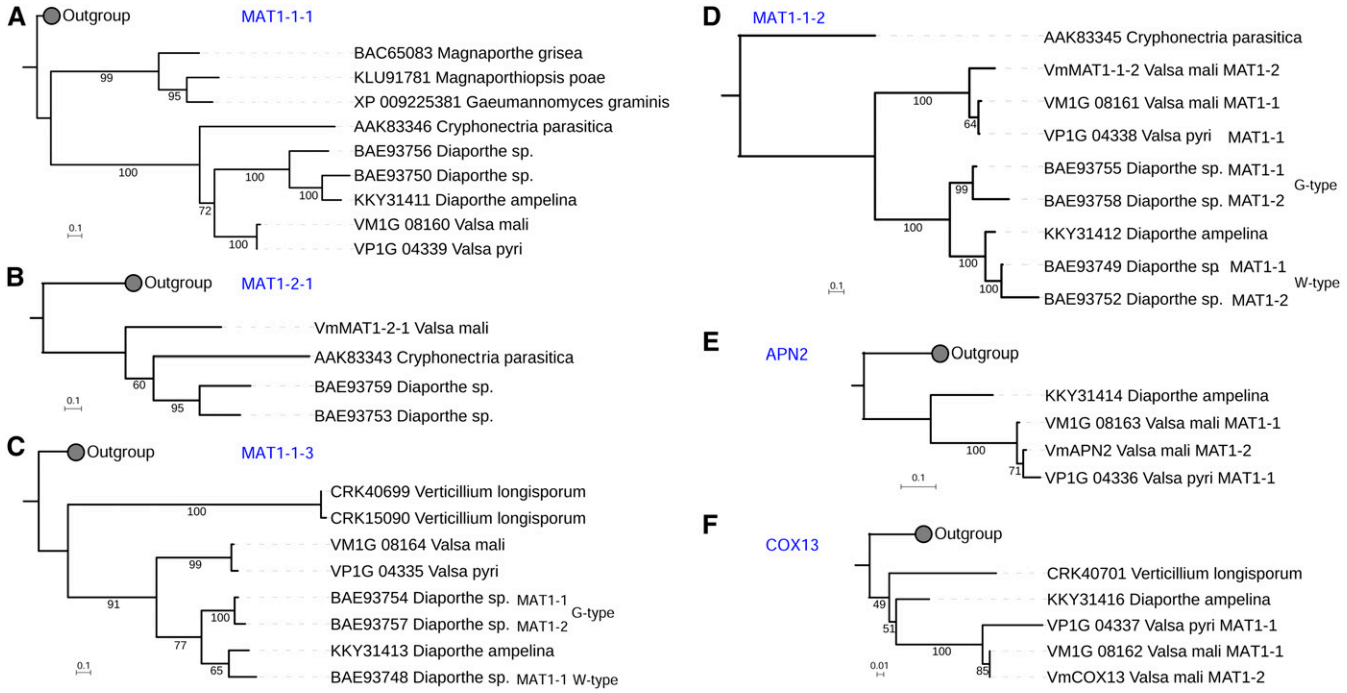

Figure 3 Phylogeny of mating type genes: (A) *MAT1-1-1*, (B) *MAT1-2-1*, (C) *MAT1-1-3*, (D) *MAT1-1-2*, (E) *APN2*, (F) *COX13*. Maximum likelihood phylogenetic trees were constructed from top 20 BLASTP hits in GenBank using IQtree. The scale bar represents substitutions per site.

flanking the *VmMAT1-1* idiomorph were used to perform BLASTN searches against genome assemblies of isolate SXLC146. Gene models of the scaffold that contains the *MAT1-2* idiomorph were then predicted using MAKER v2.31.8 (Holt and Yandell 2011). *VmMAT1-2-1* (GenBank: KX349090) contains the HMG-box, and adjacent genes includes *SLA2*, *APN2*, *COX13*, and *MAT1-1-2* (Figure 2). Similar to the *VmMAT1-1* idiomorph, the *VmMAT1-2* idiomorph also captured *APN2* and *COX13* into the *MAT* locus. The *MAT* locus organization indicates that *V. mali* is heterothallic. Unexpectedly, a mating type gene *MAT1-1-2* of the *MAT1-1* idiomorph is present in the *MAT1-2* idiomorph. *MAT1-1-2* is ubiquitous in Sordariomycetes, and is required for fruit body development (Debuchy *et al.* 2010). However, the involvement of *MAT1-1-2* (especially in the *MAT1-2* idiomorph) in sexual development of *V. mali* remains unknown.

