Comparative Genomics of the Mating-Type Loci of the Mushroom *Flammulina velutipes* Reveals Widespread Synteny and Recent Inversions

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Abstract

**Background:** Mating-type loci of mushroom fungi contain master regulatory genes that control recognition between compatible nuclei, maintenance of compatible nuclei as heterokaryons, and fertilizing body development. Regions near mating-type loci in fungi often show adapted recombination, facilitating the generation of novel mating types and reducing the production of self-compatible mating types. Compared to other fungi, mushroom fungi have complex mating-type systems, showing both loci with redundant function (subloci) and subloci with many alleles. The genomic organization of mating-type loci has been solved in very few mushroom species, which complicates proper interpretation of mating-type evolution and use of those genes in breeding programs.

**Methodology/Principal Findings:** We report a complete genetic structure of the mating-type loci from the tetrapolar, edible mushroom *Flammulina velutipes* mating type A3B3. Two matA3 subloci, matA3a that contains a unique pheromone and matA3b, were mapped 177 Kb apart on scaffold 1. The matA locus of *F. velutipes* contains three homeodomain genes distributed over 73 Kb distant matA3a and matA3b subloci. The conserved matA region in Agaricales approaches 350 Kb and contains conserved recombination hotspots showing major rearrangements in *F. velutipes* and *Schizophyllum commune*. Important evolutionary differences were indicated; separation of the matA subloci in *F. velutipes* was diverged from the Coprinopsis cinerea arrangement via two large inversions whereas separation in *S. commune* emerged through transposition of gene clusters.

**Conclusions/Significance:** In our study we determined that the Agaricales have very large scale synteny at matA (~350 Kb) and that this synteny is maintained even when parts of this region are separated through chromosomal rearrangements. Four conserved recombination hotspots allow reshuffling of large fragments of this region. Next to this, it was revealed that large distance subloci can exist in matB as well. Finally, the genes that were linked to specific mating types will serve as molecular markers in breeding.

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Introduction

The genes that regulate mating in fungi encode sets of regulatory and signaling molecules that are broadly distributed in eukaryotes. The corresponding pathways, which are often linked to pathogenicity and fruiting body formation, comparably control developmental processes in other organisms, such as pattern formation in development and sexual differentiation in animals. Studies in Agaricomycetes, that include many important mushroom-forming fungi, therefore also inform our collective understanding of cellular development in eukaryotes. Mushroom forming fungi are further important sources for food (mushrooms, fermentation), medicine (pathogens, fibres, health-promoting, anti-cancer products) and green technologies (waste recycling, fertilizers, bioremediation). As such they represent a massive economical asset and better understanding of their sexual propagation is desirable.

Mating is the beginning step in sexual development of mushroom-forming fungi. Their life cycle is characterized by haploid as well as diploid stages. Sexual, haploid spores that are dispersed by a mushroom develop into monokaryotic mycelia. Upon fusion of two genetically different monokaryons a dikaryotic mycelium is established and the different nuclei of the two mating partners coexist within the cells of the dikaryon during vegetative
growth and production of fruiting bodies. Only upon maturation of the mushroom, the different nuclei fuse in specialized reproductive cells termed basidia and new haploid spores with separate mating-types are created (for a review see [1]). The heterothallic fungi restrict their self-mating by use of one (bipolar species) or two (tetrapolar species) incompatibility loci. This system supports out-breeding and helps to promote genetic variability in populations.

Our current knowledge on the molecular genetics of mating in mushrooms is primarily based on studies in the model organisms Schizopyllum commune and Coprinus cinereus (≡ Coprinus cinereus). The mating-type loci in those species are termed A and B and control different developmental pathways that are required to maintain a fertile dikaryon (for reviews see [2,3]). Each mating-type locus consists of tightly linked subloci and encodes multiallelic genes [4,5,6]. These alleles are highly polymorphic at DNA and amino acid (AA) levels [7,8,9] due to balancing selection that favors mating-type alleles that become rare and thus extends the coalescence time between alleles [10]. The A and B subloci are functionally redundant and heterozygosity at a single sublocus is sufficient to activate the respective A or B pathway.

A mating-type loci encode pairs of divergently transcribed homeodomain genes (HD genes) and are typically accompanied by the Mitochondrial Intermediate Peptidase gene [MIP] and a Beta-fanking gene [11,12]. HD proteins are distinguished based on conserved DNA binding motifs; homeodomain 1 (HD1) and homeodomain 2 (HD2). HD1 proteins further contain two nuclear localization signals, an activation domain and only weakly bind DNA [13,14] whereas HD2 proteins lack these domains but have strong DNA binding properties [15,16]. Both HD1 and HD2 proteins possess N-terminal dimerization motifs that facilitate their interaction. Interaction of HD1 with a compatible HD2 protein generates a heterodimer that serves as a transcription factor for the A pathway [15,17].

The B mating-type loci are comprised of pheromone receptors and pheromones (recently reviewed in [3]) and each pheromone receptor is accompanied by one or several pheromones in a sublocus [9,18]. Pheromone genes encode small precursor proteins with C-terminal CAXX motifs (C = cysteine, A = aliphatic and X is any residue) that are farnesylated. They usually contain an acidic AA pair as well (ER or EH in C. cinereus) about 10–15 AA from the C-terminus. These acidic residues are highly conserved in C. cinereus and have been speculated to be the site of proteolytic cleavage [5,19,20,21,22]. They are also conserved in other basidiomycete pheromones, though not in all [23,24]. After farnesylation and proteolytic splicing, fungal pheromones typically constitute about 9–11 amino acid peptides [19,25].

Fungal pheromone receptors are classified within the Rhodopin-like superfamily and typically contain seven membrane spanning regions (7-TM). They are further characterized by a short N-terminal extracellular domain and a long cytoplasmic C-terminal tail [26]. The cytoplasmic domains are presumed to dock trimeric G-proteins that can activate the downstream B pathway after phosphorylation [26]. Phosphorylation of G-proteins is triggered by interaction of specific pheromones with extracellular pheromone receptor domains.

The fundamental composition of the A and B mating-type loci was found to be strongly conserved within basidiomycetes. The redundant subloci are a result of doubling during evolution and tetrapolar mating systems were found in all three major lineages, suggesting an ancient origin [27,28]. Moreover, bipolar and tetrapolar species have been shown to contain essentially the same genes [24,29]. On the other hand, the new availability of genome sequences reveals significant variability within mating-type loci of basidiomycetes and their numbers of pheromone receptor, pheromone and homeodomain encoding genes differ greatly between species [12,30,31,32]. To this, a new mating-type system that is not strictly bipolar or tetrapolar was discovered [30].

Flammulina velutipes, also known as Winter Mushroom and Enokitake is one of the major cultivated mushrooms in Asia. Beyond having a tetrapolar mating system with multiple alleles, the genetics of its mating-type system have remained unknown. We decided to elucidate the genetic structure of this important mushroom to map the mating-type genes and use comparative genomics to understand evolutionary relevant distinctions of the F. velutipes loci, as well as to implement this knowledge in our mushroom breeding programs. We obtained a complete map of the mating-type genes from F. velutipes KACC42780, identified the specific matA and matB loci and explain some of the events that caused the significant deviation of the matA region in comparison to the model species. Mating-type defining genes are currently used to construct haploid, monokaryotic, mushroom producing strains and primers for PCR based mating-type identification.

Materials and Methods

Strains and culture conditions

Flammulina velutipes strains were cultivated at 25°C on 100×20 mm dishes (SPL, South Korea) containing Potato Dextrose Agar (HIMEDIA Laboratories, India) for two weeks. Stocks were transferred to fresh PDA dishes every three months, sealed (Clean Wrap) and stored at 4°C after three to five days growth (storage up to one year). For genomic DNA isolation, PDA was covered with cellophane (general household) prior to inoculation. For mating experiments, small dishes (60×15 mm, SPL) were inoculated with agar plugs (5×5 mm) at 0.5 cm from the center and grown for one week. Fresh plugs from the center of the plate that contained merged mycelia were subcultured for another four to eight days prior to clamp formation analysis. Mushroom cultivation in small bottle cultures was started with mating of two monokaryons as described above. Confirmed dikaryons were grown on a 30 mm layer of sterilized sawdust (Douglas Fir)/wheat bran (Rice) mixture (4:1) in 25×45 mm glass bottles (three mycelial plugs/bottle). After 10 days incubation at 25°C the surface of colonized wood was scraped off and bottles were filled with cold distilled water for 1 hr. Following upside down drying for 30 min bottles were incubated at 15°C, 95% humidity for three weeks. Spores were collected by placing the caps of the mushrooms in small dishes for one day and eluted in sterilized water prior to plating on PDA. Strains used in this study were KACC42780 (A3B3), KACC43777 (A4B4) and dikaryon KACC43778 (A3B3A4B4) available under Korean Agricultural Culture Collection (KACC), RDA, Korea. Monokaryons for segregation studies were all derived from dikaryon KACC43778. For analysis of matB genes in F. velutipes strains from different geographical locations we used laboratory stock strains 4004-32 & 4004-23 (Korea); 4031-04 & 4031-10 (Korea, commercial strain Paengi-2); 4028-34 & 4028-38 (Taiwan); 4015-19 & 4015-11 (Japan); 4017-06 & 4017-05 (Korea); 4006-04 & 4006-01 (Korea); and 4023-01, 4023-05, 4023-29 & 4023-32, F. velutipes var. longispora (USA).

Genomic DNA extraction

For isolation of genomic DNA, 400 μl of extraction buffer (100 mM NaCl, 30 mM EDTA, 0.25 M Tris-HCl, 5% SDS), 400 μl of 2x CTAB buffer (2% CTAB, 100 mM Tris-Hcl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 1% polyvinyl pyrrolidone) and 500 μl phenol-chloroform-isooamylalcohol (25:24:1, Bioneer,
South Korea) were added to 0.1–0.5 g of lyophilized or fresh mycelium and briefly vortexed. After 5 min incubation at room temperature (RT), samples were centrifuged at 13,000 rpm, 4°C for 5 min. The supernatant was mixed with 0.7 volumes isopropanol and centrifuged for 10 min, 4°C. After washing with 70% EtOH, air dried samples were eluted in 50–100 μl TE and treated with RNase A (Bio Basic Inc., Canada) for 30 min at 60°C.

**Southern hybridization**

DNA probes (500bp) were amplified by PCR (primers; Table S2). Genomic DNA was digested to completion with SacI at 37°C, overnight. Agarose gels (0.8%) containing the digested gDNA were soaked in 0.25 M HCl for 20 min for depurination, 30 min in Denaturation buffer (0.5 M NaOH, 1.5 M NaCl pH 7.5) and 10 min in Neutralization buffer (0.5 M Tris–HCl pH 7.5, 1.5 M NaCl). DNA was transferred to a nylon membrane (Amersham) via capillary transfer with 10x SSC (1.5 M NaCl, 0.15 M sodium citrate) for 20 hrs and membranes were baked at 80°C [33]. Pre-hybridization and hybridization were done in a hybridization solution (5x SSC, 50% formamide, 5x Denhardt’s solution, 0.5% sodiumdodecylsulfate and 25 mg/ml denatured salmon sperm DNA). Pre-hybridization was performed for 3 hrs and hybridization was continued for 24 hrs with a probe labeled with [α-32P] dCTP (Ladderman TM Labeling kit, Takara, Japan) at 42°C. The membrane was exposed to an imaging screen (Fuji) for 10 hrs and DNA bands were visualized using a personal molecular imager system (Bio-Rad, USA).

**PCR analyses and sequencing**

PCRs were performed using GoTaq flexi kit (Promega Korea Ltd, South Korea) with specific primers (Bioneer, South Korea) for each gene (Table S2). Thermal cycling parameters consisted of 10 min denaturation at 94°C, 30 cycles of 94°C, 30 s denaturation, 30 s annealing (temperature 2°C lower than the specific, lowest melting temperature of primers used), 30 s extension at 72°C and a 10 min final extension at 72°C. Samples for sequencing were amplified with a BigDye Terminator cycle sequencing kit (Applied Biosystems, USA), precipitated with 0.1 volume 3 M Sodium Acetate (pH 5.8) and 2.5 volumes 100% EtOH for 10 min at ~70°C and centrifuged for 15 min, 15000rpm at 4°C. Pellets were washed with 70% EtOH, air dried and eluted in Hi-Di Formamide (Applied Biosystems, USA) and analyzed on an ABI3730 DNA Analyzer.