In order to test the null hypothesis of a 1:1 *MAT* ratio of *V. mali*, 86 isolates from four different provinces were detection by PCR using two pairs of specific primers targeting *VmMAT1-1-1* and *VmMAT1-2-1*, respectively (Supplemental Material, Table S1). Both idiomorphs were present in the four provinces, and their *MAT* ratios did not deviate significantly from 1:1 (Table 1), which suggests a relatively balanced distribution of mating-type idiomorphs in the fields.

Unconventional recombination of MAT locus in V. mali

Recombination at the *MAT* locus in ascomycetes is thought to be suppressed (Idnurm 2011). However, synteny analysis showed that the region carrying *APN2*, *COX13*, and *MAT1-1-2* in the *MAT1-2* idiomorph is a reverse insertion, probably acquired from the *MAT1-1* idiomorph by recombination (Figure 2). Additionally, *MAT1-1-2* contains many more sequence variations than *APN2* and *COX13*. Protein sequence identity of these three genes are 100% (*COX13*), 92.13% (*APN2*), and 76.89% (*MAT1-1-2*), respectively. A similar event was also reported in the closely related species *Diaporthe* spp., the *MAT1-2*

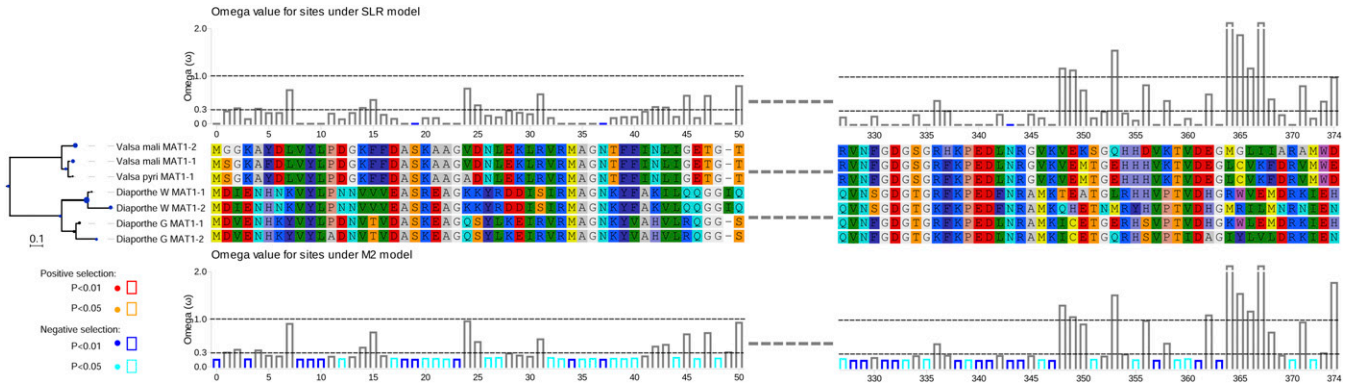

Figure 4 Evolutionary analysis of *MAT1-1-2*. The CodeML and Slr analyses were performed using the ete evol tool in ETE package. Site models M2 and SLR, and branch model fb, respectively, were used. Omega value of branch is represented in the node size and color. Small blue disk on the node of phylogenetic tree stands for low omega value.