**Identification of pheromone receptors, pheromone precursors and CLA4**

A draft genome sequence of *Flammulina velutipes* monokaryotic strain KACC42780 was screened for the presence of pheromone receptors by tblastx searches with the sequence of pheromone receptor gene Bar1 of *Schizosaccharomyces pombe* (National Center for Biotechnology Information (NCBI) Genbank accession number X77949.1), Reb1 of *Phallocrypta nameko* (AB201119.1) and Reb3 of *Coprinus cinereus* (CAA71962). The genome sequence of *F. velutipes* will become available at the Agricultural Genome information Center, RDA (http://10.30.100.11:8000/; [34] and TMHMM Server v.2.0 (CBS, Denmark, http://www.cbs.dtu.dk/services/TMHMM-2.0/; [35]). All FvSTE3 containing contigs were fully screened for pheromone precursors. Open reading frames between 40 and 100 amino acids were manually analyzed for C-terminal CAAX motifs and screened using Pfam 24.0, http://pfam.janelia.org/, [36]. CLA4 was identified in *F. velutipes* using CLA4 of *Phaeolus djamor* (Genbank AF126786.1), *C. cinereus* (J6V) A22_2-1 (NCBI Genbank X796871), *C. cinereus* a42_d1-1 (NCBI Genbank X796881). P. djamor a2-2 (NCBI Genbank KY462112.1), P. djamor a1-2 (NCBI Genbank KY462121.1), S. commune a_alpha_Y4 (EMBL-Bank M97181). Additional searches were performed with MIP proteins of *P. djamor* (NCBI Genbank KY462112.1) and *P. nameko* (NCBI Genbank BA435542.1). Gene models for FvHD1-1, FvHD2-1 and FvHD2-2 were manually annotated. Predicted proteins for FvHD1-1, FvHD2-1 and FvHD2-2 were analyzed using COILS to identify dimerization motifs [37] http://www.ch.embnet.org/software/COILS_form.html, WoLF PSORT to determine nuclear localization domains [38] http://wolfsort.org/ and 9aaTAD [39,40] http://www.es.embnet.org/Servers/EMBlactAT/hrdoc/9aatad/ to search for transactivation domains. NCBI Genbank accession numbers for the new *F. velutipes* genes are: FvHD1-1/FvHD2-2/HQ630588; FvHD2-1/MIP=HQ630589.

**Analysis of the Mat A locus**

Tblastn searches were performed against a draft genome of *F. velutipes* (see above), the genome of *S. commune* strain H4-8, available at DOE Joint Genome Institute (http://genome.jgi-psf.org/School1/School1.home.html) and the genome of *Laccaria bicolor* S238N-H82 (available under the *Coprinus cinereus* project) using predicted proteins that surround the A mating-type locus of *C. cinerea* okayama 7#130, available under the *Coprinus cinereus* sequencing project of the BROAD institute of MIT (http://www.broadinstitute.org/annotation/genome/coprinus_cinereus/GenomesIndex.html). Gene homologues that were located on F. velutipes contigs Fv01174, Fv03236 and Fv02632 were used for comparison in Chromomapper 1.0,10.26 [41].

**Phylogenetic analyses**

Pheromone receptor sequences of *L. bicolor* were obtained from the Joint Genome Institute [JGI] website according to Niculita-Hirzel et al. [31]. Sequences of *S. commune*, *P. djamor*, *C. cinereus* and *Cryptococcus neoformans* were obtained from NCBI Genbank using accession numbers as reported by James et al. (Figure 7, in [42]). Sequences of *F. velutipes* were obtained as described above. The protein sequences of pheromone receptors were aligned using ClustalW 1.64 [43] in combination with Gblocks 0.91b [44] to eliminate remaining poorly aligned sequences with a setting ‘allow smaller final blocks’. Ambiguously aligned regions were excluded from the protein sequences of pheromone receptors in the phylogenetic analyses. Maximum likelihood analyses were conducted using RAxML 7.04 [45]. A model of PROTGAMMA-WAGF was selected with an analysis of ProTest 2.4 [46] and incorporated in the analysis. Branch values from 1,000 nonparametric bootstrap replications were used for nodal support [47]. In this study, nodes were considered strongly supported when
Results

Identification of pheromone receptor genes and pheromone precursors

A draft genome of Flammulina velutipes monokaryotic strain KACC42780 (A3B3) was screened for pheromone receptor homologues and CLA4, a kinase gene shown to be linked to the B locus in other mushroom species [12]. Six contigs, five with a single and one with two pheromone receptor homologues were retrieved. CLA4 was found on a separate contig (Table S1). Pheromone receptor genes were annotated and named FvSTE3.1, FvSTE3.2 and FvSTE3.s one to five (FvSTE3.s1 to FvSTE3.s5), where ‘s’ stands for ‘similar’ to distinguish non mating-type specific pheromone receptors (see below). Repeated searches with the newly identified genes did not uncover additional pheromone receptors. Six predicted pheromone receptor proteins contained the seven-transmembrane domains that are characteristic for fungal pheromone receptors [26]. Alignments of the pheromone receptor proteins showed that the sequence of FvSte3.s2 differed in the region that corresponded with the first transmembrane receptor proteins showed that the sequence of FvSte3.s2 deviated from the seven-transmembrane domains that are characteristic for pheromone receptors (see below). Moreover, seven amino acids were deleted in this protein from a highly conserved motif, truncating its transmembrane sequence (not shown). FvSte3.s2 grouped specifically with FvSte3.s1 in maximum likelihood analyses (not shown), shared 60% base pair identity and was separated from gene FvSTE3.s1 by only 6.7 Kb. All together, this suggests that FvSTE3.s2 is a pseudogene that was derived as a copy from FvSTE3.s1. Three pheromone precursor genes named FvPP1, FvPP2 and FvPP3 were identified on contigs with pheromone receptors. FvPP1 was located 2102bp apart from pheromone receptor FvSTE3.1 and FvPP2 and FvPP3 flanked pheromone receptor FvSTE3.2 at distances of 320bp and 427bp respectively (Figure 1). Notably, FvPp1 contained a C-terminal Tryptophan (W) behind its CAAX-box (CAAXW). All three FvPp proteins were classified as fungal pheromones according to Pfam 24.0 searches [36] with E-values of 0.0013, 0.45 and 0.035 for FvPp1, FvPp2 and FvPp3. Alignment of the FvPp proteins showed that FvPp2 (54 amino acids) and FvPp3 (46 amino acids) shared 55% and 54% reciprocal similarity in contrast to FvPp1 (54 amino acids) with 37%. The three proteins contained a glutamic acid/arginine (E/R) motif at amino acid position 13/14 (FvPp1) and 15/16 (FvPp2, FvPp3) counted from the C-terminus (Figure S1). These residues are conserved in various basidiomycetes and have been speculated to be the site of proteolytic cleavage (see introduction). Proteolytic splicing at the E/R sites, together with C-terminal processing, would result in peptides of nine (FvPp1) and 11 amino acids (FvPp2 and FvPp3) which corresponds well with the size of other fungal pheromones [19,25].

Segregation analysis of FvSTE3 and FvPP genes

In order to designate the pheromone receptor and pheromone precursor genes to specific matB loci, their distribution was analyzed in dikaryon KACC43778, parental strains KACC43777 (A4B4), KACC42780 (A3B3) and two monokaryotic siblings B2 (A3B4) and B27 (A4B3). PCR with specific primers (Table S2) detected FvSTE3.1 and FvPP1 explicitly in matB3 strains. Pheromone receptors FvSTE3.2, FvSTE3.1 to FvSTE3.s5 and pheromone precursors FvPP2 and FvPP3 were detected in matB3 and matB4 mating-types (Figure 2). Correct amplification and PCR patterns of FvSTE3 and FvPP genes were confirmed by sequencing and Southern analysis (Figure 2). Analysis of 16 additional monokaryons with different mating-types showed that the observed distribution was persistent (Table 1). Monokaryons were derived as single spore colonies from dikaryon KACC43778 and mating-types were assigned based on clamp formation patterns (material and methods). Sequence alignment of the PCR products for each gene that were obtained from the 16 strains revealed two differing copies of genes FvSTE3.1, FvSTE3.2 and FvSTE3.3. This enabled segregation analysis through restriction fragment polymorphism of FvSTE3.1 and FvSTE3.2. Subtype FvSTE3.3.1-a was invariably detected together with FvSTE3.2-d while FvSTE3.3.1-b was linked with FvSTE3.2-c. Segregation of the couples was independent from the analyzed mating-type loci (Table 1). Segregation of FvPP3, FvPP2 to FvPP2 and FvPP3 remained undetermined. Taken together, the results show that only a single pheromone receptor and pheromone segregate specifically with the B3 mating-type in F. velutipes KACC42780.

Polymorphism of pheromone receptors

Mating-type specific genes in fungi are characterized by highly polymorphic alleles. We examined the distribution and polymorphism of the FvSTE3 genes in various F. velutipes strains, from different countries (materials and methods). Genomic DNA, of two compatible monokaryons for each strain, was analyzed by PCR with specific primers (Table S2). FvSTE3.1, FvSTE3.2, FvSTE3.3 and FvSTE3.4 were frequently amplified with six, five, seven and products out of 10 strains. FvSTE3.1, FvSTE3.2 and FvSTE3.5 were not detected outside the control (Table 2). New primer sets (Table S2, additional sets) resulted in frequent amplification of FvSTE3.5 but FvSTE3.1 and FvSTE3.2 remained undetected. Sequences for the amplified genes shared respectively 92–99 percent base pair similarity (Table 2). Low polymorphism of FvSTE3.1 to FvSTE3.3 showed that those genes are not mating-type specific pheromone receptors; their amino acid sequences are identical. The absence of FvSTE3.1 and FvSTE3.2 from all tested F. velutipes strains, especially in comparison with the other pheromone receptors, clearly indicates polymorphic alleles for those two genes and therefore supports a mating-type specific role.
Phylogenetic analyses of pheromone receptors

Maximum likelihood analyses based on the protein sequences of *F. velutipes* pheromone receptors and that of other basidiomycete species indicated a division into two distinct clades (Figure 3, Clade A and B). The four pheromone receptors FvSte3.1, FvSte3.s3, FvSte3.s4 and FvSte3.s5 formed a separate, strongly supported clade within clade B (Figure 3, purple shaded). FvSte3.2 was also grouped in clade B with strong support, together with pheromone receptor ScBbr2 from *S. commune*. In turn, they were strongly grouped with CcRcb2 and LbSte3.1 from *C. cinerea* and *L. bicolor* (Figure 3, yellow shaded). ScBbr2, CcRcb2 and LbSte3.1 are all mating-type specific pheromone receptors [18,31]. FvSte3.1 was a

Figure 2. Distribution of pheromone receptors and pheromones in matB3 and matB4 loci. Southern blots confirmed the PCR distribution patterns of the pheromone receptors and pheromones in dikaryon KACC43778; A384; (DK), strain KACC43777; A484; (B18), KACC 42780; A383, (B20) and two monokaryotic siblings 4019-B2; A384, (B2) and 4019-B27; A483, (B27). Pheromone receptor FvSTE3.1 and pheromone FvPP1 are exclusively detected in strains containing the matB3 locus (B20 and B27), and as a single copy in the dikaryon (DK). The small, equally sized signals for FvSTE3.s4 on the Southern blot are caused by an internal SacI restriction site in this gene. PCR detection patterns for FvSTE3.1, FvSTE3.2 and FvSTE3.s1 are inserted as panels in the bottom of the Southern analyses. Strains that contained a copy of a gene invariably generated a specific PCR product. No false products were amplified with our primers. Pheromone receptor FvSTE3.2, FvSTE3.s1 to FvSTE3.s5 and pheromone receptors FvPP2 and FvPP3 were detected in matB3 and matB4 mating-types.

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Table 1. Distribution of pheromone precursors, pheromone receptors and homeodomain genes in *F. velutipes* monokaryotic siblings.

| Strain 4019- | DK | B10 | B11 | B13 | B17 | B21 | B22 | B23 | B25 | B28 | B30 | B34 | B35 | B36 | B38 |
|-------------|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A mating-type | A3A4 | A4 | A4 | A3 | A4 | A4 | A3 | A4 | A3 | A3 | A3 | A4 | A4 | A3 | A4 |
| B mating-type | B3B4 | B4 | B3 | B3 | B3 | B3 | B4 | B4 | B3 | B3 | B4 | B4 | B3 | B4 | B4 |

Gene:
- FvSTE3.1
- FvSTE3.2
- FvSTE3.s1
- FvSTE3.s2
- FvSTE3.s3
- FvSTE3.s4
- FvSTE3.s5
- Fvpp1
- Fvpp2
- Fvpp3
- FvHD1-1
- FvHD2-1
- FvHD2-2

Distribution of pheromone receptors and pheromones was analyzed in monokaryotic F1 progeny from dikaryon KACC43778 (DK). Presence of genes is indicated by '+' and absence by '-'.

FvSTE3.s1 and FvSTE3.s2 show the distribution of their respective, non digested PCR products. Their subtypes, genes that were identified in sequence analysis of FvSTE3.s1 and FvSTE3.s2 are RFLP s1-a (a), RFLP s1-b (b), RFLP s2-c (c) and RFLP s2-d (d). Subtypes were detected by restriction length polymorphism analysis of PCR products. The table shows specific linkage of pheromone receptor FvSTE3.1 and pheromone FvPP1 with the mat B3 locus. Copies of FvSTE3.s1 and FvSTE3.s2 recombine in pairs but independent from the mating-type loci; FvSTE3.s1-a is coupled to FvSTE3.s2-d and FvSTE3.s1-b is coupled to FvSTE3.s2-c. Homeodomain genes FvHD2-1 and FvHD2-2 co-segregate specifically with the matA3 locus. Segregation of FvSTE3.2, FvSTE3.s3, FvSTE3.s4, FvSTE3.s5, FvHD1-1, FvPP2 and FvPP3 could not be determined. Copies of those genes were detected in all mating-types.