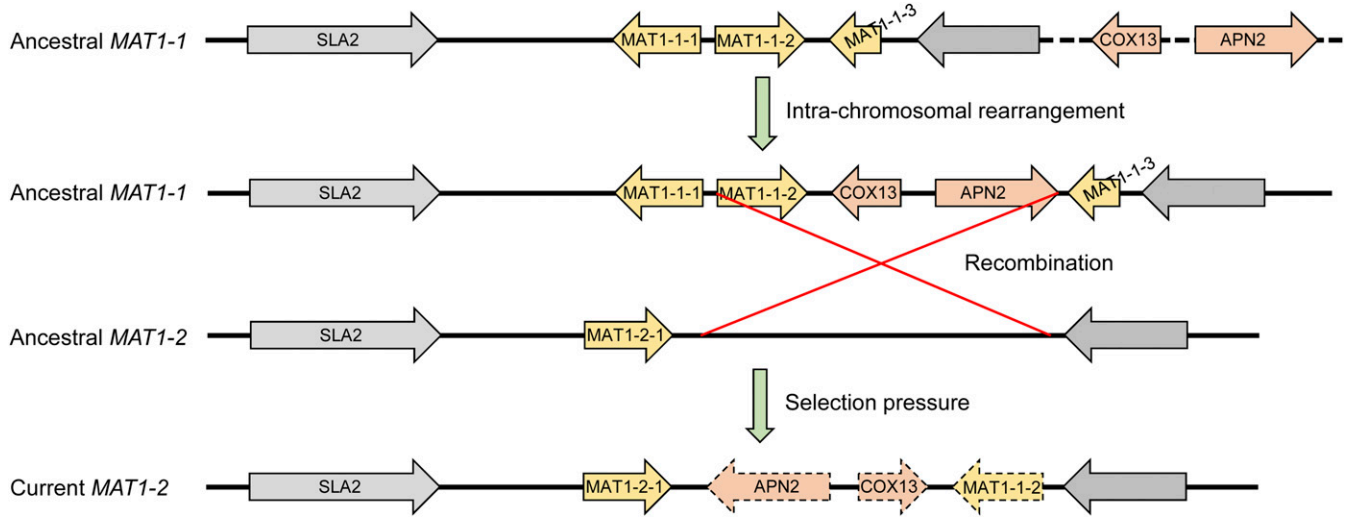

Figure 5 Proposed evolution scenario of *MAT* locus in the ancestor of *Valsa mali*.

idiomorph of which carries homologs of *MAT1-1-2* (identity: 80.95%) and *MAT1-1-3* (54.62%) in the same gene order and orientation as that in *MAT1-1* (Kanematsu *et al.* 2007) (Figure 2). These unconserved "additional" mating type genes, caused by unequal recombination, are probably also functional, because they are transcriptionally active during vegetative growth (Kanematsu *et al.* 2007). Nevertheless, future work is required to determine the functions of these genes in sexual reproduction.

Unequal recombination at the *MAT* locus has also been demonstrated in many other ascomycetes, but genes involved in those events are often fragments or truncated pseudogenes (Tsui *et al.* 2013). In *V. mali*, unequal recombination resulted in the presence of three compete genes in *MAT1-2*. The types of *MAT* structure in *Valsa* spp. (including *V. malicola*, *V. sordida*, and *V. persoonii*, Z. Yin and L. Huang, unpublished results) and *Diaporthe* spp. are unconventional