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Table 2. Presence of pheromone receptors in different *F. velutipes* strains of wide geographical distribution.

| Strain | FvSTE3.1 | FvSTE3.2 | FvSTE3.s1 | FvSTE3.s2 | FvSTE3.s3 | FvSTE3.s4 | FvSTE3.s5 |
|--------|----------|----------|-----------|-----------|-----------|-----------|-----------|
| DK     | +        | +        | +         | +         | +         | +         | +         |
| 4004 23| –        | –        | –         | +         | +         | +         | +         |
| 4004 32| –        | –        | +         | +         | +         | +         | +         |
| 4031 4 | –        | –        | +         | –         | –         | –         | +         |
| 4031 10| –        | –        | –         | –         | +         | –         | –         |
| 4028 34| –        | –        | +         | +         | +         | +         | +         |
| 4028 38| –        | –        | –         | –         | +         | –         | –         |
| 4023 23| –        | –        | +         | –         | +         | +         | –         |
| 4023 29| –        | –        | –         | –         | +         | +         | –         |
| 4023 1  | –        | –        | –         | –         | +         | +         | –         |
| 4023 5  | –        | –        | +         | –         | +         | +         | –         |

Similarity: 93–97% 92–98% 97–98% 93–99% 96–99%

The table shows a clear distinction in distribution between FvSTE3.1, FvSTE3.2 and other pheromone receptors from strain KACC42780 when analyzed in geographically distant *F. velutipes* strains. Presence was determined by PCR analysis on genomic DNA of two compatible mating types of each strain. Locations of the strains were as follows: 4004 – 23, 4004 – 32, Korea; 4031 – 4, 4031 – 10, Korea; 4028 – 34, 4028 – 38, Taiwan; 4023 – 1, 4023 – 5 & 4023 – 23, 4023 – 29, *F. velutipes* var. *longispora*, USA. FvSTE3.1 and FvSTE3.2 are uniquely amplified from the control, dikaryon KACC43778. All other pheromone receptors are regularly amplified from geographically distant *F. velutipes* strains. Sequence similarity of PCR products for each regularly amplified pheromone receptor was high showing that they are non mating-type specific. Base pair similarity is shown in percentage in the bottom line.

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close relative of ScBbr1 from *S. commune* in clade A and both were strongly grouped with LbSte3.2 and LbSte3.5 of *L. bicolor* which, except for LbSte3.5, are mating-type specific pheromone receptors as well. Close grouping of FvSte3.1 and FvSte3.2 with other known mating-type specific pheromone receptors formed a strong indication that they were also mating-type specific pheromone receptors. Interestingly, ScBBR1 and ScBBR2 are alleles at the same *matB* locus of *S. commune* [18,23].

Structure of the *matB3* locus

The pheromone receptor and pheromone precursor genes together with *CLA4* were mapped based on linkage of their respective contigs to a draft genome of *F. velutipes* KACC42780. The *MatB3a* locus containing *FvSTE3.1* and *FvPP1* was located on scaffold 1 and flanked upstream by *FvSTE3.s3* and *FvSTE3.s5*, and downstream by *FvSTE3.2*, *FvPP2* and *FvPP3* (Figure 1). Notably, the pheromone receptors were almost evenly spaced by 177 Kb, 181 Kb and 184 Kb which was considerably more distant than has been reported for other basidiomycetes [31,32]. Localization of the genes on the same fragment of scaffold 1 suggested that they might be part of the *matB3* locus. *FvSTE3.3* and *FvSTE3.s2* were mapped on scaffold 26 and *FvSTE3.s4* on scaffold 29 (not shown). *CLA4* was located at 0.98 Mb and 1.16 Mb distance from the borders of scaffold 8 (not shown). This demonstrated that *FvSTE3.3*, *FvSTE3.4*, *FvSTE3.4* and *CLA4* were not linked to the *matB3* locus.

Identification of homeodomain genes

One *F. velutipes* HD1 gene (*FvHD1-1*), two HD2 genes (*FvHD2-1*, *FvHD2-2*) and the Mitochondrial Intermediate Peptidase (*MIP*) gene were identified in the *F. velutipes* genome located on a single contig; Fv01174 [Table S1]. *MIP* was included because this gene is closely linked to HD genes in all Agaricomycetes [11,12]. Analysis of the *FvHD2-1* and *FvHD2-2* gene models (accession codes in materials and methods) showed intron-exon distributions and long C-terminal exons similar to that of homeodomain gene *a2-1* and *b2-1* of *C. cinerea* [14]. The first and the second intron interrupted the homeodomain at the exact same locations in *F. velutipes* and *C. cinerea*. The second introns have been reported to be conserved in several other basidiomycetes as well [7,14,48,49]. *FvHD1-1* (six predicted introns) showed no intron-exon resemblance to *C. cinerea* HD1 genes associated with *a2-1* and *b2-1*. No reliable coiled coils (less than 20–30% drops in the probability between weighted/non-weighted analysis) that could indicate dimerization motifs were found in the *F. velutipes* HD genes but nine-amino acid transactivation domains were detected in the N-termini of *FvHd1-1*, *FvHd2-1* and *FvHd2-2* (Table S3). These domains are generally found in mammalian and yeast transcrip-

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**Figure 3. Phylogenetic tree of mating-type specific and non mating-type specific pheromone receptor protein sequences.** The tree shows phylogeny amongst pheromone receptor proteins from *F. velutipes* (Fv), *C. cinerea* (Cc), *L. bicolor* (Lb), *S. commune* (Sc), *P. djamor* (Pd) and *C. neoformans* (Cn). Nodal supports with more than 70% bootstrap values are considered strongly supported and displayed in the tree. Known mating-type specific pheromone receptors are depicted in blue. Two major clades are distinguished, labeled A and B. The four non mating-type specific pheromone receptors of *F. velutipes* FvSte3.3 to FvSte3.5 (pseudogene FvSTE3.s2 was excluded) form a separate group (shaded purple) within clade B that is supported by strong branch values. The clade including FvSte3.1 and two other known mating-type specific pheromone receptors is shaded in orange. The clade that contains FvSte3.2 is shaded in yellow. Both these clades are supported by strong branch values. FvSte3.1 and FvSte3.2 group closest with SCBbr1 and SCBbr2, respectively. Clades that contain known mating-type specific pheromone receptors are strong evidence for mating-type specificity of other clade members. LbSte3.5 is a notable exception.

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tion factors [40]. FvHd1-1, FvHd2-1 and FvHd2-2 were further predicted to contain single and or bipartite nuclear localization signals (Table S3).

**Segregation of FvHD genes**

In order to link the FvHD genes to specific matA loci we determined their distribution in matA3 and matA4 mating-types. Specific primers for $FvHD2-1$, $FvHD2-2$ and $FvHD1-1$ were designed for exon regions that flanked the conserved homeodomain (Table S2). $FvHD2-1$ and $FvHD2-2$ showed specific linkage to the matA3 locus whereas $FvHD1-1$ was detected both in strains with matA3 and matA4 loci (Table 1). PCR products (average 450bp) obtained from matA3 and matA4 strains with $FvHD1-1$ specific primers, showed 97% base pair similarity and 100% amino acid identity (no polymorphism) demonstrating presence of a copy instead of different alleles. This means that the matA3 locus lacks a specific HD1 gene in comparison with matA4.

**Structure of the matA locus**

*MIP* was located 201bp distant from $FvHD2-1$ which is in contrast to most known basidiomycetes where *MIP* is directly flanked by a HD1 gene that is part of a HD1/HD2 couple [12]. Detailed screening of the sequence adjacent to *MIP* and $FvHD2-1$ did not reveal a $FvHD1$ gene but instead, a hypothetical gene with no orthologues near matA subloci in *L. bicolor* (373Kb distant), *C. cinerea* (different chromosome) and *S. commune* (absent). Gene $FvHD1-1$ and $FvHD2-2$ that are 199bp apart, showed divergent, outward transcription directions (Figure 5) which is typical for matA loci whereas $FvHD1-1$ was detected both in strains with matA3 and matA4 loci (Table 1). PCR products (average 450bp) obtained from matA3 and matA4 strains with $FvHD1-1$ specific primers, showed 97% base pair similarity and 100% amino acid identity (no polymorphism) demonstrating presence of a copy instead of different alleles. This means that the matA3 locus lacks a specific HD1 gene in comparison with matA4.

**Synteny of matA regions**

To unravel the events that altered the organization of the matA region of *F. velutipes* we mapped synteny of 200 successive genes surrounding the matA locus of *C. cinerea* (chromosome 1, bp2471986-2934538) with that of *L. bicolor*, *F. velutipes* and *S. commune*. The latter was included because this fungus also has separated matA subloci and is taxonomically closer to *F. velutipes* than *C. cinerea* and *L. bicolor* [50]. Since there had been no annotation of the *F. velutipes* genome, *C. cinerea* genes were acknowledged as “syntenic” in *F. velutipes* when similar protein sequences with expect values equal to or smaller than 10$^{-9}$ were obtained by tblastn. Loss of syntenic genes in the matA region from *C. cinerea* was indicated by 11 unique genes, and 21 (*L. bicolor*), 17 (*F. velutipes*) and 21 (*S. commune*) genes from *C. cinerea* that were not detected in one or two of these species. The selected *C. cinerea* region covered the matA containing *F. velutipes* contig Fv01174 (304 Kb) as well as parts of contig Fv02632 and Fv03236, the last which was found to contain the missing *Beta-flanking gene*. The three contigs were linked to scaffold 3 (3.3 Mb) of the *F. velutipes* draft genome where Fv01174 was flanked upstream at 2.1 Kb by Fv03236 and downstream by Fv02632 at 26 Kb (Figure 4). Our analysis identified synteny between *C. cinerea* (Cc) and *L. bicolor* (Lb) is shown in Figure 4. Syntenic mapping of the matA regions from *F. velutipes* (Fv), *C. cinerea* (Cc) and *S. commune* (Sc) in Chromomapper [41] reveals significant differences in gene arrangement. Distances are shown in kilo base pair (Kb) and are indicated by blue and purple lines respectively. Dashed light purple lines in panel A) are conserved sites of recombination in all four species. The gene order in *F. velutipes* and *S. commune* is changed by different events. *F. velutipes* shows many inversions of gene clusters when compared to *C. cinerea*. *S. commune* shows rearrangements of larger, different gene groups. The overall gene orders of *F. velutipes* and *S. commune* are very similar to that of *C. cinerea* and *L. bicolor* which strongly suggests that these latter models represents an ancestral organization.

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Figure 5. Gene order near the matA locus of *F. velutipes* before and after inversion. A detailed overview of the synteny map in Figure 4, shows the individual genes of the matA loci from *F. velutipes* and *C. cinerea*. Homeodomain gene FvHD2-1 (green), the Beta flanking gene (BFG, purple) and the Mitochondrial Intermediate Peptidase (MIP, blue) are presently separated, flanking both sides of the FvHD1-1/FvHD2-2 gene couple (bottom gene bar). The top gene bar shows the ancestral gene order in *F. velutipes*. Apart from a different number of HD genes, the matA locus is identical to that of *C. cinerea*. The synteny map clearly demonstrates that two inversions have caused separation of the matA subloci in *F. velutipes*. Several additional inversions are indicated in the ancestral *F. velutipes* locus. Notably, FvHD2-2 and FvHD1-1 maintained their position during all changes.

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cinerea and *L. bicolor* over a remarkably large segment of 350 Kb (Figure 4, panel A), much larger than previously demonstrated [31]. In addition, we identified the boundaries of this syntenic segment, which are clearly denoted by genes from *C. cinerea* that have no local homologue in *L. bicolor*, were differently distributed in *L. bicolor*, or were inverted (Figure 4, panel A). Those boundaries coincide with the ends of the syntenic part of the matA region from *F. velutipes* (Figure 4). The matA region in *F. velutipes* showed highest synteny over the first 250 Kb of the 350 Kb segment and somewhat lower in the last 100 Kb (Figure 4, black line, 250 Kb; start to red mark, 100 Kb; red mark to end). Though the specific gene order of *F. velutipes* in comparison to *C. cinerea* was changed by inversions, the overall gene order as found in *C. cinerea* was shown to be strongly preserved. None of the inverted gene groups was translocated (Figure 4). In *S. commune* the syntenic 350 Kb matA region was also recovered, albeit in three different sections with 140 Kb and 200 Kb interval distances (Figure 4, purple and dashed light purple lines). Synteny was highest in the two segments corresponding to the first 250 Kb of the 350 Kb matA region and lower for the third fragment, resembling *F. velutipes*. Moreover, synteny of the fragments in *S. commune* ended at the same relative locations that represented the synteny boundaries in *C. cinerea*, *L. bicolor* and *F. velutipes*. Finally, the gene order within the three segments was strongly conserved, indicating a high level of gene conservation in this entire region for all Agaricomycetes. Inside the 350 Kb segment, two spots showed high recombination in all four species. The first spot was the matA locus itself, the second spot formed a small gap in synteny located between *C. cinerea* genes CC1G_01873.3 and CC1G_01877.3 (both hypothetical genes) and their respective orthologues. Exemplary, one of the few genes that was repositioned in *L. bicolor* in comparison to *C. cinerea* was inserted in this gap. In *F. velutipes* and *S. commune* gene clusters were separated by inversion or translocation at both of these locations (Figure 4, 5, 6). Detailed comparison of the *F. velutipes* gene order to that of *C. cinerea* showed that the matA locus of *F. velutipes* was separated by inversion of two 70 Kb fragments directly left and right of FvHD1-1 and FvHD2-2 (Figure 4, 5). Modeling a reversion of those clusters reunited the Beta-flanking gene, FvHD2-1, MIP, FvHD1-1 and FvHD2-2 in a similar distribution as found in *C. cinerea* and *L. bicolor*. Notably, FvHD2-2 and FvHD1-1 were never moved during the rearrangements (Figure 5). Synteny mapping also revealed important differences between the matA regions of *F. velutipes* and *S. commune*. First, all *S. commune* homeodomain genes were repositioned, whereas FvHD2-2 and FvHD1-1 retained their position during rearrangements. This shows that the respective matA loci were split between different genes. Second, the *S. commune* fragments representing the first part of the high syntenic 350 Kb region, and whose rearrangement caused separation of the matA subloci, constituted 140 Kb and 260 Kb (Figure 4, purple lines). This was significantly larger than the 70 Kb in *F. velutipes* (Figure 4, blue lines) and shows that different fragments were rearranged. Third, the *S. commune* fragments were wedged by sections not syntenic to the 350 Kb *C. cinerea* matA region (Figure 4, dotted light purple lines), which was not observed in *F. velutipes*.