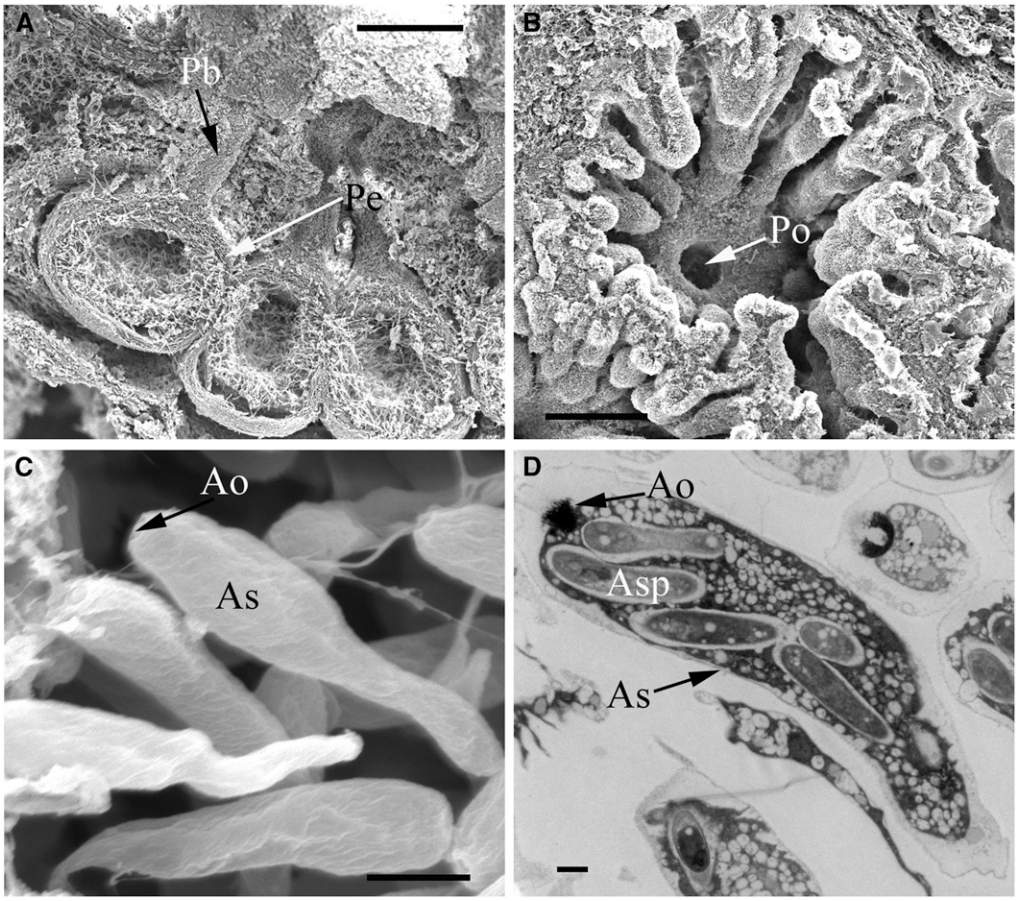

Figure 6 Scanning electron micrographs and TEM graphs of the perithecium, ascus, and ascospore of *V. mali*. (A) longitudinal section of the perithecium (Pe) with a single cavity and beak (Pb) under apple bark. (B) Transverse section of perithecium (Pe) showed a single orifice (Po) on the perithecial beak. (C) Clavate ascus (As) in perithecium with ascus orifice (Ao). (D) TEM graph of asci (As) with ascospores (Asp). Bars, (A) 200 μm; (B) 200 μm; (C) 5 μm; (D) 1 μm.

and distinct from known heterothallic filamentous ascomycetes. In addition, another closely related heterothallic species, *C. parasitica*, which causes chestnut blight, has the typical *MAT* structure in heterothallic Sordariomycetes, carrying three genes, *MAT1-1-1*, *MAT1-1-2*, and *MAT1-1-3* in the *MAT1-1* idiomorph and only one gene, *MAT1-2-1*, in the *MAT1-2* idiomorph (McGuire *et al.* 2001) (Figure 2). This finding suggests that this unconventional recombination occurred evolutionally from specific families in Diaporthales. Given that homothallism has likely evolved from heterothallic ascomycetes (Billiard *et al.* 2011; Dyer *et al.* 2016), the *MAT* structures of *V. mali* and *Diaporthe* spp. will provide clues for unveiling the evolutionary history of the mating systems in Sordariomycetes. However, to confirm this hypothesis, it is necessary to determine the *MAT* loci of additional species (especially homothallic) in Diaporthales.