To specify the events that split the *S. commune* matA locus we extended the synteny map between *C. cinerea* and *S. commune* in both directions, using on average each next 5th gene on the *C. cinerea* genome (Figure 6). Model A (Figure 6A) showed highest similarity to the gene order in *C. cinerea*, with all major *S. commune* gene clusters in an equal orientation. Model B (Figure 6B) with the *S. commune* chromosome in an opposite orientation, showed inversion of all but two major gene clusters. Both models indicated transposition of two (model A) or three (model B) high syntenic fragments corresponding to the 350 Kb region (Figure 6, blue and purple lines respectively). In addition, the two transposed fragments in model A were exchanged in order in comparison to *C. cinerea*. Repositioning of the two fragments representing the high syntenic 250 Kb region (discussed above) in model A, between the genes flanking the 350 Kb region and the genes representing the last 100 Kb of the 350 Kb region on the other side (Figure 6A, C, black lines), reconstituted a matA subloci, with all major *S. commune* gene clusters in an equal orientation. Model B clearly indicated inversions as a cause for separation of the *S. commune* matA subloci. Indeed reversion of the genes underscored by the first (390 Kb) and second (210 Kb) purple line reunited the matA subloci (Figure 6D). However, the *S. commune* matA region that resulted from those inversions still contained a large non syntenic fragment (Figure 6, red dotted line) and differed considerably in gene order from *C. cinerea*.
Discussion

With the fast growing range of sequenced genomes, new information of mating-type genes quickly becomes available. However, complete analyses of the genetic structure of mating-type loci in mushroom forming fungi remain rare. Our study focused on the genetic structure of the mating-type loci from *Flammulina velutipes* (Winter Mushroom or Enoki). This led to the finding of several characteristics for *F. velutipes* as well as new facts that will help gaining insight in mating-type locus evolution, especially in Agaricales.

The *B* mating-type genes

Three pheromone precursors were identified near pheromone receptors in *F. velutipes*. As we limited our screen for pheromones to the six contigs that contained pheromone receptors (total 446 Kb), more pheromones can be expected to be found in the genome [31]. However, those pheromones will not be closely associated with the pheromone receptors and are unlikely to perform a mating-type specific role. *FvPp1*, the only pheromone precursor that was specifically linked to the matB3 mating type is unique, ending with a tryptophan (W) after the CAAX-box. We suggest that this extra tryptophan originates from the former STOP codon (Stop = TAA, TAG or TGA). A single base pair substitution would suffice to change TAG or TGA to TGG (TGG = W). It is uncertain if the additional tryptophan impairs pheromone processing; yet deviant farnesylation signals have previously been demonstrated to be functional [23].

We identified two mating-type specific pheromone receptors (accompanying pheromones, phylogenetetic grouping with other mating-type specific pheromone receptors and high sequence polymorphism) named *FvSTE3.1* and *FvSTE3.2*, which represented two of the three fungal pheromone receptors families. No genes of the third pheromone receptor family were detected in the matB3 strain, though this family might be expected in other *F. velutipes* matB loci. The identification of multiple non mating-type specific pheromone receptors in *F. velutipes* is in line with recent discoveries in *L. bicolor*, *C. cinerea* and *S. commune* [3,31,32]. We demonstrated a clear distinction between the two pheromone receptor types based on phylogenetic distribution and sequence polymorphism. Different sequence polymorphism indicates that these genes are subjected to different selection mechanisms. Somewhere in evolution, non mating-type specific pheromone receptors must have been functionally and selectively separated from mating-type specific ones. At the moment it is uncertain if non mating-type specific pheromone receptors are functional and what role they perform in the fungus. At least, their role is not mating-type specific (this study, [31,32]).

Structure of the matB locus

Mating-type specific pheromone receptor *FvSTE3.2*, together with pheromone precursor *FvPp2* and *FvPp3*, was positioned in the same region of scaffold 1 as the matB3a locus containing *FvSTE3.1* and *FvPp1* (Figure 3). Arguably, *FvSTE3.2* and the accompanying pheromone precursors comprise a functional
second sublocus (yet our strain is just homozygous) since this specific receptor was not detected in any of the *F. velutipes* strains from different locations. The 177 Kb distance between both subloci makes the matB locus of *F. velutipes* exceptionally large in comparison to other higher basidiomycetes [29,31,32]. To this, non mating-type specific pheromone receptors have been demonstrated to be linked to matB loci [3,32] meaning that *FvSTE3.s3* and *FvSTE3.s5* could be part of matB3 as well. This would increase the matB3 locus to over 500 Kb. Identification of additional *F. velutipes* matB loci should reveal if this large distance is consistent in *F. velutipes* and if *FvSTE3.s3* and *FvSTE3.s5* are truly connected.

The presence of *FvSTE3.s1* and *FvSTE3.s2* on different subloci corresponds with their phylogenetic separation into two clades that were derived through duplication [12]. It is surprising that their closest homologues, *ScBBR1* and *ScBBR2*, are alleles on the same locus (matB8) in *S. commune* [18,23]. The diversity between *F. velutipes* and *S. commune* as well as the large distance in the first, shows that the genomic organization of *matB* loci should be considered more flexible than previously has been assumed.

The A mating-type genes

Three homeodomain genes distributed over two distant subloci were identified in *F. velutipes*. Both subloci were specifically linked to mating-type A3 (FvHD2-1 and FvHD2-2) yet the only present HD1 gene (FvHD1-1) was found in matA3 and matA4 mating types. MatA4 thus contains a different matA allele, consisting of FvHD1-1 either combined with another HD2 gene than FvHD2-2 or as a single gene. This means that FvHD1-1 or FvHD2-2, that form a mating-type gene couple in matA3, have been independently recombined in other mating types. Lack of a matA3 specific HD1 gene dictates existence of at least one other HD1 gene in matA4, presumably in the matA8 locus.