Evolutionary analyses of mating type genes

The same *MAT* structure in *V. mali* and *V. pyri*, as well as the scenario in *Diaporthe* spp., indicates that recombination predates speciation (Figure 2). The three foreign genes (*APN2*, *COX13*, and *MAT1-1-2*) in the *MAT1-2* idiomorph were unlikely to have been acquired independently, and probably diverged independently. We were thus interested in the phylogeny of these genes. For each mating type gene of *V. mali*, the protein sequences of the top 20 blast hits in the GenBank nr database were aligned, and subjected to maximum likelihood phylogenetic tree construction. The tree of the *COX13* gene shows that this gene in the *VmMAT1-2* idiomorph is more closely related to the homolog in *VmMAT1-1* than to that in the *MAT1-1* idiomorph of *V. pyri* (Figure 3), which suggests that *COX13* was acquired before the divergence of *Valsa* species. Likewise, the *MAT1-1-2* and *MAT1-1-3* genes are present in both idiomorphs of the *Diaporthe* sp. group within G-type and W-type species, respectively. The *VmCOX13* genes are highly conserved in both idiomorphs, and ancestral *VmAPN2* and *VmMAT1-1-2* genes are thus likely to have the same phylogenetic relationship as *VmCOX13*. However, the *VmMAT1-1-1* gene in *MAT1-1* is closely related to that in *MAT1-1* of *V. pyri*, while *VmAPN2* in *MAT1-2* groups with that in *MAT1-1* of *V. pyri* (Figure 3). This result suggests that the *APN2*, *COX13*, and *MAT1-1-2* genes in the different idiomorphs diverged independently after acquisition.

Selection pressure analysis of the three acquired genes showed that *MAT1-1-2* has been under purifying selection at interspecific level, indicating that this gene is preserved for proper function (Figure 4). Many sites of *MAT1-1-2* under purifying selection are likely responsible for the dramatically divergence. In contrast, one site (60E) in the nuclease domain of *APN2* is under positive selection, while the *COX13* gene may go through neutral evolution without any site under positive or purifying selection. Thus, we can speculate that the different selection pressure of these three genes results in different levels of sequence divergence. Collectively, a possible scenario of the evolution of *MAT* loci in *Valsa* spp. is that ancestral *MAT1-1* first coopted *APN2* and *COX13* into the *MAT* locus by intrachromosomal rearrangement, and the ancestral *MAT1-2* then acquired *MAT1-1-2*, *COX13* and *APN2* by unequal recombination; finally these three genes diverged independently due to different selection pressure (Figure 5).

Cryptic sexuality

In nature, sexual reproduction of *V. mali* is occasionally observed, often on dead apple trunks in autumn. The matured ascostromata could be identified on apple barks by the exposed surface, which has many minute black papillae (Figure 1J). Each papilla has an opening of a long neck connected with a perithecium. A longitudinal section of the ascostroma shows that spherical or subglobose perithecia are partly immersed in bark (Figure 6A). When fully matured, the cavity of the perithecium is closely packed with eight asci, which are formed from the basal inner wall of the perithecium. The asci are clavate-oblong or clavate-fusiform, rounded or truncate at the apex, and subsessile (Figure 6C). Ascospores are produced in sausage-shaped asci (Figure 6D) (Ideta 1909; Wang *et al.* 2011), and discharged into the air during wet weather (Agrios 2005).

Attempts to induce self-fertilization of *V. mali* in the laboratory failed. Mycelial plugs of two isolates with opposite mating types (03-8 and SXLC146) were placed at opposite sides of a sterile apple twig embedded on agar according to successful mating tests in *C. parasitica* (Marra and Milgroom 2001) and *Diaporthe* spp. (Kanematsu *et al.* 2000). However, selfing of *C. parasitica* and *Diaporthe* spp. is still a rare event in the laboratory, in that only some cross assays succeed (Kanematsu *et al.* 2000; Marra and Milgroom 2001). In addition, mature perithecia were more likely to develop successfully in crosses between isolates derived from a single ascus naturally formed on host twigs, compared to a randomly selected ascus (Kanematsu *et al.* 2000). Therefore, it is necessary to test more isolates of *V. mali*, especially isolates from the same ascus.

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