Structure of the matA region

We mapped the synten between *F. velutipes*, *S. commune*, *L. bicolor* and *C. cinerea* based on blast searches with successive genes of the latter. Consequently, few genes that are missing in *C. cinerea* but that might be syntenic between other species remained undetected. The high detail of our maps however, showed that the applied method was accurate for both annotated and non annotated species. *F. velutipes* and *S. commune* showed different gene orders when compared, yet both followed the overall gene order of *C. cinerea* and *L. bicolor*. This, together with non separated matA loci in *C. cinerea* and *L. bicolor* shows that they represent an ancestral organization. We identified a 350 Kb matA region that is strongly conserved amongst Agaricales. Notably, synteny of genes belonging to this region is preserved even if parts become separated by chromosomal rearrangements as was shown in *S. commune*. The borders of the 350 Kb region, as well as two internal hotspots (one of which is the matA locus) are conserved sites of recombination in two major clades of the Agaricales as classified by Matheny et al. [50]. They mark the edges of important rearranged segments in *F. velutipes* as well as *S. commune*. Reasonably, one might expect rearrangements of the strongly conserved 350 Kb matA region in other Agaricales, especially in the Marasmioid clade that contains *F. velutipes* and *S. commune*.

Our segregation experiments showed that both matA3 subloci are linked despite their 73 Kb distance. Until now, far distant matA3 subloci in Agaricales were only known in *S. commune* (~450 Kb) and considered to be an exception. The matA subloci of *F. velutipes* were demonstrated to be separated by inversion of two (~70 Kb) gene clusters and are clearly derived from an ancestral locus as represented by *C. cinerea*. It has been generally assumed that the matA subloci of *S. commune* have followed a similar course leading to separation. However, our analysis showed significant differences between rearrangements in *F. velutipes* and *S. commune*. What is more, the analyses of the extended synteny map between *S. commune* and *C. cinerea* strongly indicate that the matA subloci of *S. commune* were separated by transposition of gene clusters instead of inversions. As shown in model A (Figure 6A, C) a two step transposition of two high syntenic segments corresponding to the conserved 350 Kb matA region, directly results in a gene order mostly similar to that in *C. cinerea*. Though it is possible to reconstitute the ancestral matA locus by two inversions of considerably larger fragments as shown in model B, many rearrangements including transpositions remain (Figure 6B and D). Both the smaller sizes of the rearranged fragments and the fewer steps needed to reconstitute the ancestral gene order support the transposition model.

*F. velutipes* provides a phylogenetically diverse species with an unusual mating type system (one of two components of the matA3 locus, HD2, is variable relative to matA4 while the HD1 is identical and matB3 contains a unique pheromone precursor). This enables comparative genomics to identify trends in mating type locus evolution. Studies of mating-type genes in Agaricales have shown that subloci are typically closely linked (10–20 kb) with the directly surrounding genes, especially in the matA locus, being highly syntenic. In our study we determined that the Agaricales in fact have very large scale synteny at matA (~350 Kb) and that this synteny is maintained even when parts of this region are separated through chromosomal rearrangements (*S. commune*). Four conserved recombination hotspots allow reshuffling of large fragments of this region which resulted in separation of the matA subloci of *F. velutipes* as well as of *S. commune*, by different events. This implies that separation of matA loci is not exceptional and might be expected in other Agaricales. In addition to matA, we determined that also matB loci can exist over large distances (~180 Kb) and that non mating-type specific pheromone receptors and mating-type specific ones are controlled by different selection mechanisms. Finally, the genes that were linked to specific mating types will serve as important molecular markers for breeding.

Supporting Information

**Figure S1** Alignment of the three pheromone precursor proteins from *F. velutipes* KACC42780. Conserved amino acids in the pheromone sequences are marked with *. The conserved putative proteolytic site represented by E and R is indicated by a bold line. The CAAX-box of each protein is designated by a dashed line. The C-terminal halves of FvPp2 and FvPp3 are highly similar (boxed).

**Table S1** Contigs of *F. velutipes* KACC42780 that were used in this study. The table enlists the contigs of *F. velutipes* KACC42780 that were used in this study, describing their respective size and important genes that are located on those contigs. Genes that were specifically linked to mating type loci in this study are indicated with *. Pseudogene *FvSTE3.s2* is italicized. The STE3 prefix ‘Fv’ is added for *Flammulina velutipes*, the small ‘s’ preceding STE3 numbers is added to distinguish non mating-type specific pheromone receptors (*FvSte3 similar*) from mating-type specific pheromone receptors. Gene accession numbers for pheromone receptors, pheromones, homeodomain genes and MIP are given in the material and methods.

**Table S2** Primers used in this study. List of specific primers for each pheromone receptor, pheromone and homeodomain proteins from *F. velutipes* KACC42780.
gene from *F. velutipes* KACC24780. The first primer sets for each gene were used to determine distribution of genes for segregation analysis. The 4 additional sets for *FvSTE2.1, FvSTE2.2* and *FvSTE2.3* were newly designed after amplification in additional *F. velutipes* strains failed. Primer 1′ fl matA 1–1 rv 500bp for gene *FvHd1-1* is exceptional and anneals in an intron.

(DOC)

| Table 3 | Amino acid positions of domains that were detected in proteins FvHd1-1, FvHd2-1 and FvHd2-2. |
|---------|------------------------------------------------------------------------------------------|
| 1. | Statistics of amino acid positions detected in proteins FvHd1-1, FvHd2-1, and FvHd2-2. |
| 2. | FvHd1-1, FvHd2-1, and FvHd2-2, respectively. |
| 3. | The data indicate that the distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes* gene from *F. velutipes* gene from *F. velutipes*. |
| 4. | Numbers in the table refer to the amino acid (AA) numbers in the domains of the respective proteins. |
| 5. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 6. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 7. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 8. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 9. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 10. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 11. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 12. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 13. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 14. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 15. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 16. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 17. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 18. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 19. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 20. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 21. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 22. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 23. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |
| 24. | Distribution of genes for segregation analysis in *F. velutipes* gene from *F. velutipes*. |

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3. Raudaskoski M, Kotli E (2010) Basidiomycete Mating Type Genes and Homeodomain proteins were analyzed for 9 amino acid detected in proteins FvHd1-1, FvHd2-1 and FvHd2-2. Table S3 Amino acid positions of domains that were gene were used to determine distribution of genes for segregation analysis. The *4 additional sets* for *FvSTE2.1, FvSTE2.2* and *FvSTE2.3* were newly designed after amplification in additional *F. velutipes* strains failed. Primer 1′ fl matA 1–1 rv 500bp for gene *FvHd1-1* is exceptional and anneals in an intron.

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Author Contributions

Conceived and designed the experiments: W-SK AFvP B-ML. Performed the experiments: AFvP S-YP P-GS K-YJ Y-BY YJP. Analyzed the data: AFvP W-SK G-HS TYJ. Contributed reagents/materials/analysis tools: W-SK AFvP BY-Y JF. Wrote the paper: AFvP G-HS.
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